EMERGENCY RELIEF REQUESTED

No. 24-___

In the United States Court of Appeals for the Federal Circuit

REGENERON PHARMACEUTICALS, INC.,
Plaintiff-Appellant,

v.

MYLAN PHARMACEUTICALS INC., AMGEN USA, INC., BIOCON BIOLOGICS INC., CELLTRION, INC., FORMYCON AG, SAMSUNG BIOEPIS CO., LTD.,
Defendants,

AMGEN INC.,
Defendant-Appellee,

Appeal from the United States District Court for the Northern District of West Virginia in No. 1:24-md-3103-TSK, Chief Judge Thomas S. Kleeh

APPELLANT REGENERON PHARMACEUTICALS, INC.'S NONCONFIDENTIAL EMERGENCY MOTION FOR AN INJUNCTION PENDING RESOLUTION OF APPEAL AND FOR AN ADMINISTRATIVE STAY

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Confidential Material Omitted

Pursuant to Fed. Cir. R. 25.1(e)(1)(B), the material omitted on page 11 relates generally to Amgen's statements made in its regulatory application seeking approval for its biosimilar product, which Amgen has maintained as confidential. Regeneron has no objection to the public disclosure of this information.

TABLE OF AUTHORITIES

CASES

STATUTES

RULE 27(a)(2) STATEMENT

Pursuant to Federal Rule of Appellate Procedure 8, plaintiff-appellant Regeneron Pharmaceuticals, Inc. ("Regeneron") seeks an emergency injunction barring defendant-appellee Amgen, Inc. ("Amgen") from launching its generic version of Regeneron's Eylea® product pending resolution of this appeal, which Regeneron seeks to expedite in a motion filed concurrently.

Regeneron also respectfully requests an immediate administrative stay to preserve the status quo while the Court considers this application. *See, e.g.*, *Marine Polymer Techs., Inc. v. Hemcon, Inc.*, 395 F. App'x 701 (Fed. Cir. 2010). Regeneron has learned that, absent an administrative stay, Amgen will immediately begin distributing its competing biosimilar product, *see* Add716-717 (Clark September 23, 2024 Decl. ¶¶ 3-5), harming Regeneron irreparably and potentially impairing this Court's ability to provide relief to Regeneron.

Regeneron attempted to confer with Amgen before filing this motion, but Amgen did not respond. Regeneron therefore is filing this as an opposed motion.

RULE 8(c) STATEMENT

Federal Circuit Rule 8(c) permits parties to apply directly to this Court for an injunction pending appeal when moving first in the district court is "not

practicable." Fed. Cir. R. 8(c); *see* Fed. R. App. P. 8(a)(2)(A)(i) (party need not first file in district court if doing so is "impracticable"). That standard is amply satisfied in this case. As explained below, a premature biosimilar launch would fundamentally and irrevocably alter the market for Regeneron's groundbreaking Eylea® product, with devastating consequences for Regeneron and its employees. Amgen, however, has not committed to delaying its launch until this appeal is resolved. To the contrary, Regeneron has learned in recent days that Amgen has told customers its biosimilar product will be available as early as *October 1st*, indicating that Amgen intends to begin immediate activation of distributor networks and negotiations with distributors and payors on pricing and discounts. Add716-717 (Clark September 23, 2024 Decl. ¶¶ 3-5). Given the timing and the stakes at issue, it is not practicable to await a ruling by the district court, which has not yet ruled on an earlier-filed motion to stay a preliminary injunction entered against another biosimilar applicant.

INTRODUCTION

Regeneron has been litigating in West Virginia for years against multiple entities intent on marketing biosimilar versions of Eylea®, Regeneron's pioneering vision-saving treatment. To date, Regeneron has obtained injunctions against four defendants—one after a two-week bench trial on the merits, and three at the preliminary injunction stage. *See In re Aflibercept Pat. Litig.*, No. 24-md-3103, Dkt. 188 ("Mylan Injunction Decision"), Dkt. 194 ("SB PI Decision"), Dkt. 247 ("Formycon PI Decision"), Dkt. 248 ("Celltrion PI Decision") (N.D.W. Va.).[1] Each of those injunctions is currently on expedited appeal to this Court. *E.g.*, No. 24-1965, Dkt. 38 at 4. In issuing the injunctions, the district court acknowledged the immediate, irreparable harm Regeneron would suffer in the event of a biosimilar launch, including through loss of market share and irreversible price erosion. It did not find otherwise in denying injunctive relief against Amgen.

Each injunction is based on Regeneron's U.S. Patent 11,804,865 ("the '865 patent") claiming ophthalmic formulations, including Eylea®. After issuing four injunctions, however, the district court reversed course.

[1] Unless otherwise indicated, all docket citations are to *In re Aflibercept*, No. 24-md-3103 (N.D.W. Va.).

Contravening its prior, precedentially mandated construction of the claim term "buffer" that was the basis for its injunction against Formycon, *see* Formycon PI Decision at 46-55, the court found Regeneron unlikely to prove that Amgen will infringe the '865 patent. Add621-710. In so doing, the district court violated this Court's claim-construction precedent, defied its own prior decisions, and paved the way for Amgen to destroy Regeneron's market for Eylea® irreversibly.

Amgen has not been coy about its intentions: it has conveyed to customers that it intends to launch its biosimilar product on October 1st, Add716-717 (Clark September 23, 2024 Decl. ¶¶ 3-5), all while this Court is poised to decide the appeals of the four earlier-filed biosimilar applicants. Only an injunction pending appeal can preserve the status quo while this Court reviews the district court's errant decision. If the denial of injunctive relief is reversed, the presently requested injunction will ensure that the district court's error does not alter—irreversibly—the over $5 billion Eylea® market. And if the decision somehow is affirmed following expedited review, Amgen will be able to commercialize its product only a few months later, still long before its enjoined biosimilar competitors.

Each of the injunctive relief considerations supports relief; the district court did not rule otherwise with respect to factors other than likelihood of success. And the district court's likelihood-of-success decision is self-evidently wrong. The decision rested solely on Amgen's argument that its proposed biosimilar does not contain a "buffer" as claimed in the '865 patent. Add621-623, 648-649. But it was undisputed that Amgen's biosimilar *does* have a buffer: the protein aflibercept at a concentration of 40 mg/mL. Both parties' experts expressly agreed on that point. Add127-128 (Trout Decl. ¶ 279); Add606 (Chamow Dep. 316:10-12). And, critically, the district court already has construed the term "buffer" in the claims of the '865 patent to include "proteins like aflibercept." Formycon PI Decision at 54. As the district court previously found, that construction of "buffer" is supported by "both the intrinsic and extrinsic evidence," *id.*, including nearly a century of scientific literature. Formycon chose not to challenge that well-founded construction of "buffer" on appeal. No. 24-2009, Dkt. 20 at 57 n.15. Nor did the district court disturb its construction of "buffer" in its *Amgen* decision; rather, it expressly declined to revisit its construction. Add699 ("the Court declines to construe the term 'buffer' at this stage"). The court identified no error in its "buffer" construction, and yet—in direct contravention of its earlier, settled

construction of "buffer" to include "proteins like aflibercept"—concluded inexplicably that the POSA "would not consider a therapeutic fusion protein like aflibercept to be a 'buffer' in the context of the '865 patent." Add685. The court's facially contradictory construction is an abuse of discretion. Under the district court's own construction of "buffer" as including "proteins like aflibercept"—a construction that is supported by the testimony of both parties' experts in this case—Amgen undisputedly would infringe.

The district court reached the contrary result by invoking a supposed prohibition on one substance (aflibercept) meeting multiple claim limitations. But this Court's decisions abjure any such categorical rule. *Google LLC v. EcoFactor, Inc.*, 92 F.4th 1049, 1058 (Fed. Cir. 2024). That is especially true where, as here, the undisputed intrinsic and extrinsic evidence reflects that multiple substances recited in the specification—including the active protein and buffer aflibercept—were recognized by the POSA to meet multiple claim limitations. The court's error is particularly glaring given its own construction of "buffer" that includes "proteins like aflibercept." Formycon PI Decision at 54. The court's "buffer" construction indicates that, in the context of the '865 patent, aflibercept is both a buffer and a VEGF antagonist protein. And yet the court's new, contrary construction requires that "the claimed 'VEGF

antagonist' and the claimed 'buffer' are separate components." Add657. This Court's jurisprudence does not countenance, and manifestly does not compel, such an absurd result. The court's infringement determination, based on an irreconcilable claim construction, is flawed. Add657.

The remaining injunction factors likewise weigh decisively in Regeneron's favor. As the district court repeatedly has recognized, *see* Mylan Injunction Decision at 25-42, 51-67; SB PI Decision at 117-67; Formycon PI Decision at 135-88; Celltrion PI Decision at 124-69, the launch of a biosimilar version of Eylea®—exactly what Amgen has announced it intends imminently absent injunction—will alter the market for Eylea® immediately and irreversibly. In contrast, any lost sales to Amgen from maintaining the status quo while this Court adjudicates an expedited appeal pale in comparison to Regeneron's hardship, and in any event would be compensable by Regeneron's bond.

The status quo today is that Regeneron is exclusively marketing Eylea®, its blockbuster product undisputedly disclosed and claimed in the '865 patent. Without this Court's intervention, however, Amgen will upset that status quo irreversibly. This Court should not condone that catastrophic result on the

basis of the district court's indefensible claim-construction and infringement rulings below.

BACKGROUND

I. Regeneron's Invention of Eylea®

Regeneron invented and developed Eylea®, the "revolutionary," leading treatment for the most common causes of blindness, including wet age-related macular degeneration. *Regeneron Pharms. v. Mylan Pharms.*, --- F. Supp. 3d ----, 2024 WL 382495, at *13, *60 (N.D. W. Va. Jan. 31, 2024) ("*Mylan*"). Eylea®'s active ingredient is a vascular endothelial growth factor (VEGF) antagonist fusion protein called aflibercept. *Id.* at *13. The '865 patent is directed to ophthalmic formulations of aflibercept, including Eylea®, at a concentration of 40 mg/mL. *Id.* at *15. The asserted claims recite "ophthalmic formulation[s]" comprising, inter alia, 40 mg/mL of a VEGF antagonist and "a buffer." Add914 ('865 patent, claim 2).

II. Prior Eylea® Litigations

Since October 2021, several applicants have sought FDA approval under the BPCIA to market biosimilars of Eylea®. *See In re Aflibercept Pat. Litig.*, 2024 WL 1597512, at *1 (J.P.M.L. Apr. 11, 2024). The first was Mylan, against whom Regeneron proceeded to trial in June 2023. *Mylan,* 2024 WL 382495, at

*2. The district court found that Mylan infringed the '865 patent, *id.* at *31-33, and that the asserted claims were not invalid, *id.* at *41-70. The court issued a permanent injunction against Mylan, finding that Regeneron would be irreparably harmed by launch of Mylan's biosimilar and that the balance of equities and public interest favored injunctive relief. *See* Mylan Injunction Decision at 25-42, 51-67.

SB, Formycon, and Celltrion (the "PI Defendants") followed Mylan in seeking approval of aflibercept biosimilars, and Regeneron sued each last fall. Regeneron moved to preliminarily enjoin the PI Defendants. The district court again sustained the '865 patent and granted Regeneron's preliminary injunction motions, finding that Regeneron would be irreparably harmed by biosimilar launch and that the balance of equities and public interest favored injunctive relief. *See* SB PI Decision at 54-177; Formycon PI Decision at 69-199; Celltrion PI Decision at 61-178.

As relevant here, Formycon asserted noninfringement based on a narrowed construction of "buffer." In view of the specification's disclosure and the common understanding that proteins are buffers, the court rejected Formycon's construction, construing "buffer" "according to its ordinary meaning to the POSA: 'a substance that resists changes to pH upon addition

of an acid or base within an optimal pH range through a proton-donating component and/or a proton-accepting component, including, for example, histidine, phosphate, and proteins like aflibercept.'" Formycon PI Decision at 46-63. The court found that Formycon infringed the buffer limitation under this construction, *id.*, which Formycon has not appealed.

III. Amgen Litigation

The fifth applicant to seek approval for an aflibercept biosimilar was Amgen, which Regeneron sued in the Central District of California in January 2024. Regeneron then successfully moved under 28 U.S.C. § 1407 to centralize the five actions in the Northern District of West Virginia. *See In re Aflibercept,* 2024 WL 1597512, at *1.

Regeneron sought a preliminary injunction against Amgen, advancing the same construction of "buffer" as including "proteins like aflibercept" that the district court adopted (over Formycon's objection) in *Formycon*. Dkt. 157-1 at 6-13. On September 23, the court denied Regeneron's motion, determining that Regeneron failed to show a likelihood of success in proving infringement (largely adopting Amgen's proposed order), because aflibercept did not meet the "buffer" limitation of the claims. Add648-649. Specifically, the district court determined that the 40 mg/mL aflibercept in Amgen's

biosimilar could not meet both the "VEGF antagonist" and "buffer" limitations, and Regeneron thus was not likely to succeed on infringement. Add701. The court otherwise "decline[d] to construe the term 'buffer' at this stage," Add699, apart from concluding, in direct conflict with its *Formycon* construction, that it could not be a VEGF antagonist like aflibercept. Add653-699.

Regeneron noticed its appeal the same day the court's decision issued, Add711-714, and filed this motion immediately thereafter.

LEGAL STANDARD

In analyzing a motion for injunctive relief under Rule 8, this Court evaluates: "(1) whether the [movant] has made a strong showing that [it] is likely to succeed on the merits; (2) whether the [movant] will be irreparably injured absent a stay; (3) whether issuance of the stay will substantially injure the other parties interested in the proceeding; and (4) where the public interest lies." *Standard Havens Prods., Inc. v. Gencor Indus., Inc.*, 897 F.2d 511, 512 (Fed. Cir. 1990) (cleaned up); *Eli Lilly & Co. v. Actavis Elizabeth LLC*, 2010 WL 3374123, at *1 (Fed. Cir. Aug. 26, 2010) (granting motion for injunction pending appeal). A movant can satisfy the first factor by

establishing a "substantial case on the merits provided that the harm factors militate in its favor." *Eli Lilly*, 2010 WL 3374123, at *1.

ARGUMENT

I. Regeneron Is Likely To Succeed on the Merits

The asserted claims recite "an ophthalmic formulation" that comprises a VEGF antagonist protein and "a buffer." Add914-915 ('865 patent, claims 1, 26). The specification instructs that "all technical and scientific terms and phrases used herein have the same meaning as commonly understood by [the POSA]." Add907 (*Id.*, 5:39-42). As the court recognized, that meaning of "buffer" is "a substance that resists changes to pH upon addition of an acid or base within an optimal pH range through a proton-donating component and/or a proton-accepting component, including, for example, histidine, phosphate, and proteins like aflibercept." Formycon PI Decision at 46-55. Amgen's expert in this case did not dispute—and, based on nearly a century of scientific literature, could not dispute—the POSA's understanding that proteins like aflibercept may serve as buffers:

> Q. Okay. And what is that literature you're referring to?
>
> A. The literature that indicates the *proteins* are – are macro molecules that *contain ionizable groups, a number of different ionizable groups, and that those ionizable groups create a charge on the molecule that can modulate pH and provide – and allow the protein to provide buffering capacity to solutions*.

> Q. And that was literature that was *known to the POSA* for purposes of the '865 patent; right?
>
> A. *Yeah*.

Add602 (Chamow Dep. 297:7-19); *accord* Add33-34 (Trout Decl. ¶ 78).

There is also no dispute that Amgen, like the other four biosimilar applicants, infringes under this ordinary meaning to the POSA. Amgen represented to FDA that "[t]he regulatory information of the regulatory information [*i.e.*, regulatory information], due to **regulatory information**, is sufficient to regulatory information the regulatory," Add127-128 (Trout Decl. ¶ 279) (citing Add309), and both parties' experts agreed expressly that aflibercept at 40 mg/mL is a buffer in Amgen's product, Add606 (Chamow Dep. 316:10-12); Add127-128 (Trout Decl. ¶ 279) ("aflibercept serves as the buffer in [Amgen's] formulation"). Rarely does a defendant's expert admit infringement so clearly:

> Q. Right. Aflibercept does serve as a buffer in Amgen's formulation; right?
>
> A. At 40 mgs per ml.

Add606 (Chamow 316:10-12).

Nevertheless, the district court erred grievously in defying this clear, outcome-determinative admission, the specification's disclosure, the court's prior construction, and this Court's precedent. The court instead applied a rule that this Court clearly has disavowed—that a single substance (here, the

aflibercept protein) could not meet two claim limitations (here, both the "VEGF antagonist" and "buffer" limitations). *See Google*, 92 F.4th at 1058. That construction was legally erroneous, given the undisputed evidence of the POSA's understanding that substances disclosed in the patent, including aflibercept, could meet more than one claim limitation. It also was legally erroneous in light of the district court's own construction of "buffer" in the '865 patent—unchallenged by Formycon on appeal, and undisturbed by the district court's *Amgen* decision, Add699—which expressly reflects that aflibercept is both a "buffer" and a VEGF antagonist "protein," thus meeting two claim limitations. Formycon PI Decision at 54.

A. The Ordinary Meaning of "Buffer" Encompasses Proteins

"The words of a claim are generally given their ordinary and customary meaning as understood by a [POSA] when read in the context of the specification and prosecution history." *Thorner v. Sony Comp. Ent. Am. LLC*, 669 F.3d 1362, 1365 (Fed. Cir. 2012); *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312 (Fed. Cir. 2005) (en banc). The district court previously construed "buffer" as having "its ordinary meaning to the POSA: 'a substance that resists changes to pH upon addition of an acid or base within an optimal pH range through a proton-donating component and/or a proton-accepting component,

including, for example, histidine, phosphate, and proteins like aflibercept.'" Formycon PI Decision at 47. This construction is consistent with constructions adopted by other courts, including this Court. *See*, *e.g.*, *Cadence Pharms. Inc. v. Exela PharmSci Inc.*, 780 F.3d 1364, 1369 (Fed. Cir. 2015) (construing "buffering agent" as "an agent that helps the formulation resist change in pH"); *Purdue Pharm. Prods., L.P. v. Actavis Elizabeth, LLC*, 2014 WL 2624787, at *15 (D.N.J. June 11, 2014), *aff'd*, 627 F. App'x 931 (Fed. Cir. 2016) (construing "buffering agent" as "a proton-donating component or proton-accepting component used to maintain and/or achieve an approximate pH range"). Formycon did not appeal that construction. No. 24-2009, Dkt. 20 at 57 n.15. Nor did the district court revisit that construction in its *Amgen* decision, simply stating that "the Court declines to construe the term 'buffer' at this stage." Add699.

The district court acknowledged that this plain meaning encompasses all substances that meet this definition, including histidine, *see Mylan*, 2024 WL 382495, at *18, *25, and proteins that, like aflibercept, contain histidine and therefore resist changes to pH within an optimal pH range through a proton-donating component and/or a proton-accepting component, *see* Formycon PI Decision at 47; Add30-36, 128-130 (Trout Decl. ¶¶ 74-80, 280-81).

Consistent with this understanding, proteins have been known for decades to act as buffers. Add33-35 (Trout Decl. ¶¶ 78-79 (citing Add412 (WO 2006/138181 ("Gokarn")), 3:17-20)) (describing pharmaceutical formulations "that are buffered by the protein itself"); Add 513-526 (Wyman 1939); Add379-407 (Nozaki 1967); Add366-378 (Christensen 1966, entitled "Proteins as Buffers"); Add356-365 (Abe 2000). Amgen's expert Dr. Chamow agreed both that the POSA knew that proteins in general may possess "ionizable groups" that "allow the protein to provide buffering capacity to solutions," Add602 (Chamow Dep. at 297:9-15), and that aflibercept specifically "serve[s] as a buffer in Amgen's formulation" at 40 mg/mL, Add606 (Chamow Dep. 316:10-12).

Extrinsic evidence further compels a finding that the claim term "buffer" includes "proteins like aflibercept." Formycon PI Decision at 54. For example, Amgen's prior-art Gokarn publication, directed to "Self-Buffering Protein Formulations," teaches "formulations comprising a pharmaceutical protein, that are buffered by the protein itself, that do not require additional buffering agents to maintain a desired pH, and in which the protein is substantially the only buffering agent." Add409, 412 (Gokarn, 3:17-20). Dr. Chamow agreed that "Gokarn demonstrates that the buffering capacity of

proteins can be used to control pH in formulations." Add587 (Chamow Dep. 240:9-18). That testimony is fully consistent with the district court's own prior factual findings, based on the same Gokarn reference, that the POSA understood in the context of the '865 patent that histidine-containing proteins—including fusion proteins like aflibercept—"could act as buffers." Formycon PI Decision at 53-54. The district court did not explain, much less justify, its contrary conclusion as to the understanding of the POSA that led it to err here.

Amgen had no response to this evidence. But the district court simply erased it, by misreading this Court's precedent to require excising Gokarn's teaching from the POSA's knowledge because it was a prior-art reference under § 102(e). Add687-689. Controlling law plainly dictates otherwise: "reference may be made to" § 102(e) art may be used "to construe claim language." *In re Glass*, 492 F.2d 1228, 1232 n.6 (C.C.P.A. 1974). The district court inexplicably ignored this holding simply because Glass also "discusse[d] indefiniteness." Add688. The district court did not find—and could not have found—that "buffer" excludes proteins when Gokarn is included in the POSA's knowledge, as the law requires.

B. The District Court Contravened Precedent Repeatedly in Abandoning the Ordinary Meaning of Buffer

The district court identified no error in its prior construction of "buffer" as including "proteins like aflibercept." Formycon PI Decision at 54. Rather, the court simply "decline[d] to construe the term 'buffer'" in its *Amgen* decision. Add699. The court could not, and did not, offer any basis for contravening its prior determination that the ordinary meaning of "buffer" includes "proteins like aflibercept" in the context of the '865 patent. The court simply failed to apply this construction—and concluded instead, directly contrary to its prior construction of "buffer," that the POSA "would not consider a therapeutic fusion protein like aflibercept to be a 'buffer' in the context of the '865 patent." Add685. This was legal error.

The district court identified no alternative ordinary meaning of "buffer," Add698-699, and instead simply adopted a construction that abandons the ordinary meaning—without the requisite finding of lexicography or disclaimer, *Thorner*, 669 F.3d at 1365—based on a flawed understanding of this Court's precedent. Relying on *Becton, Dickinson & Co. v. Tyco Healthcare Group, LP*, 616 F.3d 1249 (Fed. Cir. 2010), the district court construed the claims to require that each claim category be met by a distinct substance. Add653-697. But this Court has subsequently clarified that *Becton*

did not establish a "*per se* rule that separately listed claim elements are distinct components." *Google*, 92 F.4th at 1058. Instead, *Becton* based its holding on the specific evidence in that case, which bears no resemblance to the evidence here. The claims in *Becton* separately recited hardware elements of a safety needle: a "hinged arm," and a "spring means connected to said hinged arm." 616 F.3d at 1254. This Court rejected the proposition that those elements could be met by a single structure, concluding it would render the claims a "nonsensical" "physical impossibility," if "the hinged arm must be 'connected to' itself and must 'extend between' itself and a mounting means." *Id.* at 1255. The Court further noted that if the separate elements were not separate structures, "then the asserted claims are clearly invalid as obvious over the prior art." *Id.* And it found that the intrinsic evidence supported the conclusion that the separate claim limitations required different structures. *Id.* at 1254-55.[2]

[2] Likewise, the other decisions relied upon by the district court simply determined that nothing in the claims or written description rebutted the presumption articulated in *Becton*. *See Kyocera v. ITC*, 22 F.4th 1369, 1382 (Fed. Cir. 2022) ("No party has identified claim language" or "any language in the written description" "overcoming the presumption that the exit end of the mechanism and the safety contact element are distinct components."); *HTC v. Cellular Comm'cns*, 701 F. App'x 978, 982 (Fed. Cir. 2017) (nonprecedential)

The scenario here, however, is the exact opposite. For nearly a century, scientists have known that proteins can serve as buffers, Add33-35 (Trout Decl. ¶¶ 78-79), and unsurprisingly, Amgen's expert Dr. Chamow never opined that there is anything nonsensical about aflibercept serving as the "buffer" in Amgen's biosimilar product, Add534 (Chamow Dep. at 25:18-26:3). To the contrary, he admitted that proteins can be buffers and—in a clear admission of infringement—that 40 mg/mL aflibercept is the buffer in Amgen's biosimilar product. Add602 *(id.* at 297:9-15) (it was known that proteins have "ionizable groups" that "allow the protein to provide buffering capacity to solutions"); Add606 (*id.* at 316:10-12) (agreeing that 40 mg/mL aflibercept "serve[s] as a buffer in Amgen's formulation"). Also unlike in *Becton*, there has never been any contention that the "buffer" distinguished the subject matter of the asserted claims from the prior art. Rather, a "buffer" was a "known structure[]." *Mylan*, 2024 WL 382495, at *67.

Crucially, the intrinsic and extrinsic evidence here make clear that substances within the claimed formulation can properly meet multiple categories. For example, the patent refers to glycerol as a "stabilizing agent,"

(noting that the specification "distinguishes the [structure performing one recited function] from the [structure performing another recited function]").

Add905 ('865 patent, 2:44-45), while other intrinsic evidence incorporated in the patent categorizes glycerol as a "non-ionic tonicity agent," U.S. Patent 6,777,429, 1:25. Likewise, the patent teaches that propylene glycol is an organic co-solvent, Add905 ('865 patent, 2:39-42), but other intrinsic evidence teaches that it is also a tonicity agent, U.S. Patent 6,676,941, 100:22-23. This intrinsic evidence expressly teaches that substances may fit multiple claimed categories. On this undisputed record, *Becton* and its progeny do not foreclose a finding of infringement—they mandate it. *See Powell v. Home Depot U.S.A., Inc.*, 663 F.3d 1221, 1231-32 (Fed. Cir. 2011) (holding that claimed "cutting box" "may also function as a 'dust collection structure'" and explaining that "prior art cited in a patent … constitutes intrinsic evidence" (citation omitted)); *Linear Tech. Corp. v. ITC*, 566 F.3d 1049, 1055 (Fed. Cir. 2009) (construing "'second circuit' and 'third circuit'" "broadly" because the "specification expressly discloses that the 'second circuit' and 'third circuit' can share common components"). The district court inexplicably ignored this undisputed evidence, which forecloses the result it reached. *Google*, 92 F.4th at 1058.

The patent's intrinsic evidence is consistent with overwhelming extrinsic evidence that, in this art, it was well-understood that a substance could meet

multiple categories. For example, Dr. Chamow agreed that histidine—undisputedly a buffer, *Mylan*, 2024 WL 382495, at *67—also "could serve as a stabilizing agent *in the context of the '865 patent*." Add562 (Chamow Dep. at 138:3-11) (emphasis added). The court likewise ignored this testimony, which Regeneron emphasized below. Dkt. 288 at 10. That was far from the only example; Dr. Chamow agreed that numerous substances recited in the '865 patent could meet multiple roles. He admitted that: (1) "Gokarn demonstrates that the buffering capacity of proteins can be used to control pH in formulations" and "discloses protein formulations in which the protein is substantially the only buffer"; (2) "trehalose is a co-solvent" as well as a stabilizing agent; (3) "sodium phosphate in the context of the '865 patent ... serve[s] as a tonicity agent" and "as a buffer"; (4) "polysorbate 80 can serve as a stabilizer" and a co-solvent; (5) "polysorbate 20 acts to stabilize biopharmaceutical formulations" and as a co-solvent; and (6) "polyethylene glycol and propylene glycol are useful as stabilizing agents" and co-solvents. Add545-546, 552, 562-564, 573-574, 587, 589-590, 606 (Chamow Dep. at 72:18-73:14, 74:22-75:10, 99:22-100:5, 139:8-140:3, 142:11-143:21, 144:2-8, 146:2-14, 183:14-185:2, 240:9-18, 248:6-250:7, 316:10-12); *see also* Add905 ('865 patent, 2:39-48). The district court blithely discarded this evidence, thereby violating

this Court's foundational principle that the claim language and intrinsic record of the specification's listed substances must be interpreted through the lens of the POSA, as the meaning to a layman—elevated by the district court over the undisputed expert testimony—is "irrelevant." *Searfoss v. Pioneer Consol. Corp.*, 374 F.3d 1142, 1149 (Fed. Cir. 2004); *Phillips*, 415 F.3d at 1313.

II. Regeneron Will Suffer Irreparable Harm Absent An Injunction

Absent the requested relief, Amgen will alter the Eylea® market imminently and irreversibly. Even before the district court's decision issued, Amgen already had begun contacting physicians about the impending availability of its Eylea® biosimilar. Add716-717 (Clark September 23, 2024 Decl. ¶¶ 3-5). Amgen informed its customers that it intends to launch its biosimilar "at risk," and that it will be available as early as October 1st. *Id.* ¶¶ 3-5. Amgen is presumably taking steps to effectuate that launch as of this writing, including setting prices and reaching sales and distribution agreements with its partners, *Id.* ¶¶ 3-5, notwithstanding that such steps infringe Regeneron's '865 patent, *see Halo Elecs., Inc. v. Pulse Elecs., Inc.*, 831 F.3d 1369, 1380 (Fed. Cir. 2016) ("We have held that 'a description of the allegedly infringing merchandise and the price at which it can be purchased' may constitute an offer to sell." (quoting *3D Sys., Inc. v. Aarotech Labs., Inc.*,

160 F.3d 1373, 1379 (Fed. Cir. 1998))). And Amgen has refused to delay its launch while this Court considers the present motion.

Amgen's launch will cause Regeneron significant irreparable harm that would be difficult to quantify and cannot be fully remedied by later monetary payments. These harms include: (1) loss of sales and market share, (2) price erosion, (3) disruption of patentee-payor relationships, and (4) reputational harm. *See* Add717-719 (Clark September 23, 2024 Decl. ¶¶ 6-11). This Court has consistently recognized each such harm as irreparable, including in the pharmaceutical context. *See Bio-Rad Labs., Inc. v. 10X Genomics Inc.*, 967 F.3d 1353, 1378 (Fed. Cir. 2020) ("increase[d] ... marketing costs"); *Mylan Institutional LLC v. Aurobindo Pharma Ltd.*, 857 F.3d 858, 872 (Fed. Cir. 2017) ("lost sales, lost research and development, price erosion, and having to directly compete with an infringer"); *Douglas Dynamics, LLC v. Buyers Prods. Co.*, 717 F.3d 1336, 1344-45 (Fed. Cir. 2013) ("lost sales and erosion in reputation"); *Celsis In Vitro, Inc. v. CellzDirect, Inc.*, 664 F.3d 922, 930-31 (Fed. Cir. 2012) ("Price erosion, loss of goodwill, damage to reputation, and loss of business opportunities are all valid grounds for finding irreparable harm."); *Sanofi-Synthelabo v. Apotex, Inc.*, 470 F.3d 1368, 1382-83 (Fed. Cir. 2006) ("price erosion" and issues with "third-party payors").

Indeed, the district court enjoined Mylan and the PI Defendants from launching their biosimilar products, finding that their market entry would inflict those harms upon Regeneron and that Regeneron demonstrated a nexus between the harms and their infringement. *See* Mylan Injunction Decision at 25-42, 51-67 (finding that "Regeneron has shown its likely harm due to lost market share and sales is not fully addressable through legal or monetary remedies," "price erosion prompted by [Mylan's biosimilar's] launch would cause Regeneron irreparable harm," Regeneron "will suffer reputational harms in the pharmaceutical community and among healthcare professionals if [Mylan's biosimilar] is permitted to launch but later is removed from the market."). The district court did not hold otherwise here.

III. The Balance of Harms Weighs in Favor of an Injunction

The balance of hardships weighs decisively in Regeneron's favor. Whereas the harm to Regeneron if Amgen launches at-risk is a near certainty, granting an injunction will harm Amgen only if this Court ultimately affirms the district court's judgment. In that event, Amgen "would only lose the ability to go on to the market and begin earning profits earlier." *Glaxo Grp. Ltd. v. Apotex, Inc.*, 64 F. App'x 751, 756 (Fed. Cir. 2003). That potential loss of sales is not irreparable harm. *See Pfizer, Inc. v. Teva Pharms., USA, Inc.*,

429 F.3d 1364, 1382 (Fed. Cir. 2005) ("Simply put, an alleged infringer's loss of market share and customer relationships, without more, does not rise to the level necessary to overcome the loss of exclusivity experienced by a patent owner due to infringing conduct."). Any harm to Amgen from lost sales, moreover, would be compensable by Regeneron's posting of bond.

In any event, any delay in Amgen's market entry would be brief: concurrently with this motion, Regeneron has filed a motion to expedite this appeal substantially by committing to expedite its briefs significantly. This Court has previously recognized that such steps will mitigate the risk of injury to a generic competitor.[3] In the interim, the injunction would "preserv[e] the

[3] *See, e.g.*, *AstraZeneca LP v. Breath Ltd.*, 2013 WL 9853383, at *1 (Fed. Cir. May 24, 2013) (granting injunction pending appeal); *AstraZeneca LP v. Breath Ltd.*, No. 13-1312, Dkt. 71 (Fed. Cir. June 5, 2013) (ordering July 1st deadline for cross-appellants' opening brief and appellees' briefs, July 12th deadline for appellants' response/reply brief, and July 19th deadline for cross-appellants' reply brief); *Eli Lilly*, 2010 WL 3374123, at *1 (granting injunction pending appeal and setting expedited deadlines of 14 days, 14 days, and 7 days for opening, responsive, and reply briefs, respectively); *cf. Jazz Pharms., Inc. v. Avadel CNS Pharms., LLC*, No. 23-1186, Dkt. 11, Dkt. 28 (Fed. Cir. 2022) (staying order and setting December 16th, January 13th, and January 20th deadlines for opening, responsive, and reply briefs, respectively); *Teva Branded Pharm. Prods. R&D, Inc. v. Amneal Pharms. of N.Y., LLC*, No. 24-1936, Dkt. 29, Dkt. 32 (Fed. Cir. 2024) (staying order and setting July 30th, August 30th, and September 11th deadlines for opening, responsive, and reply briefs, respectively).

status quo" and "the current market structure" while this Court hears Regeneron's appeal, as well as the appeals of Mylan and the three PI Defendants. *Abbott Labs. v. Sandoz, Inc.*, 544 F.3d 1341, 1362 (Fed. Cir. 2008) (affirming finding that balance of hardships weighs in favor of patentee).

IV. An Injunction Would Benefit the Public Interest

Regeneron invested years of research and millions of dollars to develop Eylea® and the inventions resulting from that development. *See* Add925-926 (Clark June 7, 2024 Decl. ¶ 19). There is a "significant public interest" in encouraging that kind of investment in drug development and "protecting the exclusionary rights conveyed in valid pharmaceutical patents," particularly in cases like this one where the patentee practices the invention. *Sanofi-Synthelabo*, 470 F.3d at 1384 (cleaned up); *Abbott*, 544 F.3d at 1362-63. As with the Hatch-Waxman Act, which creates a framework for bringing low-cost generic drugs to the market, *see Pfizer*, 429 F.3d at 1382, the BPCIA does not negate this public interest, *see Douglas Dynamics*, 717 F.3d at 1346 ("[T]he public has a greater interest in acquiring new technology through the protections provided by the Patent Act than it has in buying 'cheaper knock-offs.'").

CONCLUSION

Regeneron respectfully requests that the Court enjoin Amgen's biosimilar pending this appeal, and that it enter an administrative stay enjoining Amgen from launching its biosimilar during the pendency of this motion.

SEPTEMBER 23, 2024

Respectfully submitted,

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SEPTEMBER 23, 2024 DAVID I. BERL

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No. 24-__

In the United States Court of Appeals for the Federal Circuit

REGENERON PHARMACEUTICALS, INC.,
Plaintiff-Appellant,

v.

MYLAN PHARMACEUTICALS INC., AMGEN USA, INC., BIOCON BIOLOGICS INC., CELLTRION, INC., FORMYCON AG, SAMSUNG BIOEPIS CO., LTD.,
Defendants,

AMGEN INC.,
Defendant-Appellee,

Appeal from the United States District Court for the Northern District of West Virginia in No. 1:24-md-3103-TSK, Chief Judge Thomas S. Kleeh

ADDENDUM IN SUPPORT OF EMERGENCY MOTION FOR AN INJUNCTION PENDING RESOLUTION OF APPEAL

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Trout Ex. 121

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REVIEW

Role of Histidine-Related Compounds as Intracellular Proton Buffering Constituents in Vertebrate Muscle

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Abstract—The intracellular non-bicarbonate buffering capacity of vertebrate muscle is mainly supported by the imidazole groups of histidine residues in proteins, free L-histidine in some fish species, and histidine-containing dipeptides such as carnosine, anserine, and balenine (ophidine). The proton buffering capacity markedly differs between muscle types and animal species depending on the ability for anaerobic exercise. The capacity is typically high in fast-twitch glycolytic muscles of vertebrates adapted for anaerobic performance such as burst swimming in fishes, prolonged anoxic diving in marine mammals, flight in birds, sprint running in mammalian sprinters, and hopping locomotion in some terrestrial mammals. A high correlation between buffering capacity, concentration of histidine-related compounds in muscle, and percentage of fast-twitch fibers in all vertebrates adapted for intense anaerobic performance clearly supports the idea that proton buffering is the main physiological function of histidine-related compounds.[1]

Key words: histidine, carnosine, anserine, balenine, buffering capacity, vertebrate, muscle, anaerobic exercise

During high-intensity anaerobic exercise, a large number of protons accumulate in vertebrate muscle as ATP is hydrolyzed to ADP [1]. The proton accumulation causes a decrease in intracellular pH (pH_i) which, in turn, causes the inactivation of glycolytic enzymes such as phosphofructokinase and hence a decrease in glycolytic flux [2]. The decrease in glycolytic flux due to low pH_i is also found during prolonged anoxia of perfused rat heart [2]. The changes of pH_i are known to affect the rate of other various metabolic functions [2]. As a defense mechanism against changes in pH_i, proton buffering systems may evolve in the cell. Thus, a high buffering capacity in muscle can stabilize intramuscular pH and enhance the capability for anaerobic exercise performance or anoxia tolerance [2, 3].

The intracellular non-bicarbonate buffering of vertebrate muscle is dominated by the imidazole group which exists in histidine residues of proteins, in free L-histidine, and in histidine-containing dipeptides such as carnosine, anserine, and balenine (also known as ophidine) [1-4]. Because the p*K* values of these imidazole groups are close to pH_i, one of the two nitrogens of the imidazole ring can be protonated in the physiological range of pH. Thus, imidazole groups are utilized as potent proton buffering constituents. The regulatory process keeping pH_i close to the p*K* values of imidazole groups is called "alphastat regulation". Its role is to maintain α-imidazole relatively constant (α-imidazole being defined as non-protonated imidazole/(non-protonated imidazole + protonated imidazole)). Typical α_{Imid} is conserved at a value of about 0.55 in intracellular fluid [3]. Inorganic orthophosphate also serves as a typical inorganic buffer component in addition to imidazole compounds (Table 1).

The proton buffering capacity markedly differs between muscle types and animal species depending on the ability for anaerobic exercise [3]. This review mainly focuses on the relationship between the buffering capacities of histidine-related compounds (HRC) and the anaerobic capabilities of vertebrate skeletal muscle.

[1] Editor's note: Carnosine is now well known to be an efficient intracellular pH buffer (V. Skulachev, H. Abe), hydrophilic antioxidant (A. Boldyrev, E. Decker), heavy metal chelator (P. Trombley, E. Baran), potent anti-glycating agent (A. Hipkiss), and regulator of many specific receptors (F. Margolis, A. Fasolo, D. Miller) and enzymes (I. Severina, S. Stvolinsky). These features make this compound useful to treat ischemic brain and heart (D. Dobrota, P. Roberts, G. Zaloga) and to decelerate some senescence processes (A. Wang, S. Gallant, R. Holliday, G. McFarland). Many of these properties of carnosine are discussed in this volume.

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Table 1. Apparent p*K* values of imidazole groups (according to [3-5])

Substance	p*K*
Typical histidyl-imidazole in proteins	6.5 (25°C)
adjacent to acidic (–) group	7-8 (25°C)
adjacent to basic (+) group	5-6 (25°C)
L-Histidine	6.21 (20°C)
Carnosine	7.01 (20°C)
Anserine	7.15 (20°C)
Balenine	6.93 (20°C)
Inorganic orthophosphate	6.88 (20°C)

Note: p*K* measurements were performed at temperatures specified in parentheses.

TELEOST FISHES

Proton buffering capacity (termed β value and measured as the "slyke" unit) is defined as the μmoles of sodium hydroxide or hydrogen chloride required to change the pH of one gram of tissue by one unit, i.e., from 6 to 7 or from 6.5 to 7.5 [4, 6-8]. Table 2 shows β values, total HRC contents, and lactate dehydrogenase (LDH) activities in the white and red muscles of teleost fishes having a wide variety of anaerobic performance capabilities. Fish myotomal muscle comprises two functionally different muscle fiber types, red and white, as it is the case in other vertebrates. In fish, however, the two muscles are separated spatially. Fish red muscle exists as a thin triangular strip running longitudinally beneath the lateral line (superficial red muscle) or located more axially, extending to the vertebrae (deep-seated red muscle as in tuna fishes). Fish red muscle is an aerobic slow-twitch oxidative tissue and it is recruited during sustained, steady state swimming. In contrast, fish white muscle mainly consists of fast-twitch glycolytic fibers

Table 2. Buffering capacity (β), concentration of histidine-related compounds (HRC), and lactate dehydrogenase (LDH) activity in fish muscle (according to [4, 6, 8-13])

Species	β		HRC		LDH
	WM	RM	WM	RM	WM
Endothermic scombrids					
Katsuwonus pelamis (skipjack tuna)	122 ± 6	81 ± 34	109-148	21-42	2056
Thunnus alalunga (albacore tuna)	115 ± 13	58 ± 17	71-121	20-23	3451 ± 518
Auxis thazard (frigate mackerel)	109	—	110*	—	1186
Thunnus albacares (yellowfin tuna)	108 ± 11	82	74-91	10	2185 ± 285
Euthynnus lineatus (black skipjack tuna)	105 ± 8	83 ± 15	73-105**	11**	1572 ± 448
Active pelagic ectotherms					
scombrids					
mean for 4 species	99 ± 14	67 ± 7	16-31***	7-13***	1574 ± 267
non-scombrids					
mean for 11 species	63.3	—	<40****	<12****	455 ± 189
Deep-sea and demersal fishes					
mean for 9 species	46 ± 3	—	trace	—	59 ± 53

Note: Values (means ± SD) are expressed as μmole NaOH per pH unit per gram of wet weight of muscle (pH 6-7) in case of β, as μmole per gram of wet weight of muscle in case of HRC, and as activity units per gram of wet weight of muscle in case of LDH. Abbreviations: WM, white muscle; RM, red muscle; –, not determined.

* Values for *Auxis tapeinocephalus*.

** Values for *Euthynnus affinis*.

*** Values for chub mackerel *Scomber japonicus*.

**** Values for sardine *Sardinops melanostictus*.

and is recruited during anaerobic burst locomotion only lasting for short times.

The white muscle shows much higher β values and HRC contents than red muscle in a given species. HRC contents in red muscle correspond generally to one-tenth to one-quarter of the contents in the white muscle of various fish species [4]. The β values and HRC contents are typically high in endothermic scombrids such as tuna fishes. Tunas have a large mass of deep-seated red muscle surrounded by the "rete mirabile" (dense capillary network) used as a vascular countercurrent heat exchanger that maintains muscle temperature above ambient temperature by up to 10°C [3]. Tunas contain 60-100 μmol/g muscle wet weight of free L-histidine and 15-60 μmol/g muscle wet weight of anserine in their white muscle [4], and show over 100 slykes of proton buffering capacity (Table 2). In the white muscle of skipjack tuna, for instance, total HRC content reaches about 150 mM. A large amount of free L-histidine is also found in salmonids and cyprinids and is thought to have the same physiological function as histidine-containing dipeptides [4, 9]. Tunas also have the highest LDH activity (Table 2) found in any animals including mammals and they show very high capacities for anaerobic glycolysis. These observations clearly support the idea that high muscle buffering capacity is necessary to support the high speed, long duration, and increasing frequency of burst swimming typically seen in tuna fishes.

As seen in Table 2, ectothermic scombrids such as chub mackerel or Pacific bonito have lower β values and HRC contents than warm-bodied tuna [11]. Of the actively foraging pelagic ectotherms, non-scombrids such as sardine, rainbow trout, and several bass species have much lower β and HRC in their white muscle. Of these fish species, sardine *Sardinops* spp. contains 40 μmol/g of free L-histidine and rainbow trout contains about 20 μmol/g of anserine in white muscle, but other white-fleshed fishes have only trace amounts of HRC, even in their white muscle. Deep-sea and demersal fishes, with more sluggish locomotor activities, are found to have the lowest buffering capacities, HRC levels, and LDH activities [3, 8, 11].

The correlation between β values and LDH activities is shown in Fig. 1 for various fish species listed in Table 2. These two parameters are highly correlated (r = 0.83), indicating that buffering capacity is high in the white muscles having high glycolytic capacities and hence in species with high burst exercise capability [8, 11].

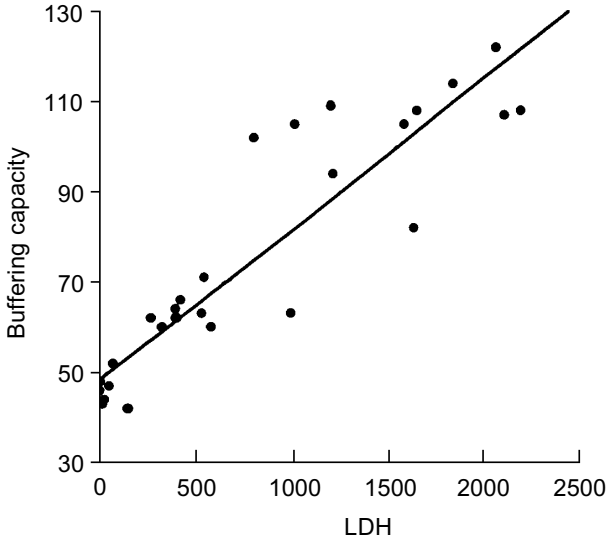

Fig. 1. Correlation between lactate dehydrogenase activity (units/g muscle) and buffering capacity (μmol NaOH per pH unit per g muscle, pH 6-7) in fish white muscle (data compiled from references [8, 11, 16]).

MARINE MAMMALS

Table 3 shows muscle β values, HRC contents, and LDH activities in several diving mammals and birds, as well as terrestrial animals for comparison. The muscles of these animals contain less than 1 μmol/g of free L-histidine but a rather large amount of one of the dipeptides. Whale muscle is unique because it contains a large amount of balenine, ranging from 20 to 80 μmol/g [14]. Whale typically shows β values as high as warm-bodied fishes. Marine diving mammals, which contain a rather large amount of carnosine in their skeletal muscle, have higher average β than terrestrial mammals [8]. LDH activity does not differ significantly between marine and terrestrial mammals, but correlates closely with β [8]. The β value also correlates strongly with muscle myoglobin concentration in these diving mammals [8]. Thus, HRC buffering may prevent a decrease in muscle pH during prolonged breath-hold diving, when oxygen stores are depleted and large amounts of lactate and protons produced.

TERRESTRIAL ANIMALS

Interspecies differences in β values and HRC contents are very large among terrestrial animals. As shown in Table 4, terrestrial mammals and birds have large amounts of carnosine or anserine, or even a small amount of balenine. Birds typically have a larger amount of anserine than carnosine, and total HRC contents are higher in white breast muscle of chicken and turkey than in red leg muscle. This distribution pattern of HRC is also found in deer muscle, which contains the highest amount of balenine in the terrestrial mammals thus far examined. Mammals containing higher anserine than carnosine are rather limited to some special species such

Table 3. Buffering capacity (β), concentration of histidine-related compounds (HRC), and lactate dehydrogenase (LDH) activity in the muscle of marine and terrestrial animals (according to [8, 15, 16])

Animals	β	HRC	LDH
Marine animals			
little-piked whale	111	68.5	—
spotter porpoise	84.1	—	1222
northern fur seal	79.1	—	1120
harbor seal	76.2	43.9	1379
Weddell seal	72.1	—	1270
sea lion	61.5	—	707
sea otter	70.6	—	801
Adelie penguin	70.0	—	2076
Terrestrial animals			
chicken (*pectoralis minor*)	82.8	43.5	—
pig (*psoas muscle*)	78.6	23.3	—
(*biceps femoris*)	63.2	15.4	615
ox (*biceps femoris*)	69.0	18.3	1016
rabbit	66.9	—	1887
dog	50.2	—	772

Note: See the note of Table 2 for units of β, HRC, and LDH activity; –, not determined.

as kangaroo, goat and sheep, rabbit, and deer which show hopping locomotion [4]. In contrast, carnosine content is higher in ox, pig, horse, and many other mammalian muscles. As seen in Table 3, buffering capacities of these terrestrial mammals and birds are as high as those of the active pelagic fishes shown in Table 2.

Rao and Gault [17] quantified the characteristics of bovine muscle for five white muscles abundant in fast fibers and seven red muscles abundant in slow fibers (Table 5). Carnosine content is significantly higher in white than in red muscle whereas no significant difference is seen in anserine content. In white muscle, inor-

Table 4. Concentration of histidine-related compounds in the muscle of terrestrial mammals and birds (according to [15])

Animals	Muscles	*n*	Carnosine	Anserine	Balenine
Ox	leg	5*	26.1 ± 3.7	5.94 ± 1.75	0.103 ± 0.026
Pig	leg	6*	29.5 ± 9.6	1.42 ± 0.29	1.77 ± 0.71
Horse	leg	3**	42.6 ± 12.6	0.176 ± 0.030	0.019 ± 0.004
Deer	leg	5**	3.35 ± 0.68	13.9 ± 1.93	3.91 ± 0.74
Chicken	leg	3*	5.70 ± 1.7	17.1 ± 3.7	0.055 ± 0.028
	breast	4*	10.4 ± 1.3	32.0 ± 1.4	0.197 ± 0.031
Turkey	leg	3**	4.53 ± 0.68	20.5 ± 1.9	0.077 ± 0.009
	breast	2**	11.2 ± 1.3	46.0 ± 0.8	0.810 ± 0.023

Note: Values are expressed as μmol/g wet weight (means ± SD).
* Average from the same muscle from different animals.
** Average from different muscles of one animal.

Add360

ganic phosphate is also significantly higher than in red muscle. After 48 h post-mortem, these muscles show an intracellular pH below 6, lower in white than in red. They exhibit no significant difference in buffering capacity from pH_i to 5 (Table 5), but significantly differ from each other in β from pH_i to more acidic pH values [17]. Thus, a strong correlation exists between carnosine and inorganic phosphate contents, and buffering capacity in bovine muscle.

Mammalian muscle is a mixture of three fiber types, slow-twitch oxidative red fiber (type I), fast-twitch oxidative-glycolytic white fiber (type IIA), and fast-twitch glycolytic white fiber (type IIB). Table 6 shows the HRC and taurine contents in the middle gluteal muscle of camel [18]. The carnosine and anserine contents of both type IIA and IIB fibers are significantly higher than those of type I fiber but there is no significant difference between type IIA and IIB. In contrast, taurine is much more concentrated in type I fiber in camel, as is the case in fish muscle; taurine content is much higher in fish red muscle than white and is thought to contribute to red muscle buffering [6].

Figure 2 represents muscle homogenate buffering and the major compounds contributing to buffering in the pH range of 6.5-7.5 [4, 16]. The relative contribution of contractile proteins to total buffering is a few percents, while soluble proteins contribute from 9 to 38%. The contribution of soluble proteins is rather high for dark-fleshed fishes containing a lot of myoglobin such as tuna and mackerel. The contributions of HRC in whale skeletal muscle, skipjack tuna white muscle, and marlin white muscle are as high as 25, 40, and 60%, respectively. Blue marlin, *Makaira nigricans*, contains 120 μmol per g of anserine in white muscle on average [4, 6]. The contribution of HRC is also high for bovine, porcine, and chicken muscle, ranging from 12 to 23%, but it is only 1 to 6% for carp and flounder white muscle that contain only a small amount of HRC. In contrast, the contribution of inorganic phosphate to total muscle β is high in the white muscle of trout, carp, and flounder, ranging from 50 to 80%. The concentration of inorganic phosphate is rather species independent and includes phosphate liberated from organic phosphates such as phosphocreatine and ATP. In the case of mammalian muscle, a rather high contribution is attributed to unknown compounds, which may include some nucleotides, organic acids, and taurine. These data indicate that the large variation in muscle β is, therefore, mainly due to changing levels of HRC. Thus, the accumulation of HRC in muscle increases the muscle β and, hence, the muscle anaerobic capability. This strategy is used by animals such as tunas, billfishes such as marlin, and marine mammals that show an elevated capacity for burst anaerobic swimming or for anoxia tolerance.

Table 5. Characteristics of bovine white and red muscle (according to [17])

Parameter	White muscles	Red muscles
White fiber**, %	58.0 ± 2.7	37.5 ± 3.6
Red fiber**, %	41.9 ± 2.7	59.8 ± 4.3
Carnosine*, g per 100 g wet weight	0.41 ± 0.06	0.29 ± 0.04
Anserine, g per 100 g wet weight	0.052 ± 0.018	0.043 ± 0.010
Inorganic phosphate*, g per 100 g wet weight	0.18 ± 0.02	0.15 ± 0.01
pH_i*	5.48 ± 0.06	5.89 ± 0.19
β (pH_i 5.0), μmol H^+ per g wet weight	46.4 ± 4.8	41.8 ± 5.1

Note: Fiber types were based on myosin ATPase staining method. pH_i means intracellular pH. Value of β (pH_i 5.0) means the buffering capacity (milliequivalents HCl per 100 g muscle required to lower the muscle pH from pH_i to 5.0). Values are means ± SD for five different white muscles and for seven different red muscles.
* $p < 0.01$.
** $p < 0.001$.

Table 6. Concentration of histidine-related compounds and taurine in the *middle gluteal* muscle of camel (according to [18])

Compound	Type I	Type IIA	Type IIB
Carnosine	23.6 ± 5.3	37.2 ± 8.6*	45.6 ± 8.0*
Anserine	28.4 ± 5.4	38.4 ± 9.8**	35.6 ± 6.4*
Total HRC	52.0 ± 8.6	75.5 ± 12.7*	81.2 ± 10.8*
Taurine	41.9 ± 10.1	24.1 ± 7.9*	23.3 ± 11.5*

Note: Values are means ± SD for four post-mortem muscles as mmol per kg dry weight. Type IIA and IIB are significantly different compared with type I muscle.
* $p < 0.05$.
** $p < 0.01$.

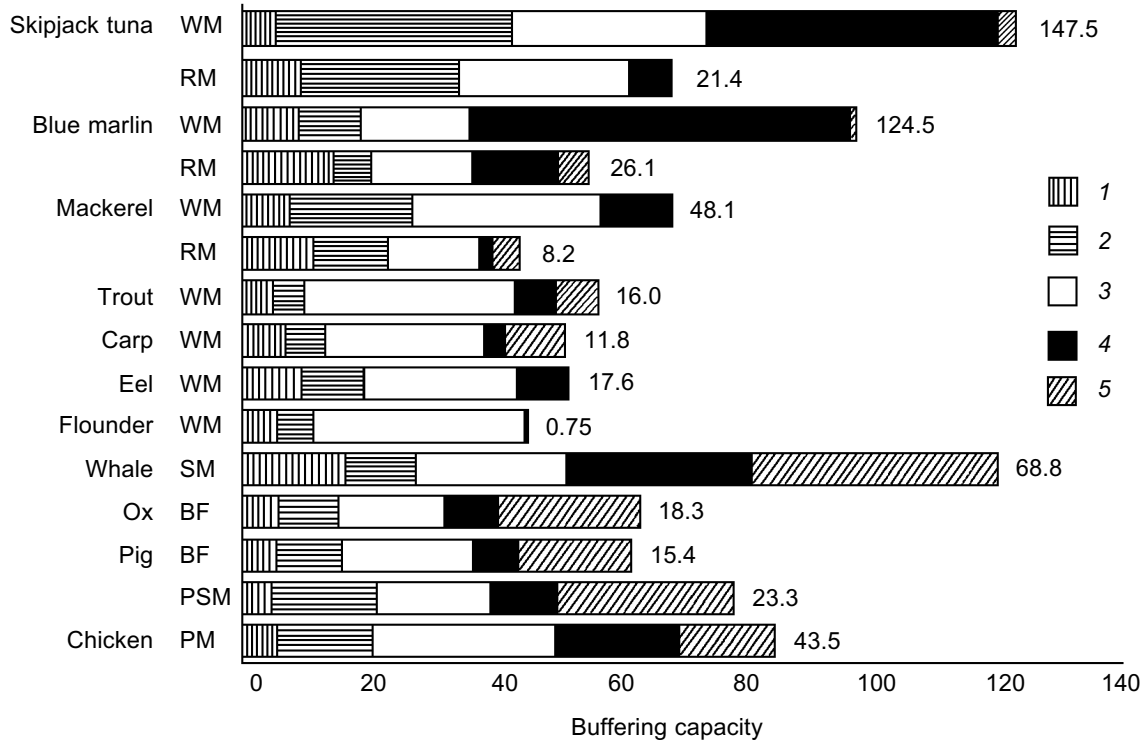

Fig. 2. Contribution of proteins (contractile (*1*) and soluble (*2*)), inorganic orthophosphate (*3*), histidine-related compounds (*4*), and unknown compounds (*5*) to the buffering capacity of muscle homogenate from several vertebrates. Buffering capacity is expressed as μmol NaOH per pH unit per g muscle over pH 6.5-7.5. Values indicated on the bars represent the concentration of total histidine-related compounds (μmol/g muscle). WM, white muscle; RM, red muscle; SM, skeletal muscle; BF, *biceps femoris*; PSM, psoas muscle; PM, *pectoralis minor* (from reference [4]).

MAMMALIAN SPRINTERS

The white muscle fibers (type IIA and IIB) are especially abundant in the muscle of outstanding mammalian sprinters such as thoroughbred horse (Table 7). The superficial portion of the middle gluteal muscle contains many type IIB fibers and the deep portion many type IIA and I fibers [19]. Estimated carnosine levels are over two times higher in type IIB than in IIA (Table 7). The contribution of carnosine to the total buffering

Table 7. Fiber types, carnosine content, and buffering capacity of the *middle gluteal* muscle of thoroughbred horse (according to [19])

Parameter	Type I	Type IIA	Type IIB
Fiber section area, %			
superficial (<4 cm, n = 10)	5.1 ± 4.2	28.9 ± 6.1	66.0 ± 7.8
deep (<9 cm, n = 10)	21.3 ± 6.0	46.0 ± 5.5	32.7 ± 7.9
Estimated carnosine, μmol per g dry weight	54 ± 15	85 ± 15	180 ± 15
β_{total}	88	98	130
$\beta_{carnosine}$	18	28	60
$\beta_{carnosine}/\beta_{total}$, %	20	29	46

Note: Buffering capacity (β) is microequivalent of H^+ per g muscle dry weight required to lower the pH from 7.1 to 6.5.

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Table 8. Effects of breed of horse on muscle fiber types and carnosine content (according to [20])

Parameter	Quarter Horses (n = 6)	Thoroughbreds (n = 6)	Standardbreds (n = 5)
Type I fiber, %	12.2 ± 1.1[a]	15.0 ± 1.8[b]	16.2 ± 0.6[b]
Type IIA fiber, %	49.0 ± 1.8[a]	61.0 ± 2.8[b]	60.0 ± 1.2[b]
Type IIB fiber, %	38.8 ± 1.0[a]	24.0 ± 1.8[b]	23.8 ± 1.2[b]
Carnosine, μmol per g wet weight	39.2 ± 1.8[a]	31.3 ± 2.9[b]	27.6 ± 0.6[b]

Note: Percentages of fibers are based on the staining negative for succinate dehydrogenase for fast twitch glycolytic fiber and for myosin ATPase for slow twitch oxidative fiber.
[a, b] Values in the same horizontal line with different superscript letters are significantly different ($p < 0.05$).

capacity in muscle is estimated to be 46% in type IIB fiber.

The muscle fiber type distribution and carnosine levels also differ between breeds of horses (Table 8). Of the middle gluteal muscle of Quarter Horses (sprinter), thoroughbreds, and standardbreds, type IIA fibers are lowest and type IIB are highest in Quarter Horses. Carnosine content is also the highest in Quarter Horses. From these results, we can conclude that horses selected for sprinting have a higher percentage of fast-twitch glycolytic fibers and require more muscle buffering capacity [20].

As shown in Table 9, the β value is also high in the muscles of another sprinter, the greyhound dog, compared with human muscle [21]. Significant differences in β_{total} exist between man, horse, and dog ($p < 0.001$). Total HRC is also high in greyhound as well as thoroughbred horse. The contribution of HRC to β_{total} reaches the high value of 25% in greyhound dogs.

HUMANS

As seen in Table 9, human muscle contains a rather small amount of HRC, which contributes little to β_{total}. The carnosine level in the *quadriceps femoris* muscle of human volunteers is significantly higher ($p < 0.05$) in male than in female subjects of similar age and training status (Table 10), although large inter-individual differences are found in both sexes [22]. As shown in Table 11, the carnosine level is twice as high in type II fibers of human *vastus lateralis* muscle compared with type I [23]. In contrast, the taurine level is four times higher in type I than type II muscle fibers as described earlier in camel and fish. This is also true in thoroughbred horses [24], where the contribution of carnosine to muscle β_{total} is 9.4% in type II but only 4.5% in type I fibers.

Parkhouse et al. [25] characterized the effects of exercise training on muscle fiber types, HRC levels, and β values in human *vastus lateralis* muscle (Table 12). Four groups of five subjects were compared: highly trained 800-m sprinters, rowers (varsity oarsmen), endurance trained marathon runners, and untrained controls. The marathon runners were significantly ($p < 0.05$) older than the other groups. Body weight and fat

Table 9. Dipeptide content and buffering capacity in the muscle of thoroughbred horse, greyhound dog, and human (according to [21])

Parameter	Horse*	Dog**	Human***
Carnosine, μmol per g dry weight	108.3 ± 15.9	33.0 ± 19.1	16.0 ± 7.2
Anserine, μmol per g dry weight	n.d.	48.6 ± 18.4	n.d.
β_{total}	117.7 ± 8.5	105.2 ± 9.1	79.5 ± 8.0
$\beta_{dipeptide}$	36.0 ± 5.3	26.0 ± 10.1	5.3 ± 2.4
$\beta_{dipeptide}/\beta_{total}$, %	30.6	24.7	6.7

Note: See the legend of Table 7 for buffering capacity; n.d., not detected.
* *Middle gluteal* muscle (n = 20).
** Mean ± SD for five different muscles in four dogs.
*** *Vastus lateralis* muscle (n = 20).

Table 10. Carnosine content in the *quadriceps femoris* muscle of male and female humans (according to [22])

Parameter	Male (n = 33)	Female (n = 17)	All subjects (n = 50)
Carnosine, μmol per g dry weight			
means ± SD	21.3 ± 4.2	17.5 ± 4.8	20.0 ± 4.7
range	12.5-30.7	7.2-27.7	7.2-30.7
Age, years	22.5 ± 3.4	21.7 ± 4.0	22.4 ± 3.8
Body mass, kg	78.0 ± 11.0	65.1 ± 7.7	73.6 ± 11.7

Note: Means ± SD of carnosine content are significantly different between male and female ($p < 0.05$).

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Table 11. Buffering capacity and carnosine and taurine contents in the human *vastus lateralis* muscle (n = 4) (according to [23])

Parameter	Type I	Type II
Taurine, μmol per g dry weight	39.2 ± 17.8	9.6 ± 2.6
Carnosine, μmol per g dry weight	10.5 ± 7.6	23.2 ± 8.1
β_{total}	77.5	81.7
$\beta_{carnosine}$	3.5	7.7
$\beta_{carnosine}/\beta_{total}$, %	4.5	9.4

Note: See the legend of Table 7 for buffering capacity.

reserves were also significantly higher in untrained controls than in the other groups (data not shown). With respect to anaerobic speed test (high-intensity running performance), the sprinters performed significantly better than the rowers who also performed significantly better than the marathoners and untrained controls. Post-exercise blood lactate differed significantly between the sprinters, rowers, and the other two groups. Significant differences in the fast-twitch percentage were found between the groups ($p < 0.05$). Muscle buffering capacity was significantly higher in the sprinters and rowers than in the marathoners and untrained subjects. Results show no significant difference in histidine levels, but carnosine levels are significantly elevated in the sprinters and rowers. Significant ($p < 0.05$) correlations are found between β and carnosine levels ($r = 0.69$), and β and fast-twitch percentage ($r = 0.51$). Overall, these data suggest that repetitive high-intensity anaerobic exercise causes muscle adaptations that include an increase in percent fast-twitch fibers and muscle β. The observed increase in carnosine levels may contribute, at least partly, to the increase of muscle β values.

Since the early works of Bate-Smith [7] and Davey [26], the importance of non-bicarbonate intracellular buffering of vertebrate muscle has been demonstrated. In this review, I show that the buffering capacity is typically high in the fast-twitch glycolytic and anaerobic white muscle of vertebrates adapted for anaerobic performance such as burst swimming, prolonged breath-hold diving, flight, sprint running, and intense hopping locomotion. These activities are especially necessary for animals dwelling in the open oceans, grassy plains, or in the sky where they allow them to escape predators or to catch their prey. Imidazole buffer systems appear to have evolved in the muscles of vertebrates requiring high burst exercise capabilities or high anoxia tolerance for their survival. The fact that buffering capacity, HRC content, and percent fast-twitch fibers are highly correlated for a wide variety of vertebrate muscles supports the idea that the main physiological function of HRC is proton buffering. Inorganic phosphate, histidine residues in proteins, and organic phosphate compounds also contribute to proton buffering. However, the cellular concentration of these other buffering components

Table 12. Buffering capacity and histidine-related compounds in human *vastus lateralis* muscle with special reference to different exercise training (according to [25])

Groups	Age, years	Anaerobic speed test, sec	Post-load blood lactate, mM	Fast twitch, %	β, μmol per g per pH unit	Histidine, μmol per g	Carnosine, μmol per g
Sprinters (n = 5)	20.6 ± 2.3	115 ± 18**	21.9 ± 1.5**	56.6 ± 7.0	30.03 ± 5.6*	0.64 ± 0.06	4.93 ± 0.76*
Rowers (n = 5)	20.6 ± 1.8	76 ± 9*	13.9 ± 0.9*	50.4 ± 12.3	31.74 ± 7.2*	0.71 ± 0.10	5.04 ± 0.72*
Marathoners (n = 5)	37.8 ± 9.3	53 ± 15	10.1 ± 3.1	33.0 ± 12.2	20.83 ± 4.4	0.63 ± 0.14	2.80 ± 0.74
Untrained (n = 5)	22.6 ± 0.9	38 ± 9	10.1 ± 2.6	50.6 ± 9.9	21.25 ± 5.0	0.89 ± 0.29	3.75 ± 0.86

Note: Values are means ± SD. Buffering capacity (β) is μmol H^+ per g muscle wet weight required to change the pH by one unit over the pH range 7.0-6.0.
* Significantly higher ($p < 0.01$) than in marathoners and untrained.
** Significantly higher ($p < 0.01$) than in all other groups.

may be constrained because their primary physiological roles have nothing to do with buffering. In contrast, HRC, and especially the metabolically inert dipeptides among them, can be stored in large amounts without harmful side effect to the cell. Finally, combining varying amounts of different HRC constituents, each with their own p*K* values (ranging from 6.21 to 7.15, see Table 1) may provide the necessary flexibility to cope with the particular physiological challenges of each vertebrate species.

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CURRENT CONCEPTS OF ACID-BASE MEASUREMENT

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CURRENT CONCEPTS OF ACID-BASE MEASUREMENT*

Consulting Editor
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Honorary Conference Chairmen
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CONTENTS

PROTEINS AS BUFFERS

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It was a strange choice to ask me, who has not contributed to the description of the titration behavior of proteins, to discuss that subject. Nevertheless I am pleased to attempt to summarize the state of knowledge for our present purposes. Our best objective in this monograph is surely to make ourselves and each other better informed; we will be examining the meaning of terms for that purpose and not, I hope, to attempt to legislate their use. In that spirit the present assignment certainly promises to be informative to me.

Buffering refers to the minimization of *p*H change on the addition of acid or alkali. We evaluate the extent of buffering by seeing how much acid or alkali is required to change the *p*H; that is, by titrating. The steepness of the titration curve at any point yields the buffer value at that *p*H. About one-third of the amino acid residues in proteins contribute titratable groups; the number of such groups in many common proteins ranges from about 20 to over 200. The common groups include the carboxyl groups, contributed by the sidechains of glutamic and aspartic acid; the amino groups on lysine sidechains; the guanidinium groups contributed by arginine sidechains; the imidazolium groups of histidyl residues; the phenolic groups contributed by tyrosyl residues; and the sulfhydryl groups of cysteine residues. Hemoglobin contains also the propionate sidechains of heme, and an acidic water molecule on each iron atom. In addition, nearly all proteins contain terminal α-amino and α-carboxyl groups on their polypeptide chains.

FIGURE 1 shows a titration curve obtained for β-lactoglobulin by Nozaki.[1] The ordinate label illustrates that the titratable groups may be counted from any desired reference point. The figure shows how the titration curve may be divided into regions; in this case the titration has been terminated before the guanidinium groups were deprotonated, or we should see a fourth region. The groups titrating in the acid range may tentatively be identified as carboxyl groups, those in the middle range as imidazole and terminal amino groups, and those titrating between *p*H 8.5 and 11.5 as sidechain amino, phenolic and sulfhydryl groups. Independent determinations of the number of terminal carboxyl and amino groups permit subtraction of these quantities from the first and second titration regions, respectively, where each is likely to be titrated, to give us a presumptive count of sidechain carboxyl and imidazole groups.

We may then determine how many phenolic groups have been titrated in the upper range, by performing a separate spectro-photometric titration; that is by observing the location and extent of a spectral change at 295 mμ

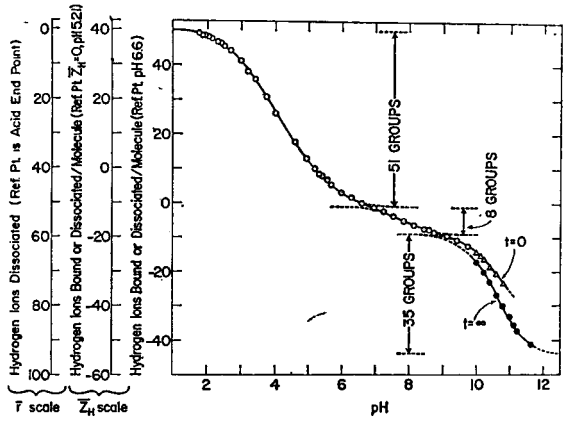

FIGURE 1. Titration curve of β-lactoglobulin at ionic strength 0.15 and 2.50. See text for discussion. From the data of Nozaki, cited by Tanford. 1962. *Advances in Protein Chemistry* **17:** 69 with permission.

characteristic of the deprotonation of each such group. We may also refine our division of our titration curve among the contributing groups by performing the titration at two different temperatures (FIGURE 2). As Jeffries Wyman and his associates showed,[2] this step will have characteristically little effect on the *p*H at which carboxyl groups titrate; but a characteristic shift, corresponding to a characteristic heat of dissociation, will occur at the point where the titration of the carboxyl groups is nearly completed and that of imidazole

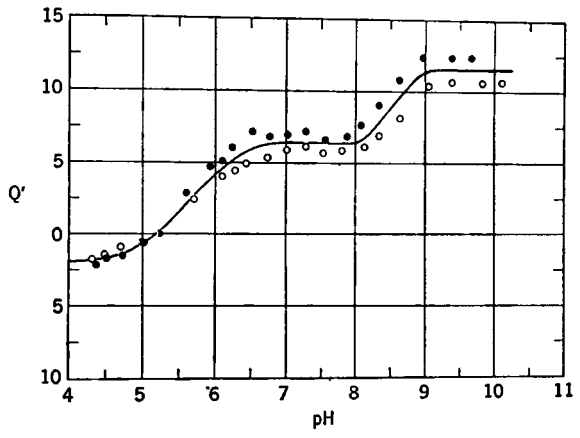

FIGURE 2. Apparent heat of dissociation of oxyhemoglobin as a function of *p*H. From Jeffries Wyman, 1939. *J. Biol. Chem.* **127:** 1 with permission.

groups becomes dominant. The step to a higher heat of dissociation as the amino groups begin to be titrated is not a clearcut one, unfortunately, because a distinction between imidazole, phenolic and sulfhydryl groups is not possible on this basis; nor that between terminal α-amino groups and the sidechain amino groups. An additional method that has been used for discriminating some of the nitrogenous groups from tyrosyl and sulfhydryl groups is to observe the effect of the presence of an excess of formaldehyde on the titration curve. Titrations may also be made in the presence of a chelating cation to assist in identifying dissociating groups.

Although the results obtained by these methods correspond rather well to the predictions from the known amino acid analysis of the protein, there are important deviations from the predictions. One such deviation is seen in FIGURE 1. Above *p*H 9.75, the *p*H values observed immediately on the addition of alkali are substantially higher than those observed when time is permitted for the *p*H to stabilize. If the titration is repeated with protein denatured at *p*H 12.5, the curve in this region becomes stable and reversible.

This phenomenon could arise from the gradual release from a "buried" position in an internal, hydrophobic region, either of uncharged phenolic or sulfhydryl sidechains, or of the unprotonated, apolar form of the sidechain of histidine. It is especially common that phenolic groups cannot be titrated as long as the native conformation of a protein is retained. All of the phenolic groups in ovalbumin, and half those in ribonuclease and chymotrypsinogen, for example, are not titrated at all in the normal position. Alternatively, but less probably, the phenolic groups might be hydrogen-bonded to some other structure. The titration behavior of native β-lactoglobulin in this region has not been entirely rationalized.

For hemoglobin, about half (16–22) of the imidazole groups do not titrate in the normal range, but are only gradually titrated when the *p*H is brought below 4.5. FIGURE 3 shows the results when the cyanide complex of ferrihemoglobin was titrated by Jacinto Steinhardt and his associates.[3] This complex is far more stable to acid denaturation than oxyhemoglobin, and by quick measurements on flowing solutions (lower curve) a considerable discrepancy in the number of groups titrated in the native and denatured (upper curve) forms could be observed. But what this figure shows is that this discrepancy develops in the neutral region, not in the acidic region. Apparently about 22 imidazole groups are restrained in their uncharged state in the native protein and cannot be titrated until denaturation occurs. Hence for the native protein their titration is an accompaniment of denaturation.

The mode of restraint of four of these imidazole groups is presumed to be well understood. Each of these four is bonded to an iron atom of a heme group; and we cannot expect them to be protonated until the hydrogen ion concentration is high enough to displace the iron. The remaining inaccessible imidazole groups must be restrained in some different way.

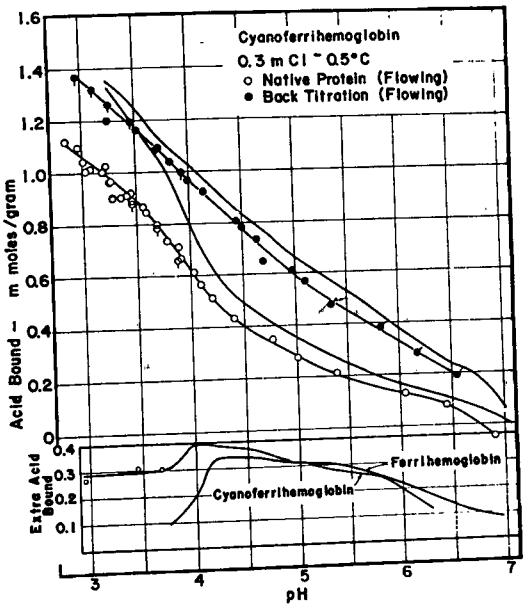

FIGURE 3. Titration data for native and denatured (back titration) carbonyl hemoglobin at 0.5° in the presence of 0.3 *M* chloride and with air excluded. Lower curves, differences in acid bound. From Steinhardt, Ona & Beychok. 1962. *Biochemistry* **1**: 29 with permission.

In addition to the possibility that a group will not titrate in the native protein until the native conformation is altered, either because the uncharged form of the group is buried or because one form of the group is bonded to another structure, we have an important factor of *electrostatic effects* in altering titration behavior. This factor can also be modified by conformational changes. Each dissociation reaction occurs in an environment containing many other charged groups. Furthermore the numbers of these groups is changed by the titration itself. In general electrostatic effects tend to stablize the charged form of a dissociating group. Thus the intrinsic *p*K of carboxyl groups is moved downward, and that of amino groups upward. In addition the titration curve tends to be made less steep. These effects can be minimized by raising the ionic strength. Linderstrøm-Lang has treated the matter quantitatively under the simplifying assumption that the charges are spread on the surface of a spherical molecule not penetrated by the solvent. If the solvent is allowed to penetrate the sphere, the dissociating group needs to lie only a short distance inside the molecular surface to have the electrostatic effect greatly accentuated (Tanford).[4] Under the generalized conditions electrostatic effects would occur on all dissociating groups and should not

cause positional anomalies for the titration of only certain specific groups. If, however, we consider that a dissociating group might emerge from or penetrate into a region with a high concentration of charged groups, then the charging electrostatic interactions might cause an unusually large shift in the acid strength of that dissociating group.

For our purposes in this monograph, the most significant deviation from simple titration behavior is that presented by the Bohr effect of hemoglobin. It has been established by several independent methods during the past dozen years, as summarized recently by Reinhold and Ruth Benesch,[5] that hemoglobin undergoes a large, reversible change in conformation when it is oxygenated and deoxygenated. As a result of this change in conformation, its reactivity with many substances, in addition to the hydrogen ion, is strongly modified. Conversely the addition of the hydrogen ion to certain groups, and its removal from them, must be assumed to cause essentially the same reversible conformational change, as can be observed by measuring in turn the affinity for oxygen.

You will recall that Jeffries Wyman showed that the shift in the titration curve on oxygenation of horse hemoglobin could be accounted for by the fall in the pK' of one imidazole group per Hb subunit from 7.93 to 6.68, and by a smaller rise in the pK' of a much more acidic imidazole group. Recently a rather similar downward shift has been calculated for human hemoglobin, from 8.25 to 6.95.[6] These four imidazole groups presumably occur one in each of the four peptide chains. The α and β chains are now of course known not to be identical, and it would be strange if the four imidazole groups had exactly the same intrinsic pK' values. Although an abnormal hemoglobin (hemoglobin H) containing four β chains fails to show any Bohr effect,[7] this result does not necessarily indicate that the oxygen-sensitive imidazole groups are restricted to the α-chains. Rather, it must be the relation established between the chains that is decisive to the Bohr effect.

We must suppose that these oxygenation-sensitive imidazole groups find themselves in a new environment when the reversible conformational change occurs. It is interesting that ionizable dye residues introduced into the hemoglobin molecule at some distance from the heme groups also show abnormally high pK' values in the deoxygenated state, as Irving Klotz and Luisa Tosi showed.[8] The new comparison with x-ray diffraction by Perutz and associates of crystalline reduced and crystalline oxygenated horse hemoglobin, each with two mercury atoms attached to the reactive sulfhydryl groups as markers (FIGURE 4), shows that on reduction the distance between two mercury atoms is increased from 30.0 to 37.3 angstroms.[9] This increase in separation occurs almost entirely in the axis describing the separation of the two β chains, which appear to be completely out of contact in the reduced form. That being the case, one cannot suppose that the change in this distance is caused by an interaction between the two β chains.[10]

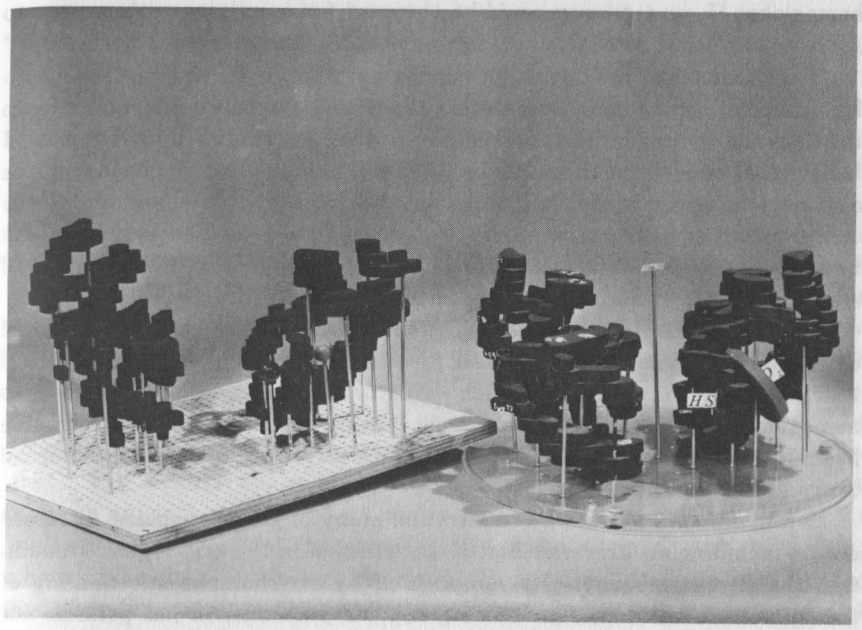

FIGURE 4. View of the two pairs of β-chains, showing the widening of the gap between them in the human reduced form *(left)*, as compared with horse oxyhemoglobin *(right)*. From Muirhead & Perutz. 1964. Nature **199:** 633 with permission.

In that connection it is interesting that results of Eraldo Antonini and his associates at The University of Rome and the Regina Elena Cancer Research Institute in Rome, appear to require that the half molecule, $\alpha\beta$, be intact for a Bohr effect; there is, however, no discontinuity in the change in magnitude of the Bohr effect as the ionic strength attains high enough levels to cause dissociation of hemoglobin to the half molecule.[11] Furthermore, the abnormal hemoglobin H containing four β chains, which shows the shape of a rectangular hyperbola for its oxygen dissociation curve, and no Bohr effect, suffers no change in the separation of the mercury atom markers on deoxygenation.[12]

One of the most interesting observations of the reversible structural change of hemoglobin is that made by Quentin Gibson,[13] who showed that carbon monoxide-hemoglobin, dissociated by flash protolysis, is able for a short interval of time to react with oxygen or carbon monoxide at a much higher rate than ordinary deoxyhemoglobin. After the CO has been removed a measurable interval is required for the usual change in conformation to occur to the more stable form of deoxyhemoglobin. Again, in the case of

hemoglobin H, no tendency could be observed for reactivity with oxygen to decrease for a time after flash protolysis of its carbon monoxide derivative.

The character of the change in the environment of the sensitive dissociating groups of hemoglobin, and the way in which this environmental change modifies the hydrogen-ion dissociation so sharply, remains undetermined. I believe that none of the three factors discussed above, namely a change in the electrostatic environment, a change in the hydrophobic character of the environment, or a change in specific bonding of these imidazole groups, can be entirely excluded at this time. Nevertheless, in the present intense phase of study, the effect seems likely soon to be largely explained.

The Bohr effect is in any case the first observed and best documented case in which structural change of a protein has been linked with biological function. The importance of such structural changes appears likely, as hemoglobin illustrates, to extend well beyond the subjects of contractile behavior and biological transport, but probably also to the physiological control of enzymatic reactivity.

Serum albumin also shows on titration many of the phenomena discussed above, including an irreversibility of the titration in the acid region. In addition the titration behavior is complicated by its characteristic binding of the anion of the acid used in titrating it. This phenomenon has perhaps not been studied as much as it deserves to be, for its relation to the subject of CO_2 transport and the acid-base balance.

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[84] Examination of Titration Behavior

By YASUHIKO NOZAKI and CHARLES TANFORD

I. Introduction

It is believed that all enzymes belong to the class of globular proteins. Not a single case to the contrary is known. The chemical structure of such protein molecules is, as far as titration is concerned, a number of dissociable side-chain groups attached to a peptide chain, in addition to an amino group residing at one end and a carboxyl group at the other end of the chain. The titration behavior of a protein therefore will be similar to that of a mixture of the constituent amino acids with their α-amino and α-carboxyl groups blocked, except for the terminal amino acids.

The information obtained from titration curves of enzymes based on this fact is therefore of an analytical nature. There is, however, an important difference between the titration of a protein and the corresponding mixture of blocked amino acids. Deviation of the former from the latter tells us the nature and magnitude of interactions affecting the titration; therefore, information about the conformation of protein molecules is obtained. A comprehensive review of the subject has recently been published,[1] to which the reader is referred for detailed information to supplement the brief remarks in this section.

Electrometric Titration Curve. An electrometric (direct) titration curve, strictly speaking an electrometric hydrogen-ion titration curve, represents the relation between pH and the number of moles of protons bound by, or removed from, 1 mole of protein in the reaction,

$$PH_i + rH^+ \rightleftharpoons PH_{i+r} \quad \text{or} \quad PH_i - rH^+ \rightleftharpoons PH_{i-r} \qquad (1)$$

where PH_i represents a protein species at the beginning of the titration, and r is the number of hydrogen ions bound by, or removed from, the protein. Experimental r values are obtained as the difference between the number of moles of strong acid or base added to a solution containing 1 mole of protein to bring its pH from initial to final values, and the number of moles of strong acid or base added to the solvent only to bring about the same pH change with other conditions (ionic strength, volume, etc.) being the same as for the protein solution. In practice, the number of moles of free hydrogen or hydroxyl ions is calculated from the pH values measured in the presence of protein, rather than being determined

[1] C. Tanford, *Advan. Protein Chem.* **17**, 69 (1962).

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each time from measurements on a control solution without protein. The procedure is based on the following assumptions:

(1) Hydrogen ion activity can be known with sufficient accuracy from pH measurement,[2] with the aid of the assumption of single ion activity.[3-5] It means that we can identify the experimental pH with the negative logarithm of hydrogen ion activity, a_H, which in turn can be considered the product of hydrogen ion concentration, C_H, and an activity coefficient, γ'_H. Thus,

$$\mathrm{pH} = \mathrm{p}a_H = \mathrm{p}C_H + \mathrm{p}\gamma'_H \tag{2}$$

where p in each symbol stands for the negative logarithm.

(2) We maintain the idea of a constant ion product, K_w, for the dissociation of water, and extend the idea of single ion activity to hydroxyl ions, so that we may write:

$$\mathrm{pOH} = \mathrm{p}K_w - \mathrm{pH} = \mathrm{p}C_{OH} + \mathrm{p}\gamma'_{OH} \tag{3}$$

A prime mark accompanies both activity coefficients to indicate that they are apparent activity coefficients, since the pH as defined by the experimental measurement, and therefore pOH also, is somewhat different from $\mathrm{p}a_H$ or $\mathrm{p}a_{OH}$ defined by more rigorous procedures.[3]

(3) The apparent activity coefficients remain the same, at constant ionic strength, in the presence and absence of protein in the solution.

In the calculation of ionic strength we take into account the concentrations of added salt and acid or base, ignoring the possibility that some of the added ions are bound by the protein. It is found that $\mathrm{p}\gamma'_H$ and $\mathrm{p}\gamma'_{OH}$ values are not affected very much by the equipment used, including the kind of glass electrode, nor by technique, such as stirring or static titration. They remain constant within a narrow range to very low and high pH. Typical values of $\mathrm{p}\gamma'_H$ and $\mathrm{p}\gamma'_{OH}$ for KCl solutions are listed in Table I.

Apparent activity coefficients obtained by titrating guanidine hydrochloride solutions are also known to depend only on the concentration of guanidine hydrochloride (GuCl) but not on pH.[6] The values at several GuCl concentrations are listed in Table II for comparison with those values listed in Table I.

The number of moles of bound hydrogen ions per mole of protein,

[2] C. Tanford, *in* "Electrochemistry in Biology and Medicine" (T. Shedlovsky, ed.), p. 251. Wiley, New York, 1955.
[3] R. G. Bates, "Electrometric pH Determinations," Wiley, New York, 1954.
[4] R. G. Bates and E. A. Guggenheim, *Pure Appl. Chem.* **1**, 166 (1960).
[5] H. S. Frank, *J. Phys. Chem.* **67**, 1554 (1963).
[6] Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967).

TABLE I
APPARENT ACTIVITY COEFFICIENTS OF HYDROGEN AND HYDROXYL IONS IN KCl SOLUTIONS AT 25°

Ionic strength (KCl-HCl or KCl-KOH)	$p\gamma'_H$	$p\gamma'_{OH}$
0.01	0.054	0.056
0.1	0.076[a]	0.116
0.15	0.073	0.143
0.25	0.074	0.143
1.0	0.022	0.162

[a] The pH value assigned to 0.01 *M* HCl–0.09 *M* KCl by Bates is 2.073; this would give a $p\gamma'_H$ value of 0.073 [R. G. Bates, *J. Res. Natl. Bur. Std.* **45**, 418 (1950)].

calculated with the aid of such activity coefficients, is plotted against pH. Typical titration curves are shown in Fig. 1. The $\bar{r}$ scale shows the number of moles of hydrogen ions dissociated from 1 mole of protein in its most acidic form, i.e., where all dissociable groups are present in the acidic form, and the protein molecule carries the maximum possible number of bound hydrogen ions.

Starting from the zero point on the $\bar{r}$ scale (i.e., the point of maximum net proton charge), as an amount of alkali is added the first groups which lose their hydrogen ions are those with lowest p*K*, presumably

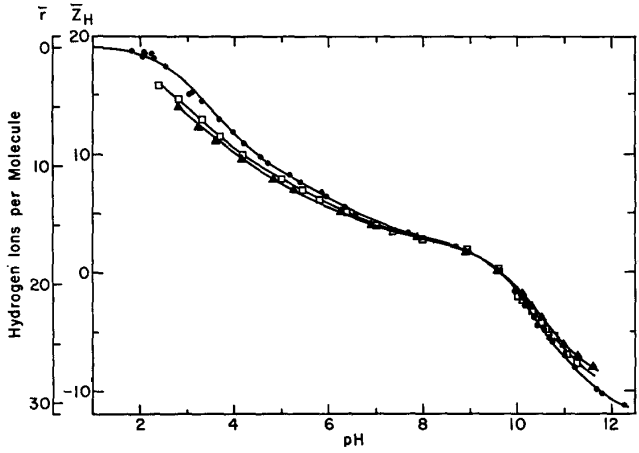

FIG. 1. Titration curves of ribonuclease in KCl solutions at 25° [C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.* **78**, 5287 (1956)]. Ionic strengths are 0.15 (●), 0.03 (□), and 0.01 (▲).

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TABLE II
APPARENT ACTIVITY COEFFICIENTS OF HYDROGEN AND HYDROXYL IONS IN GuCl SOLUTIONS AT 25°

GuCl concentration (mole/liter)	$p\gamma'_H$	$p\gamma'_{OH}$
4.0	(−0.34)	—
4.5	(−0.41)	—
5.0	−0.495	0.975[a]
5.5	−0.592	1.011[a]
6.0	−0.686	1.046[a]

[a] The large positive values reflect hydrolysis of GuCl with formation of the free base.

carboxyl groups,[7] and the net proton charge on the protein molecule decreases as a result of the negative charges introduced thereby. As more alkali is added and the pH rises, the more basic groups, among which the imidazole groups of histidine will usually be the first, will lose their hydrogen ions and their positive charges. Both effects are to reduce the number of proton charges and somewhere along the curve there will be a pH at which the protein molecules carry, on the average, zero net proton charge. This is the zero point on the $\bar{Z}_H$ scale. It is noted that the number of moles of protons bound from this point to the point of maximum net proton charge ($\bar{r} = 0$) is equal to the number of all basic groups (maximum acid-binding capacity, ΣN^+) on the protein molecule. The $\bar{Z}_H$ scale shows the number of protons bound by or removed from 1 mole of protein originally at the point of zero net proton charge. The pH at zero net proton charge ($\bar{Z}_H = 0$) is ordinarily close to the isoionic point of the protein.

Isoionic Point. The definition of the isoionic point adopted in this chapter is an operational one as follows: the pH of a solution which contains solvent water and the protein, free from ions other than hydrogen and hydroxyl ions. Such a solution can be prepared easily by the method described in Section IV below. The isoionic point is not a unique function but may depend on the protein concentration. $\bar{Z}_H$ at the isoionic point is usually close to zero, but may differ from zero if the pH is far from neutrality. Deviation from zero in that case simply reflects the fact that the protein has to acquire a net charge in order to compensate the charges of opposite sign due to excess H^+ or OH^- ions. In any event, $\bar{Z}_H$ at the isoionic point can always be calculated from the relation

$$\bar{Z}_H = (C_{OH} - C_H)/C_P \quad (4)$$

where C_{OH}, C_H, and C_P are the concentrations of free hydroxyl ions, hy-

[7] Dissociating groups such as, say, carboxyl or imidazole cannot be positively identified without supporting evidence. See the argument in footnote 1 (p. 71).

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drogen ions, and protein, respectively. (We may set pH = $-\log C_H$, and pOH = $-\log C_{OH}$, if the total ion concentration is low enough to permit equating activity coefficient to unity.) The difference between the isoionic point and the pH where $\bar{Z}_H$ is actually zero can then be measured from the titration curve directly. An unequivocal reference point of $\bar{Z}_H = 0$ can thus always be defined.

In practice a neutral salt is added to an isoionic protein solution to make up the ionic strength. It usually displaces the isoionic point slightly in a manner described by Scatchard and Black.[8] Whenever large pH displacement occurs, it is customary to seek its interpretation in ion binding. However, it should be added that Eq. (4) is valid even in the presence of added neutral salt, whether its ions are bound by the protein or not, and $\bar{Z}_H$ can be calculated by Eq. (4) at any ionic strength.

Spectrophotometric Titration of Tyrosine Groups. It is desirable to have methods that measure the titration of specific groups, in addition to the electrometric titration so far described, which gives the total number of groups titrating. Titration of tyrosine groups by the change in absorption spectrum due to the dissociation of the phenolic hydroxyl groups is the only such method of discriminating titration in common use.[9] In Fig. 2 are shown the spectrophotometric titrations of tyrosine phenolic groups of ribonuclease carried out under different conditions.

Group Counting. It is assumed that pK's of titratable groups on protein molecules are not completely different from pK's of similar groups on small molecules in a similar environment, especially with respect to electrostatic effects of neighboring groups. Those effects and eventually the assignment of reasonable pK values to the titratable groups on proteins have been the subject of many investigations. Nevertheless, we are still in an awkward position as to the assignment of accurate "normal" pK values to various titratable groups on a protein molecule. There are two approaches to the problem. One is to examine the pK values of model compounds that contain the same titratable groups as proteins do, and then to correct them for statistical factors and interactions, in order to evaluate an unperturbed or intrinsic pK (pK^{int}) for each kind of group. The second approach is to average pK's extracted from protein titration curves, after correcting for electrostatic interactions, and to assume that deviations from "normal" behavior are eliminated by the averaging process (i.e., it is assumed that positive and negative deviations occur with equal frequency). A list of values obtained in both ways is given in Table III.[10]

[8] G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.* **53**, 88 (1949).

[9] For recent progress in other methods, see footnote 1 (p. 80).

[10] Footnote 1, Table I (pp. 73–74), Table V (pp. 112–113). Readers are also referred to J. T. Edsall *in* "Proteins, Amino Acids and Peptides" (E. J. Cohn and J. T.

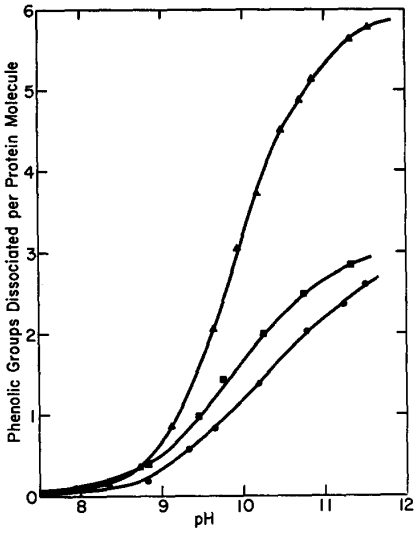

FIG. 2. Spectrophotometric titration of phenolic hydroxyl groups of ribonuclease at 25° [Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967); also see C. Tanford, J. D. Hauenstein, and D. G. Rands, *J. Am. Chem. Soc.* **77**, 6409 (1955)]. Conditions: KCl, ionic strength, 0.01 (●); KCl, ionic strength, 1.0 (■); 6 *M* GuCl (▲). Only the reversible portion of the data in KCl is shown.

It can be seen from Fig. 1 that the titration curve of a protein can usually be divided into three sigmoid regions, the transitions between them being at about pH 6 and pH 9. We may tentatively identify these regions as corresponding to the titration of (1) carboxyl groups, (2) imidazole plus α-amino groups, and (3) phenolic hydroxyl plus ϵ-amino groups. Thiol groups of cysteine residues, if they titrate, will be included in the last region. Guanidine groups of arginine are not titrated because of their high pK, although they are included in ΣN^+.

The numbers of groups in each region of the titration curve can be obtained by direct counting. Because of overlap, the results are not very accurate but are useful for the first analysis of the curve, together with a knowledge of ΣN^+ if the isoionic point is known. We usually start from the acid end of the curve at, say, pH 2, where all carboxyl, thiol, and phenolic hydroxyl groups are uncharged, and all basic groups have

Edsall, eds.), Chap. 4, 5, 20. Reinhold, New York, 1943; see also J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Chap. 8, 9. Academic Press, New York, 1958.

TABLE III
INTRINSIC pK's OF TITRATABLE GROUPS AT 25°

Type of group	Model compounds[a]	Proteins[b]	
		Dilute salt solution	GuCl 6 M
α-Carboxyl	3.8	3.6	3.1
Aspartyl carboxyl	4.0	(4.6)[c]	3.9
Glutamyl carboxyl	4.4	(4.6)[c]	4.35
Imidazole	6.3	6.6	6.4
α-Amino	7.5	[d]	7.4
Thiol	9.5	[d]	9.5
Phenolic	9.6	9.8	9.95
ϵ-Amino	10.4	10.0	10.3
Guanidyl	12	12	12

[a] Obtained from pK values of small model compounds (see reference, footnote a, Table I). Corrections for electrostatic and other effects have been made to yield pK values representative of the unperturbed group.

[b] Obtained from the titration of proteins, by application of Eq. (9). Values obtained for different proteins differ considerably, and the values listed represent somewhat arbitrary choices.

[c] Protein titration curves in dilute salt solutions have heretofore been analyzed without regard for the anticipated difference between aspartyl and glutamyl COOH groups.

[d] No reliable data have been obtained.

protonic charges. As stated previously, the first groups to titrate are carboxyls, up to about pH 6. The number should be the same as the total number of aspartic and glutamic acid residues of the protein molecule minus amide groups plus α-carboxyl groups. In the neutral region we find the number of histidine residues plus the number of α-amino groups. The number of groups titrated in the alkaline region is of least accuracy, because the alkaline end of the titration curve usually does not level off as the acid end does. The reason for this is that the titration of amino groups extends to well above the highest pH (pH 12) which can yield reliable data. However, the number of groups titrated in this region can be obtained with the aid of a short extrapolation. It should be equal to the sum of the numbers of tyrosine and lysine residues. The former can be obtained from spectrophotometric titration, and the latter can then be obtained as the difference. The transition from one region to the next is sharper at higher ionic strength for the same protein conformation, since the electrostatic interaction which makes the transitions less sharp diminishes as the ionic strength increases, as will be described below. Titration curves in concentrated guanidine hydrochloride show extremely sharp transitions, as will be seen in Section II below.

Of course, the actual groups titrated in any region is rarely exactly what it should be on the basis of the predictions just made, and therein lies the usefulness of titration curves as a tool in conformational analysis. Groups which do not titrate where expected must be in some special environment in the structure of the protein molecule.

Thermodynamic Interpretation. We shall confine ourselves to the very imprecise semi-empirical thermodynamic treatment in common use. A more sophisticated model has been presented elsewhere.[11] It should be emphasized that any thermodynamic treatment applies only to reversible portions of the titration curve, while group counting can be done on both reversible and irreversible portions, because it deals with chemical stoichiometry.

The heart of the approximate treatment is to assume that all groups with the same pK^{int} titrate simultaneously. Thus we may treat a titration curve as a combination of a small number of titration equilibria instead of having as many equilibria as there are titratable groups.

The nature of interactions between groups is considered to be solely electrostatic. A classical interpretation of this interaction is by the Linderstrøm-Lang theory,[12] which is based on the following assumption: a protein molecule is represented by a sphere impenetrable to the solvent water and salt ions, and the charges on the molecule are smeared evenly over the surface of this sphere.

For dissociation of any one titratable group of type i, the equilibrium constant K_i is given by

$$K_i = a_H x_i/(1 - x_i) \qquad \text{or} \qquad pK_i = pH - \log x_i/(1 - x_i) \tag{5}$$

where x_i is the average degree of dissociation and a_H is the thermodynamic hydrogen ion activity. As a consequence of the protein model described above, the interaction encountered by a hydrogen ion which is being added to the protein molecule is dependent on the net charge only, and can be calculated as the work that must be done to increase the net charge by one unit, against the opposing force of the charges already present. The mode of dependence is found to be

$$K_i = K_i^{int} e^{2w\bar{Z}} \qquad \text{or} \qquad pK_i = pK_i^{int} - 0.868\, w\bar{Z} \tag{6}$$

where K_i^{int} is the dissociation constant of titratable groups of type i in the absence of interaction, at $\bar{Z} = 0$ by assumption. The parameter w of Eq. (6) is given by

[11] C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.* **79**, 5333 (1957).
[12] K. Linderstrøm-Lang, *Compt. Rend. Trav. Lab. Carlsberg* **15(7)**, 1 (1924); C. Tanford, "Physical Chemistry of Macromolecules," p. 537. Wiley, New York, 1961.

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \tag{7}$$

where ϵ is the electronic charge, D is the dielectric constant of the medium, k is Boltzmann's constant, T is the temperature, b is the radius of the sphere which represents the protein molecule, a is the distance of closest approach of ions to the center of the sphere, i.e., the sum of the radii of the sphere and average small ions which make up the ionic strength, and κ is the Debye-Hückel parameter expressed by

$$\kappa = \left(\frac{8\pi\mathfrak{N}\epsilon^2}{1000\,DkT}\right)^{1/2} I^{1/2} \tag{8}$$

where $\mathfrak{N}$ is Avagadro's number and I is the ionic strength expressed by $I = \Sigma c_i z_i^2/2$, where c_i is the concentration of ion i as moles per 1000 ml and z_i is its charge. The value of w can be calculated from Eq. (7) with reasonable values for b and a, which are calculable either from the molecular weight and assumed hydration or from hydrodynamic data.[13]

It suffices here to point out that w depends on the ionic strength, has the same value for all titratable groups, and is constant as long as the protein molecule does not change its size and shape.

Combining Eqs. (5) and (6), we obtain

$$\text{pH} - \log x_i/(1 - x_i) = \text{p}K_i^{\text{int}} - 0.868\,w\bar{Z} \tag{9}$$

It is assumed, at least initially, that chemically identical groups have the same $\text{p}K_i^{\text{int}}$ which, as was mentioned earlier, means that all groups of a given kind titrate to the same degree simultaneously at any pH. Thus Eq. (9) becomes applicable to a number of groups of the same type i instead of one group.

Equation (9) provides a way to evaluate $\text{p}K_i^{\text{int}}$ and w experimentally by plotting the left-hand side against $\bar{Z}$. As long as $\text{p}K_i^{\text{int}}$ and w remain the same, the plot will be a straight line and will give $\text{p}K_i^{\text{int}}$ as the intercept at $\bar{Z} = 0$, and 0.868 w as the slope. Such logarithmic plots are shown in Fig. 3. The w values thus obtained are often a little smaller than predicted from the spherical model by Eq. (7). This might be due to possible deviations of the actual shape from a sphere, as evidenced by the myoglobin molecule.[14] On the other hand, when much too small w values have been obtained, as for α-corticotropin by Leonis and Li,[15] one must assume a molecule entirely different from a sphere. In this case the molecule is probably a random coil. Sometimes a straight line is not ob-

[13] Footnote 1 (p. 101).

[14] J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore, *Nature* **190**, 666 (1961).

[15] J. Léonis and C. H. Li, *J. Am. Chem. Soc.* **81**, 415 (1959).

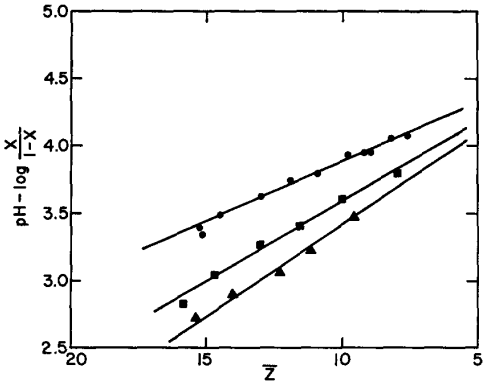

FIG. 3. Logarithmic plots of carboxyl titrations of ribonuclease at 25°. Data are from Fig. 1. Ionic strengths are 0.15 (●), 0.03 (■), and 0.01 (▲).

tained at all. This anomaly can mean heterogeneity among the groups being titrated, the wrong assumed number of groups, and/or configurational change in the pH range where curvature occurs. At any rate, one should not attempt to relate the w value to the actual conformation of the protein too strictly, since the spherical model itself is an oversimplification in the first place, but rather regard w as an empirical parameter.

II. Titration in Denaturing Media

Since almost all the titration curves of native proteins contain some anomalies, it is desirable to have a reference titration curve with which to compare the titration curve of the native protein, in order to pick out differences that point to involvement of titratable groups in the internal structure of the native molecule. The natural choice is the titration curve in a denaturing medium in which the protein is in the unfolded form, with virtually all titratable groups exposed to the solvent. The preferred denaturing agent is guanidine hydrochloride, because of its greater ability to unfold proteins than other reagents, such as urea, and because of its stability in solution (in contrast to urea, which is unstable[16]). In addition, guanidine hydrochloride is a strong electrolyte which should reduce electrostatic interactions almost to the vanishing point when concentrated solutions are used.

An example is provided by Fig. 4, which represents the titration of ribonuclease in 6 M guanidine hydrochloride. The results of the group

[16] G. R. Stark, W. H. Stein and S. Moore, *J. Biol. Chem.* **235**, 3177 (1960).

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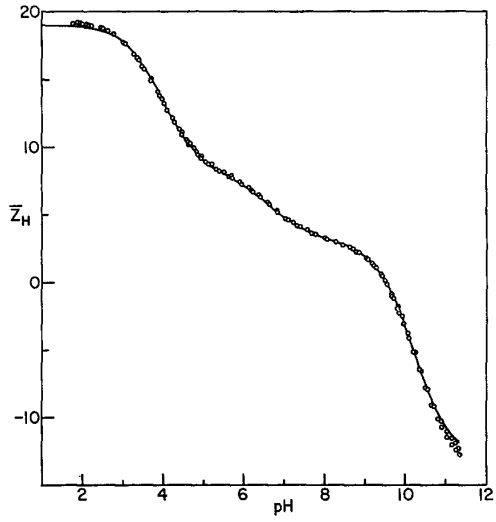

FIG. 4. Titration curve of ribonuclease in 6 *M* GuCl at 25° [Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967)]. *Solid line* represents the theoretical curve which was constructed by using the following parameters: $w = 0$; 1 α-COOH, pK 3.4; 5 aspartyl COOH, pK 3.8; 5 glutamyl COOH, pK 4.3; 4 imidazole, pK 6.5; 1 α-NH_3, pK 7.6; 3 phenolic hydroxyl, pK 9.75; 3 phenolic hydroxyl, pK 10.15; 10 lysyl-NH_3, pK 10.35; 4 guanidinyl, p$K > 12.5$.

counting procedure in this case are almost, but not quite, what is expected. The pH of zero net proton charge is 9.54, and ΣN^+ is close to 19 groups. This corresponds to the expected number (4 arginine, 10 lysine, 4 histidine plus an α-amino group). The number of groups titrated in the most acidic region is about 11, equal to the content of carboxyl groups (5 aspartic acid, 5 glutamic acid plus an α-carboxyl group). The number of groups titrated in the neutral region is 5, again as expected (4 histidine plus an α-amino group). The number of groups titrated in the alkaline branch of the curve, however, is more than expected on the basis of 10 lysine plus 6 tyrosine residues. This difference turned out to be due to the release of an extra proton in the β-elimination reaction of cystine residues, which leads to the eventual formation of lysinoalanine by a condensation with a neighboring lysine, as first reported by Patchornik and Sokolovsky[17] and Bohak.[18]

[17] M. Sokolovsky and A. Patchornik, *J. Am. Chem. Soc.* **86**, 1859 (1964); A. Patchornik and M. Sokolovsky, *ibid.* 1860 (1964).
[18] Z. Bohak, *J. Biol. Chem.* **239**, 2878 (1964).

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Preliminary experiments with other proteins in guanidine hydrochloride suggest that the same or similar reactions occur there also. The covalent structure of a protein (at least if it contains disulfide bonds) appears not to be stable at high pH, and reactions occur which result in the appearance of titratable groups. Apart from such anomalies at high pH, group counting in a medium such as 6 M guanidine hydrochloride should give the expected result for all proteins, i.e., it should be equivalent to a true analysis for the numbers of titratable groups on a protein molecule.

The spectrophotometric titration curve of the phenolic groups of ribonuclease in guanidine hydrochloride is shown in Fig. 2. It is seen that all six tyrosines are titrated together, as expected.

When the groups of any one kind, for instance the phenolic hydroxyls as shown above or side-chain carboxyls, are taken,[19] and Eq. (9) is applied to their titration, values of w close to zero are obtained, as expected. The value of w is actually not quite equal to zero, if it is assumed that all groups of either kind titrate simultaneously. (For ribonuclease, $w = 0.021$ has been obtained from either phenolic hydroxyl groups or β,γ-carboxyl groups.) As far as the side-chain carboxyl groups are concerned, the non-zero value of w turns out to be simply the result of assuming, falsely, that the pK's for aspartyl and glutamyl side chains are identical. When proper account is taken of the pK difference between these groups (see Table III) the acidic branch of the titration curve can be assigned a value of w which is zero within experimental error. The non-zero value of w for the phenolic groups, however, represents a real deviation from the ideally expected result. This probably does not mean that there continues to be, under these conditions, a general electrostatic interaction between all the charges on the molecule, as visualized in the Linderstrøm-Lang treatment, but rather the existence of specific local effects which invalidate the idea that all groups of the same kind titrate altogether simultaneously. The titratable groups on a protein molecule, even when it is unfolded, have different environments by virtue of having different neighbors on adjacent residues. For instance, location of a charged group on a neighboring residue could affect the pK value even at high ionic strength. Or a neighboring disulfide bond could provide a somewhat hydrophobic environment, because separation of all parts of the chain from each other would not be complete at that point. In ribonuclease some such effect clearly influences the titration of the phenolic groups. Within the experimental uncertainty of the data of Fig. 4, no similar effect need be invoked for any of the other groups of the protein.

[19] See footnote 1 (p. 99) for method of correcting for overlaps of the titration of groups of one kind with those of another.

III. Titration of Native Proteins

The simple result obtained for proteins in denaturing media will certainly not hold true for most native proteins, because they provide more complicated interactions due to their special, tightly folded structure of peptide chains. A considerable number of groups can always be expected to titrate anomalously in the native protein. The easiest way to demonstrate this is to measure the titration curve of a native protein at high ionic strength, where electrostatic effects must be grealy reduced. Nevertheless, large differences from the titration curves in 6 M guanidine hydrochloride can still be found, as is demonstrated dramatically by the data for β-lactoglobulin shown in Fig. 5. That these differences truly

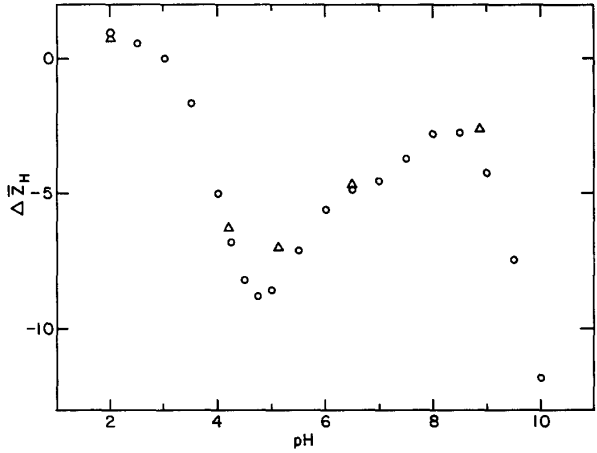

FIG. 5. Difference between the titration curves of β-lactoglobulin in 6 M GuCl and 1 M KCl at 25° (C. Tanford and Y. Nozaki, in preparation): ○ represents $\Delta\bar{Z}_H = \bar{Z}_H$ in 6 M GuCl $- \bar{Z}_H$ in 1 M KCl, at the same pH; △ represents $\Delta\bar{Z}_H$ obtained by direct difference titration with a pH-stat.

reflect the difference between native and denatured conformations is demonstrated directly by difference titrations, as shown in Fig. 6. The difference is seen to arise during a steep transition over a narrow range of guanidine concentration, corresponding to the transition from native to denatured protein as observed by measurement of optical rotation or other conformation-dependent parameters.[20]

Turning next to the actual analysis of the titration curves of native proteins, we begin with group counting. We ordinarily find differences

[20] N. Pace, unpublished data.

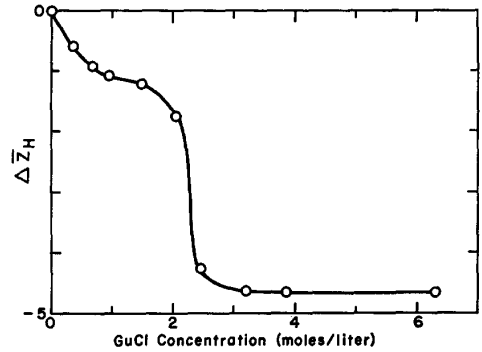

FIG. 6. $\Delta \bar{Z}_H$, measured as in Fig. 5 with a pH-stat, as a function of the concentration of GuCl at constant pH of 6.5. (C. Tanford and Y. Nozaki, in preparation). The solution at (GuCl) = 0 contained 1 *M* KCl, and the initial decrease in ΔZ_H presumably reflects the increase in ionic strength above 1 *M*. The major part of the titration curve difference closely parallels the unfolding of the protein as observed by optical rotation or difference spectra.

between the number of groups titrated in a given region, and the number expected on the basis of amino acid analysis. For ribonuclease (Fig. 1), for example, we find that the expected number of groups is titrated in the acid and neutral branches, and also the measurement of ΣN^+ continues to give the expected result, as for the titration in 6 *M* GuCl. On the alkaline side, however, three fewer groups are titrated than expected from amino acid analysis. Spectrophotometric titration (Fig. 2) shows that the three groups not titrated are phenolic groups. Only 3 of the 6 such groups can be titrated at all in the native protein, while the other three are unavailable for titration until the native conformation is altered. In hemoglobin[21] and in myoglobin,[22] by contrast, carboxyl groups are normal, but some imidazoles are titrated in the acid branch of the curve instead of the neutral branch. We regard all unavailable groups of this sort as buried in the interior of the protein molecule, so that they are not titrated at all unless a conformational change in the protein molecule exposes them to the solvent. In many cases the conformational change required is drastic and irreversible. The buried groups in most cases are probably present as uncharged species. For instance, the three tyrosine groups of ribonuclease,[23] the buried histidine groups of hemoglobin and

[21] J. Steinhardt and E. Zaiser, *J. Am. Chem. Soc.* **75**, 1599 (1955).
[22] E. Breslow and F. R. N. Gurd, *J. Biol. Chem.* **237**, 371 (1962).
[23] C. Tanford, J. D. Hauenstein, and D. G. Rands, *J. Am. Chem. Soc.* **77**, 6409 (1955).

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myoglobin, and the two abnormal carboxyl groups of β-lactoglobulin[24] are all present in the uncharged form. However, the existence of buried charged carboxylate groups has been reported for lysozyme.[25]

There are also anomalies in pK which are not so drastic as the above examples, although they are related to the special conformation of protein molecules. For instance, in β-lactoglobulin two carboxyl groups titrate reversibly in the neutral region of the curve instead of the acidic region.[24] They appear to be buried in the native protein, and to become exposed at neutral pH in a conformational change which, although it can be detected by changes in optical rotation and other parameters, is much less drastic than complete unfolding. The behavior of these two groups, plus other anomalies not yet identified, lead to the large difference in titration behavior between native and denatured proteins shown in Fig. 5.

More subtle anomalies are usually observed when the groups titrated in the normal range are subjected to analysis by Eq. (9). From the logarithmic plots of Eq. (9) against $\bar{Z}$ for the carboxyl groups of ribonuclease, w values were found that were much higher than predicted by a spherical model for the protein. In this case the anomalous w values were found to stem from the fact that there must be at least two kinds of β,γ-carboxyl group with different pK^{int} values.[26,27] The w value found for lysozyme is also much higher than predicted by a spherical model.[28] High w values are obtained quite often, and it is possible that most of them can be explained by the involvement of heterogeneous pK^{int} values among chemically identical groups.

Even when the analysis itself does not show obvious anomalies, there must be effects of conformation on the titration curve which are hidden by the averaging process that occurs when attempts are made to reconcile the experimental data on the assumption that all groups of a given kind titrate simultaneously. In hemoglobin, for example, there are at least two groups which must have different pK's in the oxygenated and deoxygenated forms.[29,30] The pH-dependence of the dissociation of hemoglobin into half-molecules requires that there must also be groups which have a different pK in the whole molecule and in the half-mole-

[24] C. Tanford, L. G. Bunville, and Y. Nozaki, *J. Am. Chem. Soc.* **81,** 4032 (1959).
[25] J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.* **82,** 2154 (1960).
[26] C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.* **78,** 5287 (1956).
[27] J. Hermans, Jr. and H. A. Scheraga, *J. Am. Chem. Soc.* **83,** 3293 (1961).
[28] C. Tanford and M. L. Wagner, *J. Am. Chem. Soc.* **76,** 3331 (1954).
[29] E. Antonini, J. Wyman, M. Brunori, C. Fronticello, E. Bucci, and A. Rossi-Fanelli, *J. Biol. Chem.* **240,** 1096 (1965).
[30] J. Wyman, Jr., *Advan. Protein Chem.* **19,** 223 (1964).

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cule.[30] These anomalies cannot be detected with any assurance by examination of the titration curve per se, and no satisfactory method for identifying them exists.

Most of these anomalies have not yet been explained in terms of structure. All that can be said is that they are characteristic of the native conformation, and that they disappear when the native conformation is lost. In some instances, as in the case of oxy- and deoxy-hemoglobins, they point to a difference in conformation between two forms of the native protein.

IV. Experimental Technique

a. Glass Electrode. Requirements for a glass electrode are: (1) quick and stable pH response—a good electrode reaches the final pH reading quickly and shows no pH drifts for several hours thereafter; (2) $p\gamma'_H$ and $p\gamma'_{OH}$ values are constant over wide pH ranges, at least slightly beyond the pH limits within which titrations are intended, and ordinarily $p\gamma'_H$ should be constant to pH 1.5 and $p\gamma'_{OH}$ to pH 12.5, when determined in KCl solutions; and (3) durability, that is, the qualities described remain satisfactory for a long time. A good electrode will deteriorate eventually and rejuvenation methods usually do not restore its lost performance. Unfortunately not all commercial electrodes meet these requirements. Only one out of about three behaves satisfactorily.

b. Calomel Electrode. Calomel electrodes with finely drawn, U-bent capillary tips, as shown in Fig. 7B, are recommended for continuous titration. Commercial calomel electrodes that have a wick, porous plug, or sleeve to make the liquid junction are not satisfactory for protein solutions. The use of the U-bent tip was recommended by Jacobsen *et al.*[31] and is commercially available. It is important, however, to make the capillary of the U-tip very fine,[32] so that the amount of KCl flowing into the solution can be ignored.

c. Syringe Burette. The major problem with syringe-type microburettes is leakage. If the reagent is leaking into the sample solution (leaking forward), too flat a titration curve will be obtained; backward leakage will result in too steep a titration curve. In order to prevent leakage in the case of all-glass syringe burettes, the end piece and the barrel are fused together. In addition, a drop of glycerol is applied on the plunger at the rear end of the barrel after filling the syringe with a standard solution. A U-shaped wire is useful to prevent the plunger from

[31] C. F. Jacobsen, J. Léonis, K. Linderstrøm-Lang, and M. Ottesen, *Methods Biochem. Anal.* **4**, 171 (1957).

[32] Approximately 15/100 mm in diameter, or about the size of the bore of an AGLA syringe tip or Gilmont microburette glass tip.

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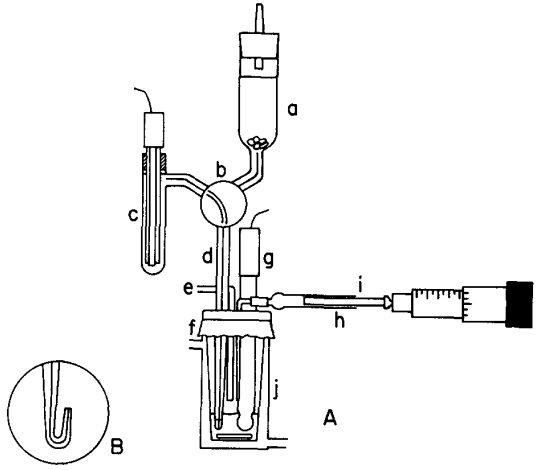

FIG. 7. Apparatus for continuous titration: (a, b, c, d) calomel electrode assembly (the salt bridge tip is shown in insert B). A commercial calomel electrode is cut near its tip and secured inside (c) with Tygon tubing (*shaded*). The three-way glass capillary stopcock is shown in working position. (e) Nitrogen gas inlet. (f) Rubber sheet (dental dam). (g) Glass electrode. (h) Syringe burette assembly. (i) Place of application of sealant glycerol. (j) Conical titration vessel with water jacket and a stirring bar. The whole assembly is placed on a magnetic stirrer.

slipping. Usually an L-shaped delivery tube is attached to the syringe through a short piece of Tygon tubing. The delivery tube is brought into the titration vessel only when there is no sign of leakage, forward or backward. Forward leakage can be detected by touching the syringe tip with the fingernail, on which a wet spot will be seen if the syringe is leaking. An air bubble will be noticed in the syringe tip, if backward leakage occurs.

In the case of other types of syringe burette, in which the plunger passes through plastic gaskets, possible leakage can be minimized by filling the syringe bubble-free.

d. Vessels for Continuous Titration. The titration vessels currently being used in the laboratory of the authors are made from solid plastic rod, so that any possibility of leakage is excluded. An interchangeable water jacket can be screwed on the inner vessel, and the sample can be weighed directly in the inner vessel before assembly. The smallest vessel for 2.5–3-ml samples is shown in Fig. 7A. Thorough mixing is essential to successful titration. It becomes difficult when a small-size, say 2.5-ml, sample is to be titrated, since a part of the solution trapped in the narrow

space might not be mixed well with the main body of the solution. Dixon and Wade have designed a novel titration vessel which is rotated by a constant temperature water jet.[33] Another small vessel has been designed by Leonis and Li for use with a 1-ml sample.[15]

e. Guanidine Hydrochloride. At present the quality of commercial guanidine hydrochloride is not satisfactory for titration work. It is recommended that the salt be recrystallized by the following procedure: 250 g GuCl is dissolved in 1 liter of hot absolute ethanol, and the solution is decolorized with charcoal and filtered through a heated large funnel if necessary. Benzene (500 ml) is added to the still hot ethanol solution, and the mixture is kept in the cold for several hours before the crystals (needles) are collected. Sometimes a yellow, sticky impurity can be removed from the original sample by washing with acetone.

The recrystallized sample should be further recrystallized either from methanol by dissolving in nearly boiling methanol and chilling in Dry Ice-acetone mixture, or from water by vacuum evaporation of an aqueous solution nearly saturated at 40°.

An alternative procedure is to neutralize a slurry of guanidinium carbonate, which has been recrystallized from 50% ethanol, with 20% distilled hydrochloric acid to pH 4. The solution is evaporated to obtain crystals of guanidine hydrochloride. The product is fairly pure, but still requires recrystallization as described above.

Criteria of purity are: (1) The absorption spectrum of a 6 *M* solution should show gradually increasing absorbance and no peak from 350 mμ to near 230 mμ, where a sharp rise is usually observed to approximately 0.15 absorbance unit at 225 mμ. (2) A minute amount of standard acid or base should cause a large pH shift. Quantitatively, titration of 6 *M* guanidine hydrochloride solution should produce $p\gamma'_H$ and $p\gamma'_{OH}$ values comparable with the values given in Table II.

f. Titration Procedure. Generally speaking, continuous titration is liable to systematic error, but the accuracy can approach that of batchwise titration if proper care is taken. It has the great advantage of saving time and material. The following comments apply mostly to continuous titration, although some are also relevant to batchwise titration technique.

It is recommended that a blank titration be performed occasionally, so that irregularities in electrodes or standard solutions can be detected before a protein sample is titrated. Attempts to determine $p\gamma'$ values with too low a concentration of acid or base added should be avoided. The concentration should be at least 3×10^{-3} moles per liter to maintain sufficient buffer capacity.

[33] G. H. Dixon and R. D. Wade, *Science* **127**, 338 (1958).

Choice of concentration of protein for titration will vary over a wide range, but the most convenient concentration is 0.5–1%, except at very low pH (<2.5) and high pH (>11), where the concentration of free H^+ and OH^- ions reaches the same order of magnitude as of bound ions and the accuracy of the titration will decrease unless a high protein concentration, say 3% or even higher, is used. This is especially desirable for accurate $\bar{Z}$ values near the acid end of titration curves, where the important value of ΣN^+ is obtained.

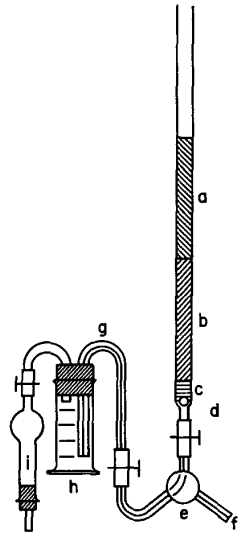

FIG. 8. Apparatus for deionization of protein solutions. (a) Mixture of ammonium and acetate forms of ion-exchange resins. (b) Mixture of hydrogen and hydroxyl forms of ion-exchange resins. (c) Short layer of hydrogen form of cation-exchange resin. (d) A glass bead. (e) Three-way Teflon capillary stopcock. (f) Capillary outlet. (g) Capillary U-tube. (h) Receiver. (i) Ascarite tube.

It is advisable to shift the pH of an initial sample solution in the direction opposite to that of the intended titration, so that at least a portion of different titration curves will be superimposed in a particular pH region. It is also recommended that nitrogen gas be passed over the solution at slightly acidic pH for a while until the pH reading becomes constant before a continuous titration starts, since it is possible that a sample of high pH will have absorbed CO_2 from the air.

g. Deionization and Isoionic Point Measurement. Dintzis[84] has introduced a convenient procedure to deionize proteins which has become

[84] H. Dintzis, Ph.D. thesis, Harvard University, Cambridge, Massachusetts, 1952.

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standard. A diagram of the apparatus believed to be most convenient is shown in Fig. 8.

After packing the column with three layers of resins, as shown in the figure, it is rinsed repeatedly with water. The conductivity of the rinse water can be determined by attaching a flow cell to *f*, while the receiver *h* may be flushed with nitrogen gas. The sample solution is placed on the column when the rinse water is about to disappear. The effluent does not contain an appreciable amount of protein until the volume reaches roughly half the apparent volume of the resin column. Usually an amount of effluent is collected equal to about 1.5 times the initial volume, depending on the retention of protein by the resin, to recover nearly all the protein. Roughly speaking, 9 ml resin (2 ml each of hydrogen, hydroxyl, ammonium, and acetate cycles, plus 1 ml hydrogen resin at the bottom) is sufficient to retain 10^{-3} equivalent of salt. The flow rate is kept rather slow, about 1 ml per minute for a column containing 9 ml resin.

It is recommended that untreated samples as well as samples adjusted to acid and alkaline pH, if the quantity of protein permits, be deionized to confirm the validity of the pH values obtained. If the column is working properly, the final pH will be independent of the initial pH.

[85] Hydrogen Exchange

By GIOVANNI DI SABATO and MARTIN OTTESEN

I. Introduction

It is known that when proteins are dissolved in deuterium oxide some of the hydrogen atoms will exchange for deuterium. Linderstrøm-Lang developed a quantitative method to determine this exchange, and demonstrated that in simple peptides and in denatured proteins the hydrogens bound to nitrogen, to oxygen, or to sulfur would exchange rapidly.[1] In native proteins, structural factors were present that slowed down the exchange. In most cases some of the exchange was so slow that some potentially exchangeable hydrogens remained unexchanged after several days in deuterium oxide solution at ordinary temperatures. Therefore, measurements of the rates and extent of hydrogen exchange in native proteins provide information about their structure. In the present article the mechanism of the hydrogen exchange is not discussed in detail, but

[1] I. M. Krause and K. Linderstrøm-Lang, *Compt. Rend. Trav. Lab. Carlsberg*, **29**, 367 (1955).

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(54) Title: SELF-BUFFERING PROTEIN FORMULATIONS

(57) Abstract: The invention herein described, provides, among other things, self-buffering protein formulations. Particularly, the invention provides self-buffering pharmaceutical protein formulations that are suitable for veterinary and human medical use. The self-buffering protein formulations are substantially free of other buffering agents, stably maintain pH for the extended time periods involved in the distribution and storage of pharmaceutical proteins for veterinary and human medical use. The invention further provides methods for designing, making, and using the formulation. In addition to other advantages, the formulations avoid the disadvantages associated with the buffering agents conventionally used in current formulations of proteins for pharmaceutical use. The invention in these and other respects can be productively applied to a wide variety of proteins and is particularly useful for making and using self-buffering formulations of pharmaceutical proteins for veterinary and medical use, especially, in particular, for the treatment of diseases in human subjects.

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SELF-BUFFERING PROTEIN FORMULATIONS

Reference to Related Applications

This application is a continuation-in-part of and claims full priority benefit of U.S. Provisional Application Serial No. 60/690,582 filed 14 June 2005, which is incorporated herein by reference in its entirety.

Field of the Invention

The invention relates to the formulation of proteins, especially pharmaceutical proteins. In particular, it relates to self-buffering biopharmaceutical protein compositions, and to methods for designing, making, and using the compositions. It further relates to pharmaceutical protein compositions for veterinary and/or for human medical use, and to methods relating thereto.

Background of the Invention

Many aspects of pharmaceutical production and formulation processes are pH sensitive. Maintaining the correct pH of a finished pharmaceutical product is critical to its stability, effectiveness, and shelf life, and pH is an important consideration in designing formulations for administration that will be acceptable, as well as safe and effective.

To maintain pH, pharmaceutical processes and formulations use one or more buffering agents. A variety of buffering agents are available for pharmaceutical use. The buffer or buffers for a given application must be effective at the desired pH. They must also provide sufficient buffer capacity to maintain the desired pH for as long as necessary. A good buffer for a pharmaceutical composition must satisfy numerous other requirements as well. It must be appropriately soluble. It must not form deleterious complexes with metal ions, be toxic, or unduly penetrate, solubilize, or absorb on membranes or other surfaces. It should not interact with other components of the composition in any manner which decreases their availability or effectiveness. It must be stable and effective at maintaining pH over the range of conditions to which it will be exposed during formulation and during storage of the product. It must not be deleteriously affected by oxidation or other reactions occurring in its environment, such as those that occur in the processing of the composition in which it is providing the buffering action. If carried over or incorporated into a final product, a buffering agent

must be safe for administration, compatible with other components of the composition over the shelf-life of the product, and acceptable for administration to the end user.

Although there are many buffers in general use, only a limited number are suitable for biological applications and, of these, fewer still are acceptable for pharmaceutical processes and formulations. As a result, it often is challenging to find a buffer that not only will be effective at maintaining pH but also will meet all the other requirements for a given pharmaceutical process, formulation, or product.

The challenge of finding a suitable buffer for pharmaceutical use can be especially acute for pharmaceutical proteins. The conformation and activity of proteins are critically dependent upon pH. Proteins are susceptible to a variety of pH sensitive reactions that are deleterious to their efficacy, typically many more than affect small molecule drugs. For instance, to mention just a few salient examples, the side chain amides of asparagine and glutamine are deamidated at low pH (less than 4.0) and also at neutral or high pH (greater than 6.0). Aspartic acid residues promote the hydrolysis of adjacent peptide bonds at low pH. The stability and disposition of disulfide bonds is highly dependent on pH, particularly in the presence of thiols. Solubility, flocculation, aggregation, precipitation, and fibrillation of proteins are critically dependent on pH. The crystal habit important to some pharmaceutical formulations also is critically dependent on pH. And pH is also an important factor in surface adsorption of many pharmaceutical peptides and proteins.

Buffering agents that catalyze reactions that inactivate and/or degrade one or more other ingredients, moreover, cannot be used in pharmaceutical formulations. Buffers for pharmaceutical use must have not only the buffer capacity required to maintain correct pH, but also they must not buffer so strongly that their administration deleteriously perturbs a subject's physiological pH. Buffers for pharmaceutical formulations also must be compatible with typically complex formulation processes. For instance, buffers that sublime or evaporate, such as acetate and imidazole, generally cannot be relied upon to maintain pH during lyophilization and in the reconstituted lyophilization product. Other buffers that crystallize out of the protein amorphous phase, such as sodium phosphate, cannot be relied upon to maintain pH in processes that require freezing.

Buffers used to maintain pH in pharmaceutical end-products also must be not only effective at maintaining pH but also safe and acceptable for administration to the subject. For instance, several otherwise useful buffers, such as citrate at low or high

concentration and acetate at high concentration, are undesirably painful when administered parenterally.

Some buffers have been found to be useful in the formulation of pharmaceutical proteins, such as acetate, succinate, citrate, histidine (imidazole), phosphate, and Tris. They all have undesirable limitations and disadvantages. And they all have the inherent disadvantage of being an additional ingredient in the formulation, which complicates the formulation process, poses a risk of deleteriously affecting other ingredients, stability, shelf-life, and acceptability to the end user.

There is a need, therefore, for additional and improved methods of maintaining pH in the production and formulation of pharmaceuticals and in pharmaceutical compositions, particularly in the production and formulation of biopharmaceutical proteins and in biopharmaceutical protein compositions.

Summary

Therefore, it is among the various objects and aspects of the invention to provide, in certain of the preferred embodiments, protein formulations comprising a protein, particularly pharmaceutically acceptable formulations comprising a pharmaceutical protein, that are buffered by the protein itself, that do not require additional buffering agents to maintain a desired pH, and in which the protein is substantially the only buffering agent (i.e., other ingredients, if any, do not act substantially as buffering agents in the formulation).

In this regard and others, it is among the various objects and aspects of the invention to provide, in certain preferred embodiments, self-buffering formulations of a protein, particularly of a pharmaceutical protein, characterized in that the concentration of the formulated protein provides a desired buffer capacity.

It is further among the various objects and aspects of the invention to provide, in certain of the particularly preferred embodiments, self-buffering protein formulations, particularly pharmaceutical protein formulations, in which the total salt concentration is less than 150 mM..

It is further among the various objects and aspects of the invention to provide, in certain of the particularly preferred embodiments, self-buffering protein formulations, particularly pharmaceutical protein formulations, that further comprise one or more polyols and/or one or more surfactants.

It is also further among the various objects and aspects of the invention to provide, in certain of the particularly preferred embodiments, self-buffering formulations comprising a protein, particularly a pharmaceutical protein, in which the total salt concentration is less than 150 mM, that further comprise one or more excipients, including but not limited to, pharmaceutically acceptable salts; osmotic balancing agents (tonicity agents); surfactants, polyols, anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; and analgesics.

It is additionally among the various objects and aspects of the invention to provide, in certain preferred embodiments, self-buffering protein formulations, particularly pharmaceutical protein formulations, that comprise, in addition to the protein, one or more other pharmaceutically active agents.

Various additional aspects and embodiments of the invention are illustratively described in the following numbered paragraphs. The invention is described by way of reference to each of the items set forth in the paragraphs, individually and/or taken together in any combination. Applicant specifically reserves the right to assert claims based on any such combination.

1. A composition according to any of the following, wherein the composition has been approved for pharmaceutical use by a national or international authority empowered by law to grant such approval preferably the European Agency for the Evaluation of Medical Products, Japan's Ministry of Health, Labor and Welfare, China's State Drug Administration, United States Food and Drug Administration, or their successor(s) in this authority, particularly preferably the United States Food and Drug Administration or its successor(s) in this authority.

2. A composition according to any of the foregoing or the following, wherein the composition is produced in accordance with good manufacturing practices applicable to the production of pharmaceuticals for use in humans.

3. A composition according to any of the foregoing or the following, comprising a protein, the protein having a buffer capacity per unit volume per pH unit of at least that of approximately: 2.0 or 3.0 or 4.0 or 5.0 or 6.50 or 8.00 or 10.0 or 15.0 or 20.0 or 30.0 or 40.0 or 50.0 or 75.0 or 100 or 125 or 150 or 200 or 250 or 300 or 350 or 400 or 500 mM sodium acetate buffer in pure water over the range of pH 5.0 to 4.0 or pH 5.0 to 5.5, preferably as determined in accordance with the methods described in Example 1 and 2, particularly preferably at least 2.0 mM, especially particularly

preferably at least 3.0 mM, very especially particularly preferably at least 4.0 mM or at least 5.0 mM, especially particularly preferably at least 7.5 mM, particularly preferably at least 10 mM, preferably at least 20 mM.

4. A composition according to any of the foregoing or the following wherein, exclusive of the buffer capacity of the protein, the buffer capacity per unit volume per pH unit of the composition is equal to or less than that of 1.0 or 1.5 or 2.0 or 3.0 or 4.0 or 5.0 mM sodium acetate buffer in pure water over the range of pH 4.0 to 5.0 or pH 5.0 to 5.5, preferably as determined in accordance with the methods described in Example 1 and 2, particularly preferably less than that of 1.0 mM, very especially particularly preferably less than that of 2.0 mM, especially particularly preferably less than that of 2.5 mM, particularly preferably less than that of 3.0 mM, preferably less than that of 5.0 mM.

5. A composition according to any of the foregoing or the following comprising a protein wherein over the range of plus or minus 1 pH unit from the pH of the composition, the buffer capacity of the protein is at least approximately: 1.00 or 1.50 or 1.63 or 2.00 or 3.00 or 4.00 or 5.00 or 6.50 or 8.00 or 10.0 or 15.0 or 20.0 or 30.0 or 40.0 or 50.0 or 75.0 or 100 or 125 or 150 or 200 or 250 or 300 or 350 or 400 or 500 or 700 or 1,000 mEq per liter per pH unit, preferably at least approximately 1.00, particularly preferably 1.50, especially particularly preferably 1.63, very especially particularly preferably 2.00, very highly especially particularly preferably 3.00, very especially particularly preferably 5.0, especially particularly preferably 10.0, particularly preferably 20.0.

6. A composition according to any of the foregoing or the following comprising a protein wherein over the range of plus or minus 1 pH unit from the pH of the composition, exclusive of the protein, the buffer capacity per unit volume per pH unit of the composition is equal to or less than that of 0.50 or 1.00 or 1.50 or 2.00 or 3.00 or 4.00 or 5.00 or 6.50 or 8.00 or 10.0 or 20.0 or 25.0 mM sodium acetate buffer in pure water over the range pH 5.0 to 4.0 or pH 5.0 to 5.5, particularly preferably determined in accordance with Example 1 and/or Example 2.

7. A composition according to any of the foregoing or the following, wherein over a range of plus or minus 1 pH unit from a desired pH, the protein provides at least approximately 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% of the buffer capacity of the composition, preferably at least approximately 75%, particularly preferably at least approximately 85%, especially

particularly preferably at least approximately 90%, very especially particularly preferably at least approximately 95%, very highly especially particularly preferably at least approximately 99% of the buffer capacity of the composition.

8. A composition according to any of the foregoing or the following, wherein the concentration of the protein is between approximately: 20 and 400, or 20 and 300, or 20 and 250, or 20 and 200, or 20 and 150 mg/ml, preferably between approximately 20 and 400 mg/ml, particularly preferably between approximately 20 and 250, especially particularly between approximately 20 and 150 mg/ml.

9. A composition according to any of the foregoing or the following, wherein the pH maintained by the buffering action of the protein is between approximately: 3.5 and 8.0, or 4.0 and 6.0, or 4.0 and 5.5, or 4.0 and 5.0, preferably between approximately 3.5 and 8.0, especially particularly preferably approximately 4.0 and 5.5.

10. A composition according to any of the foregoing or the following, wherein the salt concentration is less than: 150 mM or 125 mM or 100 mM or 75 mM or 50 mM or 25 mM, preferably 150 mM, particularly preferably 125 mM, especially preferably 100 mM, very particularly preferably 75 mM, particularly preferably 50 mM, preferably 25 mM.

11. A composition according to any of the foregoing or the following, further comprising one or more pharmaceutically acceptable salts; polyols; surfactants; osmotic balancing agents; tonicity agents; anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; analgesics; or additional pharmaceutical agents.

12. A composition according to any of the foregoing or the following, comprising one or more pharmaceutically acceptable polyols in an amount that is hypotonic, isotonic, or hypertonic, preferably approximately isotonic, particularly preferably isotonic, especially preferably any one or more of sorbitol, mannitol, sucrose, trehalose, or glycerol, particularly especially preferably approximately 5% sorbitol, 5% mannitol, 9% sucrose, 9% trehalose, or 2.5% glycerol, very especially in this regard 5% sorbitol, 5% mannitol, 9% sucrose, 9% trehalose, or 2.5% glycerol.

13. A composition according to any of the foregoing or the following, further comprising a surfactant, preferably one or more of polysorbate 20, polysorbate 80, other fatty acid esters of sorbitan, polyethoxylates, and poloxamer 188, particularly preferably polysorbate 20 or polysorbate 80, preferably approximately 0.001 to 0.1%

polysorbate 20 or polysorbate 80, very preferably approximately 0.002 to 0.02% polysorbate 20 or polysorbate 80, especially 0.002 to 0.02% polysorbate 20 or polysorbate 80.

14. A composition according to any of the foregoing or the following, wherein the protein is a pharmaceutical agent and the composition is a sterile formulation thereof suitable for treatment of a non-human or a human subject.

15. A composition according to any of the foregoing or the following, wherein the protein is a pharmaceutical agent effective to treat a disease and the composition is a sterile formulation thereof suitable for administration to a subject for treatment thereof.

16. A composition according to any of the foregoing or the following, wherein the protein does not induce a significantly deleterious antigenic response following administration to a subject.

17. A composition according to any of the foregoing or the following, wherein the protein does not induce a significantly deleterious immune response following administration to a subject.

18. A composition according to any of the foregoing or the following, wherein the protein is a human protein.

19. A composition according to any of the foregoing or the following, wherein the protein is a humanized protein.

20. A method according to any of the foregoing or the following, wherein the protein is an antibody, preferably an IgA, IgD, IgE, IgG, or IgM antibody, particularly preferably an IgG antibody, very particularly preferably an IgG1, IgG2, IgG3, or IgG4 antibody, especially an IgG2 antibody.

21. A composition according to any of the foregoing or the following, wherein the protein comprises a: Fab fragment, Fab_2 fragment, Fab_3 fragment, Fc fragment, scFv fragment, bis-scFv(s) fragment, minibody, diabody, triabody, tetrabody, VhH domain, V-NAR domain, V_H domain, V_L domain, camel Ig, Ig NAR, or peptibody, or a variant, derivative, or modification of any of the foregoing.

22. A composition according to any of the foregoing or the following, wherein the protein comprises an Fc fragment or a part thereof or a derivative or variant of an Fc fragment or part thereof.

23. A composition according to any of the foregoing or the following, wherein the protein comprises a first binding moiety of a pair of cognate binding moieties, wherein the first moiety binds the second moiety specifically.

24. A composition according to any of the foregoing or the following, wherein the protein comprises (a) an Fc fragment or a part thereof or a derivative or variant of an Fc fragment or part thereof, and (b) a first binding moiety of a pair of cognate binding moieties.

25. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is selected from the group consisting of proteins that bind specifically to one or more CD proteins, HER receptor family proteins, cell adhesion molecules, growth factors, nerve growth factors, fibroblast growth factors, transforming growth factors (TGF), insulin-like growth factors, osteoinductive factors, insulins and insulin-related proteins, coagulation and coagulation-related proteins, colony stimulating factors (CSFs), other blood and serum proteins blood group antigens; receptors, receptor-associated proteins, growth hormone receptors, T-cell receptors; neurotrophic factors. neurotrophins, relaxins, interferons, interleukins, viral antigens, lipoproteins, integrins, rheumatoid factors, immunotoxins, surface membrane proteins, transport proteins, homing receptors, addressins, regulatory proteins, and immunoadhesins,

26. A composition according to any of the foregoing or the following, wherein the protein is selected from the group consisting of: OPGL specific binding proteins, myostatin specific binding proteins, IL-4 receptor specific binding proteins, IL1-R1 specific binding proteins, Ang2 specific binding proteins, NGF-specific binding proteins, CD22 specific binding proteins, IGF-1 receptor specific binding proteins, B7RP-1 specific binding proteins, IFN gamma specific binding proteins, TALL-1 specific binding proteins, stem cell factors, Flt-3 ligands, and IL-17 receptors.

27. A composition according to any of the foregoing or the following, wherein the protein is selected from the group consisting of proteins that bind specifically to one ormore of: CD3, CD4, CD8, CD19, CD20, CD34; HER2, HER3, HER4, the EGF receptor; LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM, alpha v/beta 3 integrin; vascular endothelial growth factor ("VEGF"); growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-1-alpha), erythropoietin (EPO), NGF-

beta, platelet-derived growth factor (PDGF), aFGF, bFGF, epidermal growth factor (EGF), TGF-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, TGF-beta5, IGF-I, IGF-II, des(1-3)-IGF-I (brain IGF-I), insulin, insulin A-chain, insulin B-chain, proinsulin, insulin-like growth factor binding proteins;, such as, among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1–antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin; M-CSF, GM-CSF, G-CSF, albumin, IgE, flk2/flt3 receptor, obesity (OB) receptor, bone-derived neurotrophic factor (BDNF), NT-3, NT-4, NT-5, NT-6); relaxin A-chain, relaxin B-chain, prorelaxin; interferon-alpha, -beta, and -gamma;IL-1 to IL-10; AIDS envelope viral antigen; calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES, mouse gonadotropin-associated peptide, Dnase, inhibin, and activin; protein A or D, bone morphogenetic protein (BMP), superoxide dismutase, decay accelerating factor (DAF).

28. A composition according to any of the foregoing or the following, wherein the protein is selected from the group consisting of:Actimmune (Interferon-gamma-1b), Activase (Alteplase), Aldurazme (Laronidase), Amevive (Alefacept), Avonex (Interferon beta-1a), BeneFIX (Nonacog alfa), Beromun (Tasonermin), Beatseron (Interferon-beta-1b), BEXXAR (Tositumomab), Tev-Tropin (Somatropin), Bioclate or RECOMBINATE (Recombinant), CEREZME (Imiglucerase), ENBREL (Etanercept), Eprex (epoetin alpha), EPOGEN/Procit (Epoetin alfa), FABRAZYME (Agalsidase beta), Fasturtec/Elitek ELITEK (Rasburicase), FORTEO (Teriparatide), GENOTROPIN (Somatropin), GlucaGen (Glucagon), Glucagon (Glucagon, rDNA origin), GONAL-F (follitropin alfa), KOGENATE FS (Octocog alfa), HERCEPTIN (Trastuzumab), HUMATROPE (SOMATROPIN), HUMIRA (Adalimumab), Insulin in Solution, INFERGEN® (Interferon alfacon-1), KINERET® (anakinra), Kogenate FS (Antihemophilic Factor), LEUKIN (SARGRAMOSTIM Recombinant human granulocyte-macrophage colony stimulating factor (rhuGM-CSF)), CAMPATH (Alemtuzumab), RITUXAN® (Rituximab), TNKase (Tenecteplase), MYLOTARG (gemtuzumab ozogamicin), NATRECOR (nesiritide), ARANESP (darbepoetin alfa), NEULASTA (pegfilgrastim), NEUMEGA (oprelvekin), NEUPOGEN (Filgrastim), NORDITROPIN CARTRIDGES (Somatropin), NOVOSEVEN (Eptacog alfa), NUTROPIN AQ (somatropin), Oncaspar (pegaspargase), ONTAK (denileukin diftitox), ORTHOCLONE OKT (muromonab-CD3), OVIDREL (choriogonadotropin

alfa), PEGASYS (peginterferon alfa-2a), PROLEUKIN (Aldesleukin), PULMOZYME (dornase alfa), Retavase (Reteplase), REBETRON Combination Therapy containing REBETOL® (Ribavirin) and INTRON® A (Interferon alfa-2b), REBIF (interferon beta-1a), REFACTO (Antihemophilic Factor), REFLUDAN (lepirudin), REMICADE (infliximab), REOPRO (abciximab)ROFERON®-A (Interferon alfa-2a), SIMULECT (baasiliximab), SOMAVERT (Pegivisomant), SYNAGIS® (palivizumab), Stemben (Ancestim, Stem cell factor), THYROGEN, INTRON® A (Interferon alfa-2b), PEG-INTRON® (Peginterferon alfa-2b), XIGRIS® (Drotrecogin alfa activated), XOLAIR® (Omalizumab), ZENAPAX® (daclizumab), and ZEVALIN® (Ibritumomab Tiuxetan).

29. A composition according to any of the foregoing or the following, wherein the protein is Ab-hCD22 or a fragment thereof, or a variant, derivative, or modification of Ab-hCD22 or of a fragment thereof; Ab-hIL4R or a fragment thereof, or a variant, derivative, or modification of Ab-hIL4R or of a fragment thereof; Ab-hOPGL or a fragment thereof, or a variant, derivative, or modification of Ab-hOPGL or of a fragment thereof, or Ab-hB7RP1 or a fragment thereof, or a variant, derivative, or modification of Ab-hB7RP1 or of a fragment thereof.

30. A composition according to any of the foregoing or the following, wherein the protein is: Ab-hCD22 or Ab-hIL4R or Ab-hOPGL or Ab-hB7RP1.

31. A composition according to any of the foregoing or the following comprising a protein and a solvent, the protein having a buffer capacity per unit volume per pH unit of at least that of 4.0 mM sodium acetate in water over the range of pH 4.0 to 5.0 or pH 5.0 to 5.5, particularly as determined by the methods described in Examples 1 and 2, wherein the buffer capacity per unit volume of the composition exclusive of the protein is equal to or less than that of 2.0 mM sodium acetate in water over the same ranges preferably determined in the same way.

32. A composition according to any of the foregoing or the following comprising a protein and a solvent, wherein at the pH of the composition the buffer capacity of the protein is at least 1.63 mEq per liter for a pH change of the composition of plus or minus 1 pH unit wherein the buffer capacity of the composition exclusive of the protein is equal to or less than 0.81 mEq per liter at the pH of the composition for a pH change of plus or minus 1 pH unit.

33. A lyophilate which upon reconstitution provides a composition in accordance with any of the foregoing or the following.

34. A kit comprising in one or more containers a composition or a lyophilate in accordance with any of the foregoing or the following, and instructions regarding use thereof.

35. A process for preparing a composition or a lyophilate according to any of the foregoing or the following, comprising removing residual buffer using a counter ion.

36. A process for preparing a composition or a lyophilate according to any of the foregoing or the following, comprising removing residual buffer using any one or more of the following in the presence of a counter ion: chromatography, dialysis, and/or tangential flow filtration.

37. A process for preparing a composition or a lyophilate according to any of the foregoing or the following, comprising removing residual buffer using tangential flow filtration.

38. A process for preparing a composition or a lyophilate according to any of the foregoing or the following comprising a step of dialysis against a solution at a pH below that of the preparation , and, if necessary, adjusting the pH thereafter by addition of dilute acid or dilute base.

39. A method for treating a subject comprising administering to a subject in an amount and by a route effective for treatment a composition according to any of the foregoing or the following, including a reconstituted lyophilate.

Brief Description of the Figures

Figure 1 depicts titration data and buffer capacity as a function of concentration for sodium acetate standard buffers over the range from pH 5.0 to 4.0. Panel A is a graph that depicts the pH change upon acid titration of several different concentrations of a standard sodium acetate buffer, as described in Example 1. pH is indicated on the vertical axis. The amount of acid added to each solution is indicated on the horizontal axis in microequivalents of HCl added per ml of solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. Acetate concentrations are indicated in the inset. Panel B is a graph that depicts the buffer capacity of the acetate buffers over the acidic pH range as determined from the titration data depicted in Panel A, as described in Example 1. Buffer capacity is indicated on the vertical axis as microequivlents of acid per ml of buffer solution per unit change in pH (μEq/ml-pH). Acetate concentration is indicated on the horizontal axis in mM.

Figure 2 depicts titration data and buffer capacity as a function of concentrations for sodium acetate standard buffers over the range from pH 5.0 to 5.5. Panel A is a graph that depicts the pH change upon base titration of several different concentration of a standard sodium acetate buffer, as described in Example 2. pH is indicated on the vertical axis. The amount of base added to each solution is indicated on the horizontal axis in microequivalents of NaOH added per ml of solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. Acetate concentrations are indicated in the inset. Panel B is a graph that depicts the buffer capacity of the acetate buffers over the basic pH range as determined from the titration data depicted in Panel A and described in Example 2. Buffer capacity is indicated on the vertical axis as microequivlents of base per ml of buffer solution per unit change in pH (μEq/ml-pH). Acetate concentration is indicated on the horizontal axis in mM.

Figure 3 depicts the determination of acetate concentration in acetate buffer standards, as described in Example 3. The graph shows a standard curve for the determinations, with peak area indicated on the vertical axis and the acetate concentration indicated on the horizontal axis. The nominal and the measured amounts of acetate in the solutions used for the empirical determination of buffer capacity are tabulated below the graph.

Figure 4 is a graph that depicts the pH change upon acid titration of several different concentrations of Ab-hOPGL over the range of pH 5.0 to 4.0, as described in Example 4. pH is indicated on the vertical axis. The amount of acid added to the solutions is indicated on the horizontal axis in microequivalents of HCl added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. Ab-hOPGL concentrations are indicated in the inset.

Figure 5 is a graph that depicts the pH change upon base titration of several different concentrations of Ab-hOPGL over the range 5.0 to 6.0, as described in Example 5. pH is indicated on the vertical axis. The amount of base added to the solutions is indicated on the horizontal axis in microequivalents of NaOH added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. Ab-hOPGL concentrations are indicated in the inset.

Figure 6 shows the residual acetate levels in Ab-hOPGL solutions used for determining buffer capacity. The graph shows the standard curve used for the acetate determinations as described in Example 6. The nominal and the experimentally measured acetate concentrations in the solutions are tabulated below the graph.

Figure 7 is a graph depicting the buffer capacity of Ab-hOPGL plus or minus residual acetate in the pH range 5.0 to 4.0. The data were obtained as described in Example 7. The upper line shows Ab-hOPGL buffer capacity with residual acetate. The lower line shows Ab-hOPGL buffer capacity adjusted for residual acetate. The vertical axis indicates buffer capacity in microequivalents of acid per ml of Ab-hOPGL solution per unit of pH (μEq/ml-pH). The horizontal axis indicates the concentration of Ab-hOPGL in mg/ml. The buffer capacities of different concentrations of standard acetate buffers as described in Example 1 are shown as horizontal lines. The concentrations of the buffers are indicated above the lines.

Figure 8 is a graph depicting the buffer capacity of Ab-hOPGL plus or minus residual acetate in the basic pH range pH 5.0 to 6.0. The data were obtained as described in Example 8. The upper line depicts Ab-hOPGL buffer capacity with residual acetate. The lower line depicts Ab-hOPGL buffer capacity adjusted for residual acetate. The vertical axis indicates buffer capacity in microequivalents of base added per ml of buffer solution per unit of pH (μEq/ml-pH). The horizontal axis indicates the concentration of Ab-hOPGL in mg/ml. The buffer capacities of several concentrations of standard sodium acetate buffers as described in Example 2 are indicated by horizontal lines. The acetate concentrations are indicated above each line.

Figure 9 depicts, in a pair of charts, pH and Ab-hOPGL stability in self-buffering and conventionally buffered formulations. Panel A depicts the stability of self-buffered Ab-hOPGL, Ab-hOPGL formulated in acetate buffer, and Ab-hOPGL formulated in glutamate as a function of storage time at 4° C over a period of six months. The vertical axis indicates Ab-hOPGL stability in percent Ab-hOPGL monomer determined by SE-HPLC. Storage time is indicated on the horizontal axis. Panel B depicts the pH of the same three formulations measured over the same period of time. The determinations of protein stability and the measurements of pH are described in Example 9.

Figure 10 depicts titration curves and buffer capacities for several concentrations of self-buffering Ab-hB7RP1 formulations over the range of pH 5.0 to 4.0. Panel A shows the titration data. pH is indicated on the vertical axis. The amount of acid added to the solutions is indicated on the horizontal axis in microequivalents of HCl added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. The Ab-hB7RP1 concentrations are indicated in the inset. Panel B depicts the buffer capacities of Ab-hB7RP1 formulations. The upper line

shows the buffer capacities for the formulations including the contribution of residual acetate. The lower line shows the buffer capacities for formulations after subtracting the contribution of residual acetate based on SE-HPLC determinations as described in Example 3. Linear least squares trend lines are shown for the two data sets. The vertical axis indicates buffer capacity in microequivalents of acid per ml of buffer solution per unit of pH (μEq/ml-pH). The concentration of Ab-hB7RP1 is indicated on the horizontal axis in mg/ml. The buffer capacities of several concentrations of standard sodium acetate buffers as described in Example 1 are shown by dashed horizontal lines. The acetate buffer concentration are shown below each line. The results were obtained as described in Example 10.

Figure 11 depicts titration curves and buffer capacities for several concentrations of self-buffering Ab-hB7RP1 formulations over the range of pH 5.0 to 6.0. Panel A shows the titration data. pH is indicated on the vertical axis. The amount of base added to the solutions is indicated on the horizontal axis in microequivalents of NaOH added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. The Ab-hB7RP1 concentrations are indicated in the inset. Panel B depicts the buffer capacities of Ab-hB7RP1 formulations. The upper line shows the buffer capacities for the formulations containing residual acetate. The lower line shows the buffer capacities for formulations adjusted to remove the contribution of residual acetate. Linear least squares trend lines are shown for the two data sets. The vertical axis indicates buffer capacity in microequivalents of base per ml of buffer solution per unit of pH (μEq/ml-pH). The concentration of Ab-hB7RP1 is indicated on the horizontal axis in mg/ml. The buffer capacities of several concentrations of standard sodium acetate buffers as described in Example 2 are shown by dashed horizontal lines. The acetate buffer concentrations are shown above each line. The results were obtained as described in Example 11.

Figure 12 depicts Ab-hB7RP1 stability in self-buffering and conventionally buffered formulations at 4° C and 29° C. Panel A depicts the stability of self-buffered Ab-hB7RP1, Ab-hB7RP1 formulated in acetate buffer, and Ab-hB7RP1 formulated in glutamate as a function of storage at 4° C over a period of six months. The vertical axis depicts Ab-hB7RP1 monomer in the samples determined by SE-HPLC. Time is indicated on the horizontal axis. Panel B depicts the stability of the same three formulations as a function of storage at 29° C over the same period of time. Axes in

Panel B are the same as in Panel A. The determinations of protein stability by HPLC-SE are described in Example 12.

Figure 13 depicts pH stability in self buffer formulations of Ab-hB7RP1 at 4° C and 29° C. The vertical axis indicates pH. Time, in weeks, is indicated on the horizontal axis. Temperatures of the datasets are indicated in the inset. The data were obtained as described in Example 13.

Figure 14 depicts the buffer capacity of self-buffering formulations of Ab-hCD22 as a function of Ab-hCD22 concentration over the range of pH 4.0 to 6.0. Panel A depicts the buffer capacities of self-buffering Ab-hCD22 formulations as a function of Ab-hCD22 concentration over the range of pH 4.0 to 5.0. Panel B depicts the buffer capacities of self-buffering Ab-hCD22 formulations as a function of concentration over the range of pH 5.0 to 6.0. In both panels the vertical axis indicates buffer capacity in microequivalents of base per ml of buffer solution per unit of pH (μEq/ml-pH), and the horizontal axis indicates Ab-hCD22 concentrations in mg/ml. For reference, the buffer capacity of 10 mM sodium acetate as described in Example 1 is shown in both panels by a dashed horizontal line. The results shown in the Figure were obtained as described in Example 14.

Figure 15 depicts titration curves and buffer capacities for several concentrations of self-buffering Ab-hIL4R formulations over the range of pH 5.0 to 4.0. Panel A shows the titration data. pH is indicated on the vertical axis. The amount of acid added to the solutions is indicated on the horizontal axis in microequivalents of HCl added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. The Ab-hIL4R concentrations are indicated in the inset. Panel B depicts the buffer capacities of Ab-hIL4R as a function of concentration. The linear least squares trend line is shown for the dataset. The vertical axis indicates buffer capacity in microequivalents of base per ml of buffer solution per unit of pH (μEq/ml-pH). The concentration of Ab-hIL4R is indicated on the horizontal axis in mg/ml. The buffer capacities of several concentrations of standard sodium acetate buffers as described in Example 1 are shown by dashed horizontal lines. The acetate buffer concentrations are shown above each line. The results were obtained as described in Example 15.

Figure 16 depicts titration curves and buffer capacities for several concentrations of self-buffering Ab-hIL4R formulations over the range of pH 5.0 to 6.0. Panel A shows the titration data. pH is indicated on the vertical axis. The amount

of base added to the solutions is indicated on the horizontal axis in microequivalents of NaOH added per ml of buffer solution (μEq/ml). The linear least squares trend lines are depicted for each dataset. The Ab-hIL4R concentrations are indicated in the inset. Panel B depicts the buffer capacities of Ab-hIL4R as a function of concentration. The linear least squares trend line is shown for the dataset. The vertical axis indicates buffer capacity in microequivalents of base per ml of buffer solution per unit of pH (μEq/ml-pH). The concentration of Ab-hIL4R is indicated on the horizontal axis in mg/ml. The buffer capacities of several concentrations of standard sodium acetate buffers as described in Example 2 are shown by dashed horizontal lines. The acetate buffer concentrations are shown above each line. The results were obtained as described in Example 16.

Figure 17 depicts Ab-hIL4R and pH stability in acetate buffered and self-buffered formulations of Ab-hIL4R at 37° C as a function of time. Panel A is a bar graph showing Ab-hIL4R stability over four weeks at 37° C. The vertical axis indicates stability in per cent monomeric Ab-hIL4R as determined by SE-HPLC. The horizontal axis indicates storage time in weeks. The insert identifies the data for the acetate and for the self-buffered formulations. Panel B shows the pH stability of the same formulations for the same conditions and time periods. The pH is indicated on the vertical axis. Storage time in weeks is indicated on the horizontal axis. Data for the acetate and self-buffered formulations are indicated in the inset. The data were obtained as described in Example 17.

Glossary

The meanings ascribed to various terms and phrases as used herein are illustratively explained below.

"A" or "an" herein means "at least one;" "one or more than one."

"About," unless otherwise stated explicitly herein, means ∀ 20%. For instance about 100 herein means 80 to 120, about 5 means 4 to 6, about 0.3 means 0.24 to 0.36, and about 60% means 48% to 72% (not 40% to 80%).

"Agonist(s)" means herein a molecular entity that is different from a corresponding stimulatory ligand but has the same stimulatory effect. For instance (although agonists work through other mechanisms), for a hormone that stimulates an

activity by binding to a corresponding hormone receptor, an agonist is a chemically different entity that binds the hormone receptor and stimulates its activity.

"Antagonist(s)" means herein a molecular entity that is different from a corresponding ligand and has an opposite effect. For instance (although antagonists work through other mechanisms), one type of antagonist of a hormone that stimulates an activity by binding to a corresponding hormone receptor is a chemical entity that is different from the hormone and binds the hormone receptor but does not stimulate the activity engendered by hormone binding, and by this action inhibits the effector activity of the hormone.

"Antibody(s)" is used herein in accordance with its ordinary meaning in the biochemical and biotechnological arts.

Among antibodies within the meaning of the term as it is used herein, are those isolated from biological sources, including monoclonal and polyclonal antibodies, antibodies made by recombinant DNA techniques (also referred to at times herein as recombinant antibodies), including those made by processes that involve activating an endogenous gene and those that involve expression of an exogenous expression construct, including antibodies made in cell culture and those made in transgenic plants and animals, and antibodies made by methods involving chemical synthesis, including peptide synthesis and semi-synthesis. Also within the scope of the term as it is used herein, except as otherwise explicitly set forth, are chimeric antibodies and hybrid antibodies, among others.

The prototypical antibody is a tetrameric glycoprotein comprised of two identical light chain–heavy chain dimers joined together by disulfide bonds. There are two types of vertebrate light chains, kappa and lambda. Each light chain is comprised of a constant region and a variable region. The two light chains are distinguished by constant region sequences. There are five types of vertebrate heavy chains: alpha, delta, epsilon, gamma, and mu. Each heavy chain is comprised of a variable region and three constant regions. The five heavy chain types define five classes of vertebrate antibodies (isotypes): IgA, IgD, IgE, IgG, and IgM. Each isotype is made up of, respectively, (a) two alpha, delta, epsilon, gamma, or mu heavy chains, and (b) two kappa or two lambda light chains. The heavy chains in each class associate with both types of light chains; but, the two light chains in a given molecule are both kappa or both lambda. IgD, IgE, and IgG generally occur as "free" heterotetrameric glycoproteins. IgA and IgM generally occur in complexes comprising several IgA or

several IgM heterotetramers associated with a "J" chain polypeptide. Some vertebrate isotypes are classified into subclasses, distinguished from one another by differences in constant region sequences. There are four human IgG subclasses, IgG1, IgG2, IgG3, and IgG4, and two IgA subclasses, IgA1 and IgA2, for example. All of these and others not specifically described above are included in the meaning of the term "antibody(s)" as used herein.

The term "antibody(s)" further includes amino acid sequence variants of any of the foregoing as described further elsewhere herein.

"Antibody-derived" as used herein means any protein produced from an antibody, and any protein of a design based on an antibody. The term includes in its meaning proteins produced using all or part of an antibody, those comprising all or part of an antibody, and those designed in whole or in part on the basis of all or part of an antibody. "Antibody-derived" proteins include, but are not limited to, Fc, Fab, and Fab_2 fragments and proteins comprising the same, V_H domain and V_L domain fragments and proteins comprising the same, other proteins that comprise a variable and/or a constant region of an antibody, in whole or in part, scFv(s) intrabodies, maxibodies, minibodies, diabodies, amino acid sequence variants of the foregoing, and a variety of other such molecules, including but not limited to others described elsewhere herein.

"Antibody-related" as used herein means any protein or mimetic resembling in its structure, function, or design an antibody or any part of an antibody. Among "antibody-related" proteins as the term is used herein are "antibody-derived" proteins as described above. It is to be noted that the terms "antibody-derived" and "antibody-related" substantially overlap; both terms apply to many such proteins. Examples of "antibody-related" proteins, without implying limitation in this respect, are peptibodies and receptibodies. Other examples of "antibody-related" proteins are described elsewhere herein.

"Antibody polypeptide(s)" as used herein, except as otherwise noted, means a polypeptide that is part of an antibody, such as a light chain polypeptide, a heavy chain polypeptide and a J chain polypeptide, to mention a few examples, including among others fragments, derivatives, and variants thereof, and related polypeptides.

"Approximately" unless otherwise noted means the same as about.

"Binding moiety(s)" means a part of a molecule or a complex of molecules that binds specifically to part of another molecule or complex of molecules. The binding

moiety may be the same or different from the part of the molecule or complex of molecules to which it binds. The binding moiety may be all of a molecule or complex of molecules as well.

"Binds specifically" is used herein in accordance with its ordinary meaning in the art and means, except as otherwise noted, that binding is stronger with certain specific moieties than it is to other moieties in general, that it is stronger than non-specific binding that may occur with a wide variety of moieties, and that binding is selective for certain moieties and does not occur to as strong an extent with others. In the extreme case of specific binding, very strong binding occurs with a single type of moiety, and there is no non-specific binding with any other moiety.

"Co-administer" means an administration of two or more agents in conjunction with one another, including simultaneous and/or sequential administration.

"Cognate(s)" herein means complementary, fitting together, matching, such as, for instance, two jigsaw puzzles that fit one another, the cylinder mechanism of a lock and the key that opens it, the substrate binding site of an enzyme and the substrate of the enzyme, and a target and target binding protein that binds specifically thereto.

"Cognate binding moieties" herein means binding moieties that bind specifically to one another. Typically, but not always, it means a pair of binding moieties that bind specifically to one another. The moieties responsible for highly selective binding of a specific ligand and ligand receptor provide an illustrative example of cognate binding moieties. Another example is provided by the moieties that binds an antigen and an antibody.

"Composition" means any composition of matter comprising one or more constituents, such as a formulation.

"Comprised of" is a synonym of "comprising" (see below).

"Comprising" means including, without further qualification, limitation, or exclusion as to what else may or may not be included. For example, "a composition comprising x and y" means any composition that contains x and y, no matter what else it may contain. Likewise, "a method comprising x" is any method in which x is carried out, no matter what else may occur.

"Concentration" is used herein in accordance with its well-known meaning in the art to mean the amount of an item in a given amount of a mixture containing the item, typically expressed as a ratio. For example, concentration of a solute, such as a protein in a solution, can be expressed in many ways, such as (but not limited to): (A)

Weight Percent (i) = weight of solute per 100 units of solvent volume; (B) Weight Percent (ii) = weight of solute per 100 units of total weight; (C) Weight Percent (iii) = weight of solute per 100 units of solvent by weight; (D) Mass Percent = mass of solute per 100 mass units of solution; (E) Mole Fraction = moles of solute per total moles of all components; (F) Molarity = moles of solute per liter of solution (*i.e.*, solute plus solvent); (G) Molality = moles of solute per Kg of solvent; and (H) Volume Molality = moles of solute per liter of solvent.

"Control region(s)" is used herein in accordance with its well-known meaning in the art, and except as noted otherwise, refers to regions in DNA or proteins that are responsible for controlling one or more functions or activities thereof. For instance, "expression control region" with reference to the control of gene expression, means the regions in DNA that are required for transcription to occur properly and that are involved in regulating when transcription occurs, how efficiently it occurs, when it is stopped, and the like.

"*De novo*" is used herein in accordance with its well-known meaning in the art, to denote something made from new. For instance, a *de novo* amino acid sequence is one not derived from a naturally occurring amino acid sequence, although, such a *de novo* sequence may have similarities with a naturally occurring sequence. *De novo* amino acid sequences can be generated, for instance, by *a priori* design, by combinatorial methods, by selection methods. They can be made, for example, by chemical synthesis, by semi-synthesis, and by a variety of recombinant DNA techniques, all of which are well know to those skilled in the art.

"Deleterious" means, as used herein, harmful. By way of illustration, "deleterious" processes include, for example, harmful effects of disease processes and harmful side effects of treatments.

"Derivative(s) " is used herein to mean derived from, in substance, form, or design, such as, for instance, a polypeptide that is based on but differs from a reference polypeptide, for instance, by alterations to its amino acid sequence, by fusion to another polypeptide, or by covalent modification.

"Disease(s)" a pathology, a condition that deleteriously affects health of a subject.

"Disorder(s)" a malediction, a condition that deleteriously alters health.

"Dysfunction" means, as used herein, a disorder, disease, or deleterious effect of an otherwise normal process.

"Effective amount" generally means an amount which provides the desired local or systemic effect. For example, an effective amount is an amount sufficient to effectuate a beneficial or desired clinical result. The effective amount can be provided all at once in a single administration or in fractional amounts that provide the effective amount in several administrations. The precise determination of what would be considered an effective amount may be based on factors individual to each subject, including their size, age, injury, and/or disease or injury being treated, and amount of time since the injury occurred or the disease began. One skilled in the art will be able to determine the effective amount for a given subject based on these considerations which are routine in the art. As used herein, "effective dose" means the same as "effective amount."

"Effective route" generally means a route which provides for delivery of an agent to a desired compartment, system, or location. For example, an effective route is one through which an agent can be administered to provide at the desired site of action an amount of the agent sufficient to effectuate a beneficial or desired clinical result.

"Endogenous" (such as endogenous gene) is used herein to refer to, for instance, genes and other aspects of DNA, such as control regions, that naturally occur in a genome and organism, unless otherwise indicated.

"Exogenous" (such as exogenous gene), unless otherwise indicated, is used herein generally to mean, for instance, DNA from an outside source, such as DNA introduced to a cell and incorporated into its genome.

"FBS" means fetal bovine serum.

"Formulation(s)" means a combination of at least one active ingredient with one or more other ingredients for one or more particular uses, such as storage, further processing, sale, and/or administration to a subject, such as, for example, administration to a subject of a specific agent in a specific amount, by a specific route, to treat a specific disease.

"Fragment(s)" herein means part of a larger entity, such as a part of a protein; for instance, a polypeptide consisting of less than the entire amino acid sequence of a larger polypeptide. As used herein, the term includes fragments formed by terminal deletion and fragments formed by internal deletion, including those in which two or more non-contiguous portions of a polypeptide are joined together to form a smaller polypeptide, which is a fragment of the original.

"Fusion protein(s)" herein means a protein formed by fusing all or part of two polypeptides, which may be either the same or different. Typical fusion proteins are made by recombinant DNA techniques, by end to end joining of nucleotides encoding the two (or more) polypeptides.

"Genetically engineered" herein means produced using a deliberate process of genetic alteration, such as by recombinant DNA technology, classical methods of genetic manipulation, chemical methods, a combination of all three, or other methods.

"Homolog(s)" herein means having homology to another entity, such as a protein that is homologous to another protein. Homologous means resembling in structure or in function.

"Ionization" herein means the change of net charge on a substance by at least one, including loss or gain of charge, such as the ionization of acetic acid in low pH solution, from HOAc to OAc^- and H^+.

"k" herein denotes an equilibrium co-efficient, in accordance with its standard meaning in chemistry.

"k_a" herein denotes the dissociation constant of a particular hydrogen of a molecule, in accordance with its standard meaning in chemistry, such as, for example, the dissociation constant of the acidic hydrogen of acetic acid.

"k_d" herein denotes a dissociation constant of a pair of chemical entities (or moieties), in accordance with its standard meaning in chemistry.

"Kit" means a collection of items used together for a given purpose or purposes.

"Ligand(s)" herein means a molecular entity that binds selectively and stoichiometrically to one or more specific sites on one more other molecular entities. Binding typically is non-covalent, but can be covalent as well. A very few examples, among many others, are (a) antigens, which typically bind non-covalently to the binding sites on cognate antibodies; (b) hormones, which typically bind hormone receptors, non-covalently; (c) lectins, which bind specific sugars, non-covalently; (d) biotins, which bind multiple sites on avidin and other avidin-like proteins, non-covalently; (e) hormone antagonists, which bind hormone receptors and inhibit their activity and/or that of the corresponding hormone; and (f) hormone agonists, which similarly bind hormone receptors but stimulate their activity.

"Ligand-binding moiety(s)" herein means a molecular entity that binds a ligand, typically, a part of a larger molecular entity that binds the ligand, or a molecular entity derived therefrom.

"Ligand-binding protein(s)" herein means a protein that binds a ligand.

"Ligand moiety(s)" herein means a molecular entity that binds to a ligand-binding molecular entity in much the same way as does the corresponding ligand. A ligand moiety can be all of a ligand, or part of it, derived from a ligand, or generated *de novo*. Typically, however, the ligand moiety is more or less exclusively the aspect thereof that binds corresponding ligand-binding entities. The ligand moiety need not comprise, and the term generally does not denote, structural features other than those required for ligand binding.

"mEq" herein means milliequivalent(s).

"μEq" herein means microequivalent(s).

"Mimetic(s)" herein means a chemical entity with structural or functional characteristics of another, generally unrelated chemical entity. For instance, one kind of hormone mimetic is a non-peptide organic molecule that binds to the corresponding receptor in the same way as the corresponding hormone.

"mM" means millimolar; 10^{-3} moles per liter.

"Modified protein(s)," "modified polypeptide(s)," or "modified fragment(s)" herein means a protein or a polypeptide or a fragment of a protein or polypeptide comprising a chemical moiety (structure) other than those of the twenty naturally occurring amino acids that form naturally occurring proteins. Modifications most often are covalently attached, but can also be attached non-covalently to a protein or other polypeptide, such as a fragment of a protein.

"Moiety(s)" herein means a molecular entity that embodies a specific structure and/or function, without extraneous components. For instance, in most cases, only a small part of a ligand-binding protein is responsible for ligand binding. This part of the protein, whether continuously encoded or discontinuously, is an example of a ligand-binding moiety.

"Naturally occurring" means occurs in nature, without human intervention.

"Non-naturally occurring" means does not occur in nature or, if it occurs in nature, is not in its naturally occurring state, environment, circumstances, or the like.

"PBS" means phosphate buffered saline.

"Peptibody" refers to a molecule comprising an antibody Fc domain (i.e., C_H2 and C_H3 antibody domains) that excludes antibody C_H1, C_L, V_H, and V_L domains as well as Fab and F(ab)2, wherein the Fc domain is attached to one or more peptides, preferably a pharmacologically active peptide, particularly preferably a randomly

generated pharmacologically active peptide. The production of peptibodies is generally described in PCT publication WO00/24782, published May 4, 2000, which is herein incorporated by reference in its entirety, particularly as to the structure, synthesis, properties, and uses of peptibodies.

"Peptide(s)" herein means the same as polypeptide; often, but not necessarily, it is used in reference to a relatively short polypeptide,

"pH" is used in accordance with its well-known and universal definition as follows:

$pH = - \log [H_3O^+]$.

"Pharmaceutical" as used herein means is acceptable for use in a human or non-human subject for the treatment thereof, particularly for use in humans, and approved therefor by a regulatory authority empowered to regulate the use thereof such as , for example, the Food and Drug Administration in the United States, European Agency for the Evaluation of Medicinal Products, Japan's Ministry of Health, Labor and Welfare, or other regulatory agency such as those listed in R. Ng, DRUGS: FROM DISCOVERY TO APPROVAL, Wiley-Liss (Hoboken, NJ) (2004), which is herein incorporated by reference in its entirety, particularly as to regulatory authorities concerned with drug approval, especially as listed in Chapter 7. As used herein the phrase "wherein the composition has been approved for pharmaceutical use by an authority legally empowered to grant such approval" means an entity or institution or the like, established by law and by law charged with the responsibility and power to regulate and approve the use of drugs for use in humans, and in some cases, in non-humans. Approval by any one such agency anywhere meets this qualification. It is not necessary for the approving agency to be that of the state in witch, for instance, infringement is occurring. Example of such entities include the U. S Food and Drug Administration and the other agencies listed herein above.

As used herein, "pharmaceutical" also may refer to a product produced in accordance with good manufacturing practices, such as those described in, among others, Chapter 9 and Chapter 10, of R. Ng, DRUGS: FROM DISCOVERY TO APPROVAL, Wiley-Liss (Hoboken, NJ) (2004), which is herein incorporated by reference in its entirety, particularly in parts pertinent to good manufacturing practices for pharmaceutical protein formulations, in particular, as set forth in Chapters 9 and 10.

"Pharmaceutically acceptable" is used herein in accordance with its well-known meaning in the art to denote that which is acceptable for medical or veterinary use,

preferably for medical use in humans, particularly approved for such use by the US Food and Drug Administration or other authority as described above regarding the meaning of "pharmaceutical."

"Polypeptide(s)" see "Protein(s)."

"Precursor(s)" is used herein in accordance with its well-known meaning in the art to denote an entity from which another entity is derived. For instance, a precursor protein is a protein that undergoes processing, such as proteolytic cleavage or modification, thereby giving rise to another precursor protein (which will undergo further processing) or a mature protein.

"Protein(s)" herein means a polypeptide or a complex of polypeptides, in accordance with its well-known meaning in the art. As used herein, "protein(s)" includes both straight chain and branched polypeptides. It includes unmodified and modified polypeptides, including naturally occurring modifications and those that do not occur naturally. Such modifications include chemical modifications of the termini, the peptide backbone, and the amino acid side chains; amino acid substitutions, deletions and additions; and incorporation of unusual amino acids and other moieties, to name just a few such modifications. It also includes "engineered" polypeptides and complexes thereof, such as, but not limited to, any polypeptide or complex of polypeptides that has been deliberatively altered in its structure by, for instance, recombinant DNA techniques, chemical synthesis, and/or covalent modification, including deliberate alteration of amino acid sequence and/or posttranslational modifications.

"Protonation" means the addition of at least one hydrogen.

"Self-buffering" means the capacity of a substance, such as a pharmaceutical protein, to resist change in pH sufficient for a given application, in the absence of other buffers.

"Semi-*de novo*" herein means (a) partly designed in accordance with a particular reference and or produced from a precursor, and (b) partly designed without reference to a particular reference (such as designed solely by general principles and not based on any particular reference). For example, a polypeptide made by producing a first peptide in a bacterial expression system, producing a second peptide by chemical synthesis, and then joining the two peptides together to form the polypeptide.

"Semi-synthesis" means as used herein a combination of chemical and non-chemical methods of synthesis.

"Subject" means a vertebrate, such as a mammal, such as a human. Mammals include, but are not limited to, humans, farm animals, sport animals, and pets. Subjects in need of treatment by methods and/or compositions of the present invention include those suffering from a disorder, dysfunction, or disease, or a side effect thereof, or from a side effect of a treatment thereof.

"Substantially" is used herein in accordance with its plain and ordinary definition to mean to a great extent or degree. For example, substantially complete means complete to a great extent, complete to a great degree. By way of further illustration, substantially free of residue means to a great extent free of residue, free of residue to a great degree. Should numerical accuracy be required, depending on context, "substantially," as used herein means, at least, 80% or more, particularly 90% or more, very particularly 95% or more.

"Therapeutically effective" is used herein in accordance with its well-known meaning in the art to denote that which achieves an improvement in the prognosis or condition of a subject or that otherwise achieves a therapeutic objective, including, for instance, a reduction in the rate of progress of a disease even if a subject's condition, nonetheless, continues to deteriorate.

"Therapeutically effective amount" generally is used to qualify the amount of an agent to encompass those amounts that achieve an improvement in disorder severity. For example, effective neoplastic therapeutic agents prolong the survivability of the subject, inhibit the rapidly-proliferating cell growth associated with the neoplasm, or effect a regression of the neoplasm. Treatments that are therapeutically effective within the meaning of the term as used herein, include treatments that improve a subject's quality of life even if they do not improve the disease outcome per se.

"Treat," "treating," or "treatment" are used broadly in relation to the invention and each such term encompasses, among others, preventing, ameliorating, inhibiting, or curing a deficiency, dysfunction, disease, or other deleterious process, including those that interfere with and/or result from a therapy.

"Variant(s)" herein means a naturally occurring or synthetic version of, for instance, a protein that is structurally different from the original but related in structure and/or function, such as an allelic variant, a paralog, or a homolog of a protein.

Description of the Invention

The invention provides for the first time self-buffering protein formulations, particularly biopharmaceutical protein formulations, methods for making the formulations, and methods for using the formulations, among other things. Any protein that provides sufficient buffer capacity within the required pH range at a concentration suitable for its intended use can be prepared as a self-buffering protein formulation in accordance with the invention. The invention can be practiced with a variety of proteins, including both naturally-occurring proteins and "engineered" proteins, particularly biopharmaceutical proteins, as discussed further below.

The utility of proteins, particularly biopharmaceutical proteins, to be formulated in self-buffering compositions, particularly pharmaceutically acceptable compositions, has not been recognized prior to the invention herein disclosed. The influence of proteins in the regulation of physiological pH has been recognized and studied for some time. However, it has not heretofore been recognized that proteins, particularly biopharmaceutical proteins, can have enough buffer capacity to maintain a formulation within a desired pH range, without additional buffering agents.

Biopharmaceutical proteins for use in the United States are formulated as buffered solutions, unbuffered solutions, amorphous or crystalline suspensions, and lyophilates.

Most of the buffered solution formulations use a conventional buffering agent. Two proteins, Pulmozyme® and Humulin®, are formulated as solutions without conventional buffering agents. Neither of these proteins provides substantial self-buffering capacity in the formulations.

Pulmozyme® has a molecular weight of about 37,000 Daltons and contains 5 histidines, 22 aspartic acids, and 12 glutamic acids, among its 260 amino acids. The buffering capacity of the protein within 0.5 pH units of pH 6.3 is determined substantially by its histidine content. On this basis, the upper limit of the self-buffering capacity of the formulation is determined by the effective concentration of the histidine residues, 0.15 mM. The molar concentration of aspartic acid and glutamic acid in the formation is 0.9 mM. The total molar concentration of all three amino acids together, thus, is just a little over 1 mM, at the concentration of the formulation.

Humulin® is formulated at 3.5 g/ml. It has a molecular weight of about 6,000 Daltons and contains 2 aspartic acids, 8 glutamic acids, and 2 histidines. None of these amino acids is a particularly effective buffer at the pH of the formulation: 7.0 to 7.8.

At this concentration the molar concentration of histidines, which are closest in pK_a to the pH of the formulation, is 1.16 mM.

The biopharmaceutical lyophilates are reconstituted prior to use forming solutions or suspensions. Most of the lyophilates contain conventional buffers that maintain the proper pH of the reconstituted formulations. A few others, in which the protein concentration is low or the pH must be low (less than 3) or high (greater than 9.5), are, effectively unbuffered.

Thus, buffering is achieved in current biopharmaceutical protein formulations using conventional buffering agents. The ability of proteins by themselves to buffer pharmaceutical protein formulations has not been fully appreciated and has not been used for the manufacture of protein pharmaceuticals.

The determination of protein buffer capacity, typically, is important to developing self-buffering protein formulations in accordance with the invention. Pertinent thereto, methods for measuring buffer capacity and for determining the buffer capacity of proteins are described below. To allow ready comparability of data, protein buffer capacity must be expressed in comparable units and/or related to a buffer standard. Accordingly, the following section describes pH metrics and standards that meet these requirements, in accordance with the invention.

1. Buffering

A widely accepted definition of buffering is the resistance to change in pH of a composition upon addition of acid or base. Buffer capacity thus often is defined as the ability of a composition to resist pH change.

Typically buffer capacity is expressed in terms of the amount of strong acid or base required to change the pH of a composition a given amount. Van Slyke provided the most widely used quantitative measure of buffer capacity, according to which, for a solution, buffer capacity is expressed as the amount of strong acid or base required to change the pH of one liter of the solution by one pH unit under standard conditions of temperature and pressure.

According to this measure, for instance, the buffer capacity of 1 liter of 5 mM HOAc, 5 mM NaOAc, pH 4.76 in pure water is 4.09×10^{-3} moles of a univalent strong base (*i.e.*, 4.09×10^{-3} equivalents of base), which can be calculated as follows.

The Henderson-Hasselbalch equation for the solution is:

$$pH = \log \{[5\ mM]\ NaOAc / [5\ mM]\ HOAc\} + 4.76$$

Accordingly, the concentration, X, of a univalent strong base required to increase the pH of this buffer is:

4.76 to 5.76 is 5.76 = log {[5 mM + X mM] NaOAc / [5 mM - X mM] HOAc} + 4.76

Thus:

1.00 = log {[5 mM + X mM] NaOAc / [5 mM - X mM] HOAc}

10.0 = [5 mM + X mM] NaOAc / [5 mM - X mM] HOAc

10.0 = (5 mM + X mM) / (5 mM - X mM)

50 mM - 10X mM= 5 mM + X mM

11X mM = 45 mM

X = 4.09 mM,

which, for one liter yields:

(4.09 x 10^{-3} moles / liter)(1 liter)(1 equivalent / mole) = 4.09 x 10^{-3} equivalents

Thus, according to this measure, the buffer capacity of 1 liter of a 10mM acetate buffer containing 5 mM NaOAc and 5 mM HOAC at a pH of 4.76 in pure water is 4.09 x 10^{-3} equivalents of base per liter per pH unit. Put other ways, the buffer capacity of the solution is 4.09 milliequivalents of base per liter per pH unit, 4.09 microequivalents of base per milliliter per pH unit, 0.409 microequivalents of base per 100 microliters per pH unit, 40.9 nanomoles of base per 10 microliters per pH unit, and 4.09 nanonmoles of base per microliter per pH unit.

The same calculation yields the following buffer capacity for other concentrations of this acetate buffer at pH 4.76. A 2 mM acetate buffer as above has a buffer capacity of 0.818 mEq per liter per pH unit. At 4 mM the buffer capacity is 1.636 mEq per liter per pH unit. The capacity at 5 mM is 2.045 mEq per liter per pH unit. At 7.5 mM the capacity is 3.068 mEq per liter per pH unit. At 10 mM the acetate buffer has a buffer capacity of 4.091 mEq per liter per pH unit. At 15 mM its capacity is 6.136 mEq per liter per pH unit.

It is worth noting that an acetate buffer solution at the pK_a of acetic acid (pH 4.76) is equimolar in acetic acid and acetate base. (i.e., at the pK_a the acid and base are present in equal amounts). As a result, the resistance to change in pH (buffer capacity) of an acetate buffer at the pK_a of acetic acid is the same for addition of acid and base. The equipoise to acid and base is a general characteristic of buffering agents in buffers at a pH equal to their pK_a.

At any other pH a buffer will contain different amounts of acid and base forms and, therefore, its resistance to change (i.e., its buffer capacity) upon addition of acid will not be the same as its resistance to change upon addition of base. As a result, it is preferable to define the capacity of such buffers in terms of (i) the amount of acid required to lower the pH by one unit, and (ii) the amount of base required to raise the pH by one unit.

The partitioning in a buffer between acid and base forms in a given composition, such as a pH standard, can be calculated at any pH and buffer concentration using the procedures set forth above in describing the buffer capacity of 10 mM NaOAc at pH 4.76 plus or minus (containing equimolar amounts of acetic acid and sodium acetate). And the results can be used to define the buffer capacity of a standard for reference use.

Thus, for instance, the partitioning of acetic acid into acetic acid and acetate base in a solution at pH 5.0 can be calculated readily using the foregoing procedures, and from this the buffer capacity can be calculated for both base and for acid addition. Calculated this way, the theoretical buffer capacity of 10 mM sodium acetate buffer over the range from pH 5.0 to 5.5 is approximately 2.1 mM per 0.5 pH unit and 4.2 mM per pH unit. Put another way, the buffer capacity of the buffer, theoretically, is approximately 4.2 μEq per ml of buffer solution per unit of pH change. Similarly, the theoretical buffer capacity of 10 mM sodium acetate buffer over the range from pH 5.0 to 4.0 is 4.9 mM, and, put another way, 4.9 μEq per ml of buffer per unit of pH change over a given range of pH.

While such calculations often are quite useful in many cases, empirical standards and empirical determinations are preferred. Among particularly preferred empirical standards are sodium acetate buffers over the range of pH 5.0 to 4.0 and pH 5.0 to 5.5 as exemplified in Examples 1 and 2. Especially preferred are sodium acetate buffers in accordance therewith in which the total acetate concentration is, in particular, 10 mM, preferably 5 mM, especially 4 mM, among others as set forth elsewhere herein.

Acetate buffers at pH 5.0 are more resistant to change in pH upon addition of acid than upon addition of base, as discussed above. In a preferred empirical standard of buffer capacity, the buffer capacity of a standard acetate buffer such as these is defined as: (i) the slope of the least squares regression line calculated for base titration data for the buffer from pH 5.0 to pH 5.5, and (ii) the slope of the least squares regression line calculated for acid titration data for the buffer from pH 5.0 to pH 4.0.

The preparation of standard acetate buffers and the determination of their buffer capacities are described in Examples 1, 2, and 3. It is to be appreciated that much the same methods can be used to establish and use buffer capacity standards using other suitable buffering agents.

In measuring the buffer capacity of a self-buffering protein composition in accordance with the invention, it often is convenient to express the buffer capacity in terms of the concentration of a standard buffer at the same pH having the same buffer capacity. When a standard is used that is not at the pK_a of the buffering agent, such as a sodium acetate buffer initially at pH 5.0, in accordance with the invention the self-buffering composition is defined as having a buffer capacity equal to or greater than that of the standard, if either its buffer capacity upon base titration or its buffer capacity upon acid titration (or both) is equal to or exceeds the corresponding buffer capacity of the standard.

It is to be further appreciated that the pH of self-buffering protein compositions in accordance with the invention generally will not be at the pK_a of the self-buffering protein, or any acid-base substituent therein. Indeed proteins are polyprotic and, as discussed herein, often will have several substituents, each with a somewhat different pK_a that contribute to its buffer capacity in a given pH range. Accordingly, the buffer capacity of self-buffering protein formulations in accordance with the invention preferably is determined empirically by both acid titration and base titration over a given range of pH change from the desired pH of the composition. In preferred embodiments in this regard, the buffer capacity is determined by titrating with acid and separately with base over a change of respectively + and - 1 pH unit from the starting pH of the formulation. In particularly preferred embodiments, the titration data is collected for a change in pH of plus or minus 0.5 pH units. As described in the Examples, the buffer capacity is the slope of the least squares regression line for the data for pH as a function of equivalents of acid or base added to the composition over the range of titration.

a. Empirical Measures and Standards of Buffer Capacity

In certain preferred embodiments of the invention, the measure of buffer capacity is an empirical standard. Among preferred empirical standards in this regard are a particular volume of an aqueous solution at a particular temperature and a particular pH, containing a particular buffering agent at a particular concentration and

either no other components than water, or one or more other particular components, each at a particular concentration.

A particularly preferred specific standard for determining buffer capacity in accordance with various aspects and preferred embodiments of the invention is 10 mM sodium acetate pH 5.00 in pure water free of other constituents at 21°C in equilibrium with ambient air at 1 atmosphere, as described in Examples 1 and 2, preferably expressed in equivalents per unit volume per pH unit, such as μEq/ml-pH. Buffer capacity of the standard should be measured empirically as described in Examples 1, 2, and 3, and as further discussed elsewhere herein.

A particularly preferred specific standard for determining buffer capacity in accordance with various aspects and preferred embodiments of the invention is 10 mM sodium acetate pH 4.76 in pure water free of other constituents at 21°C in equilibrium with ambient air at 1 atmosphere, as described in Examples 1 and 2, preferably expressed in equivalents per unit volume per pH unit, such as μEq/ml-pH. Buffer capacity of the standard should be measured empirically as described in Examples 1, 2, and 3, and as further discussed elsewhere herein. According to the Henderson-Hasselbalch equation, as noted above, the calculated buffer capacity of this standard over the range of pH 4.76 plus or minus 1 pH unit is 4.09 microequivalents per milliliter per pH unit (4.09 μEq/ml-pH).

A variety of other buffers are available for use as standards in other ranges of pH in accordance with various aspects and preferred embodiments of the invention in this regard. Reference buffers are particularly preferred in this regard, such as those well-known and routinely employed for analytical chemistry determinations. A variety of such buffering agents are set forth in textbooks on analytical chemistry and in monographs on the accurate determination of pH and buffer capacity.

Also useful in the invention in this regard are biological buffers, such as those described in, among other texts: TEITZ TEXTBOOK OF CLINICAL CHEMISTRY, 3rd Ed., Burtis and Ashwood, eds., W.B. Saunders Company, Philadelphia, PA (1999), in particular in Tables 50-13 to 50-16, which are herein incorporated by reference in their entireties as to buffering agents and buffers and their use as pH and/or buffer capacity standards in accordance with the invention in this respect; THE TOOLS OF BIOCHEMISTRY, Terrance G. Cooper, John Wiley & Sons, New York, NY (1977), in particular Chapter 1, pages 1-35, which is herein incorporated by reference in its entirety as to buffering agents and buffers and their use as pH and buffer capacity

standards in accordance with the invention in this respect, most particularly as to Tables 1-3, 1-4, and 1-5 and text relating thereto, and PROTEIN PURIFICATION PRINCIPLES AND PRACTICE, 3rd Ed., Robert K. Scopes, Springer-Verlag, New York, NY (1994), in particular pages 160-164, especially therein Tables 6.4 and 6.5 and text relating thereto, Chapter 12, section 3, pages 324-333, especially therein Tables 12-4 and 12-5 and text relating thereto, and all of Appendix C: Buffers for Use in Protein Chemistry, which are herein incorporated by reference in their entirety as to buffering agents and buffers and their use in accordance with the invention in this respect.

Since some dissolved gases in water react with OH^- and/or H_3O^+, however, the empirically determined buffer capacity of the standard solution may vary somewhat from the theoretical value. Hence, the definition of the standard requires that the solution be in equilibrium with the atmosphere at a pressure of 1 atmosphere. In addition, the buffer standard must be held in and contacted only with materials that do not alter its components or its buffer capacity, such as those that leach acids, bases, or other reactants that may alter the effective concentration or activity of the acetate buffer in any way that would alter its buffer capacity. Given both of the foregoing, atmospheric equilibration and inertness of the container, buffer capacity of the standard will scale directly and linearly with its volume. Accordingly, the buffer capacity of 100 ml will be 1/10 that of 1.00 liter, and the buffer capacity of 10 ml will be 1/100 that of 1.00 liter. Accordingly, the volume of the standard can be adjusted for convenience and then normalized back to 1 liter as desired.

It may not always be convenient to make the foregoing 10 mM acetate buffer capacity standard for field use. However, a variety of other buffer capacity standards can be made and used in the same way as the acetate buffer, using a variety of other buffering agents. Provided only that the buffering standards are prepared properly, they can be calibrated against the acetate buffering standard described above and then used in the field. The results obtained with such alternative standards may then be expressed in terms of the foregoing acetate standard without substantial distortion or error.

The buffer capacity of such alternative standards also can be calibrated by calculation. To do so, the buffer capacity of the alterative standard is determined directly and expressed in mEq per unit volume per unit of pH. Determinations based on the alternative standard then can be normalized to the acetate standard using the ratio between the buffering capacities expressed in mEq per unit volume per unit of pH of the alternative and the acetate standards.

Using such methods, which are commonly employed in metrology to relate field standards back to a reference standard, the acetate buffer standard described above provides a portable, scalable, reliable, and accurate reference for determining the buffer capacity of any composition that readily can be compared with disparate measures made on other compositions using similar methods.

b. Preparation of Buffer Capacity Standards

Buffer capacity standards can be prepared using well-established methods of analytical chemistry. See for instance, ANALYTICAL CHEMISTRY, 3rd Ed., Douglas A. Skoog and Donald M. West, Holt, Rinehart and Winston, New York (1979), particularly chapter 9 (pages 186-226), chapter 10 (pages 227-233), and methods described on pages 583-588; TEITZ TEXTBOOK OF CLINICAL CHEMISTRY, 3rd Ed., Burtis and Ashwood, eds., W.B. Saunders Company, Philadelphia, PA (1999), in particular Chapter 1 regarding general laboratory techniques for preparing and calibrating buffers and Tables 50-13 to 50-16; THE TOOLS OF BIOCHEMISTRY, Terrance G. Cooper, John Wiley & Sons, New York, NY (1977), in particular Chapter 1, pages 1-35, and Tables 1-3, 1-4, and 1-5 and text relating thereto; PROTEIN PURIFICATION PRINCIPLES AND PRACTICE, 3rd Ed., Robert K. Scopes, Springer-Verlag, New York, NY (1994), in particular pages 160-164, especially therein Tables 6.4 and 6.5 and text relating thereto, Chapter 12, section 3, pages 324-333, especially therein Tables 12-4 and 12-5 and text relating thereto, and all of Appendix C: Buffers for Use in Protein Chemistry; and REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 21st Ed., Beringer *et al.* Editors, Lippincott, Williams & Wilkins, Philadelphia, PA (2005), particularly in parts relating to buffering agents, buffers, buffer capacity and the like; each of which is herein incorporated by reference in its entirety particularly as to the preparation and use of buffers and buffer capacity standards in accordance with the invention in this respect.

The water used for preparing buffer capacity standards should be highly purified, preferably Type I water, such as milliQ water, or triple distilled water. The buffer reagents should be pure and, in particular, free of any substance that can alter the pH or buffer capacity of the standard solution, such as Reference Grade or ACS Reagent Grade reagents suitable for use in demanding analytic chemical analyses, as described in the foregoing references, TEITZ and REMINGTON cited above in

particular, which are hereby incorporated by reference in their entireties particularly in parts pertinent to analytical grade water and reagents.

The exact compositions of the buffer reagents must be well established. The molecular weight of the buffer reagents must be known accurately for each buffer reagent. The molecular weights must be for the exact reagent that will be used and must include the weight of adducts such as hydrates that are present in the reagent. The effective number of hydrogen donors or hydrogen acceptors per molecule must be known accurately for each buffer reagent. The proportional distribution of different forms, such as hydrates, must be known for each reagent that contains a mixture of such forms. Concentrations of liquid buffer reagents much be known exactly, preferably in moles/volume and in moles/mass (*e.g.*, moles/liter and moles/gm or kg. Hygroscopic agents must be dried to remove moisture so that reagent can be accurately weighed.

Generally speaking, the information provided by well-established vendors of reagents and reference grade chemicals is sufficiently accurate for the preparation of buffer capacity standards as described above. And well-known standard techniques routinely employed in analytical chemistry can be used to dry "hygroscopic reagents" so that they can be weighed accurately.

As described therein, well established and routinely employed analytical chemistry methods can be employed to prepare and calibrate acid and base solutions, such as 1 N HCl and 1 N NaOH (to name just two) for titrating buffer capacity standard solutions, as well as sample protein solutions, to determine buffer capacity. It should be noted that the preparation of NaOH solutions for titration should be done so as to eliminate inaccuracies that arise from the interaction of certain dissolved gases with basic solutions, and the pH altering effects of their solvation. See for instance Skoog and West (1979) and other references cited above regarding the preparation and calibration of buffers and buffer standards, which are herein incorporated by reference in their entireties particularly in parts pertinent to the preparation of standard solutions for titration, as discussed above.

c. Empirical Measurement of Buffer Capacity

Titration of standards and samples to determine buffer capacity can be done using well- known, routine methods. Titrations can be carried out manually. They also can be carried out using an autotitrator. A wide variety of autotitrators that are suitable

for use in the invention in this regard are commercially available from numerous vendors. Methods suitable for use in the invention in this regard are the same as those described in the references cited above regarding preparation and calibration of buffer standards, each of which is incorporated herein by reference in its entirety particularly in parts pertinent to the titration of known and unknown solutions to determine their buffer capacity.

2. Buffering by Proteins and Protein Buffer Capacity

a. Determination of Protein Hydrogen Equilibria and Buffer Capacity

Proteins invariably contain many acidic and basic constituents. As a result hydrogen ion equilibrium of proteins is highly complex. In fact, a complete description of the hydrogen ion equilibria of a protein in a given environment is beyond the reach of current theoretical and computational methods. Empirical measurements of protein buffer capacities, thus are preferred. Methods developed for precise empirical measurement of protein hydrogen equilibria, which can be and are routinely employed by those skilled in the art, are well-suited to measuring the buffering properties of proteins pertinent to the development of self-buffering protein formulations in accordance with the invention. Thus, the pH titration curves of proteins can be determined in accordance with the invention by well-known methods such as those described in and exemplified by pH titration studies of Tanford and co-workers on ribonuclease. See C. Tanford, "Hydrogen Ion Titration Curves of Proteins," in T. Shedlovsky (ed.), ELECTROCHEMISTRY IN BIOLOGY AND MEDICINE, John Wiley and Sons, New York, 1955, Ch. 13; C. Tanford and J.D. Hauenstein, *J. Am. Chem. Soc.* 78, 5287 (1956), C. Tanford, PHYSICAL CHEMISTRY OF MACROMOLECULES, John Wiley and Sons, New York, 1961, particularly pages 554-567, all of which are herein incorporated by reference particularly in parts pertinent to hydrogen ion titration of proteins and to the determination of buffering action and buffer capacity of proteins.

However, the present invention does not require such precise determinations as those described in the foregoing references. Rather, the buffering properties and buffer capacity of proteins in accordance with the invention can be determined using the methods described in standard references on analytical chemistry and biochemistry, such as, for instance, Skoog (1979), Cooper (1977), and Scopes (1994), cited above, each of which is herein incorporated by reference in its entirety particularly as to the

empirical determination of titration curves, particularly of proteins within a given range of pH in accordance with the invention.

The determination of titration curves and buffer capacity in accordance with the invention is described in detail for numerous acetate buffers and a variety of pharmaceutical proteins in the Examples below. Thus, the pH titration curves of proteins can be determined empirically in accordance with such methods as described in the foregoing references over particular limited ranges of pH that are of interest to a given formulation. In many respects these methods are the same as those used in analytical chemistry for the titration of small molecules such as acetate buffers (as illustrated in the Examples). Somewhat greater care must be taken, however, in handling proteins to maintain the conformation and function required for effective formulation.

Protein titrations may be carried out manually or using automated titrators. Equipment for manual titration and automated titrators are readily available from a large number of suppliers and vendors. Methods suitable for determining pH titration curves and buffer capacity of proteins are exemplified in the Examples by reference to titration of acetate buffer standards and to titration of several different therapeutic proteins over defined ranges of pH. These methods can be employed to determine the hydrogen ionization behavior and buffer capacity of any other protein in accordance with the invention.

It is a particular aspect of the invention to determine the buffer capacity of proteins as a function of concentration in solution. In a preferred method in this regard, solutions of a given protein are prepared in a graded series of concentrations. A pH titration curve is determined for the protein at each concentration over the pH range of interest. Preferably titration curves are determined for the range of interest using both base titration and acid titration. The data are, in certain preferred embodiments, plotted on a graph of equivalents of acid or base added versus the measured pH of each solution. Typically, for ranges of about 0.5 to 1.0 pH unit, the titration data for each concentration closely fit a straight line, preferably determined by a least squares regression analysis. In preferred embodiments in this regard, buffer capacity for the protein at each concentration is equated to the slope of the regression line, expressed in units of equivalents per ml per pH unit (or fractions thereof). Also useful in the invention in this regard is the relationship between the buffer capacity of the protein and its concentration. In certain preferred embodiments, this relationship is determined

by a least squares regression analysis of the best straight line fit of the buffer capacity data determined in accordance with the foregoing plotted on a graph of buffer capacity versus protein concentration.

Empirical data on the buffer capacity of proteins in accordance with the invention preferably is related to the buffer capacity of a standard acetate buffer. That is, in particularly preferred embodiments of the invention in this regard, the buffer capacity of a given protein at a given concentration in a given formulation, determined as above, is equated to the concentration of a standard acetate buffer having the same buffer capacity.

While empirical determinations as described herein are generally a crucial aspect of formulating self-buffering compositions in accordance with various aspects and preferred embodiments of the invention, theoretical and computational methods also can be productively employed to guide the design, manufacture, and use of such compositions (in conjunction with empirical determinations), as described below.

b. Prediction of Protein Hydrogen Ion Equilibria and Buffer Capacity

The ionization of hydrogen in proteins is complex but can be broken down in general terms into pH ranges defined by the ionizable hydrogens of amino acid side chains, and the terminal amino and carboxyl groups. The pK_a of terminal carboxyls in polypeptides typically ranges around 3.1. The pK_a of the acidic hydrogens in the side chains of aspartic acid and glutamic acid range around 4.4. The pK_a of histidine in polypeptides ranges around 6.0. The terminal amino group hydrogen ionization pK_a typically ranges around 7.5. The sulfhydryl in cysteine has a pK_a range around 8.5. The tyrosine hydroxyl and the lysine amine both have pK_as ranging around 10. The pK_a of arginine ranges around 12.

Conformational folding typically partitions large polypeptides and proteins in polar solvents into exposed solvent-accessible regions and more or less non-polar core regions that have little or no contact with the ambient environment. Folding produces many environments between these two extremes. Furthermore, the micro environment around a given amino acid side chain in a protein typically is affected by one or more of: solvent effects; binding of ions; chelation; complexation; association with co-factors; and post-translational modifications; to name just a few possibilities. Each of these can influence the pK_a of a given amino acid ionization in a protein. The pK_as for

specific residues in a given protein, thus, can vary dramatically from that of a free amino acid.

Indeed, the perturbation of pK_as by microenvironments of amino acids in proteins has been used to study the folding of proteins and the disposition and charge state of specific amino acids in folded proteins. The protein titration curves reported by Tanford and others are complex with a few broad features in common. Typically only some of the ionizable protons are accounted for in the titration curves. Others apparently are located in the core and are inaccessible to solvent. The pK_as of individual side chains of the same type that can be detected in some cases can be distinguished from one another. Nonetheless, while detectably different, their pK_as generally are close to that of the free amino acid.

The strongest buffering action of proteins does not generally occur at the isoelectric point, as may be mistakenly supposed. In fact, buffering depends on the amino acid side chain hydrogens and the terminal hydrogens, and therefore occurs in ranges spanning the pK_as of the ionizable hydrogens in the free amino acids, as discussed above. The most important of these, for formulating compositions of proteins, especially certain pharmaceutical proteins that are more soluble and/or more stable, among other things, at weakly acidic pH (pH 4 to 6), is buffering action that occurs in the range of the pK_as of the carboxyl hydrogen of the amino acids aspartic acid and glutamic acid; that is, pH 4.0 to 5.5, particularly around 4.5.

There are a variety of ways available for estimating the buffer capacity of a given protein in a given solution at a given pH. Methods range from highly technical and complex computer models to those that can be carried out on a hand calculator. None of the methods is complete or entirely accurate; but, they can in some instances provide useful estimates.

For instance, a potentially useful idea of buffer capacity in some instances may be calculated for a protein in a solution based on its amino acid composition, the pK_as (in the solvent in question) of the terminal amine and carboxy groups and the amino acid side hydrogen donors and acceptors, the concentration of the protein, and the pH of the solution.

For example, a potentially useful estimate of the buffer capacity of a protein at pH in the range of the pK_a of the side chain carboxyl hydrogen of glutamic acid (as a free amino acid), can be gained from the molecular weight of the protein and the number of glutamic acid residues it contains. Dividing the former by the latter provides

the weight per equivalent of glutamic acid and, therefore, the weight per equivalent of ionizable hydrogen at the pK_a of glutamic acid. Since glutamic acid and aspartic acid side chain carboxyl groups have nearly the same pK_as, results of such calculations for the two should be added together to yield an estimate of buffer capacity in a range around both their pK_as. The estimated buffer capacity of a solution of the protein at the pK_a can be calculated from the protein's concentration in the solution and the intrinsic factor just provided, namely weight per equivalent of ionizable hydrogen. Dividing the concentration by the weight per equivalent yields an estimate for the buffer capacity in units of Eq/volume. Such estimates often will be too high, since some residues usually are sequestered in regions of the protein not accessible to the solvent, and, therefore, do not contribute to its actual buffer capacity. It may be possible in certain instances to account for the effect of sequestering on buffer capacity. For instance, a fractional coefficient that reflects theoretical or empirical estimates of sequestering can be applied to adjust the original calculation.

Such calculations generally will be of less utility and less accurate than empirical determinations of protein buffer capacity, in accordance with the methods described elsewhere herein. But they can be useful to provide rough maximum estimates of the buffer capacity of proteins in solution.

3. Proteins

The invention herein disclosed may be practiced with any protein that provides sufficient buffer capacity in a desired pH range within the parameters of protein concentration and the like required for a desired formulation. Among preferred proteins in this regard are pharmaceutical proteins for veterinary and/or human therapeutic use, particularly proteins for human therapeutic use. Also among preferred proteins are proteins that are soluble in aqueous solutions, particularly those that are soluble at relatively high concentrations and those that are stable for long periods of time. Additionally, among preferred proteins are those that have a relatively high number of solvent accessible amino acids with side chain hydrogen ionization constants near the pH of the desired buffering action.

Further among preferred proteins of the invention are proteins for pharmaceutical formulations that do not induce a highly deleterious antigenic response following administration to a subject. Preferred in this regard are proteins for

veterinary and/or human medical use, particularly, regarding the latter, humanized and human proteins.

Further among preferred proteins of the invention are proteins that bind selectively to specific targets, including ligand-binding proteins and protein ligands. Antigen-binding proteins, proteins derived therefrom, and proteins related thereto are among the particularly preferred embodiments of the invention in this regard. Highly preferred proteins of the invention in this regard are antibodies and proteins derived from antibodies or incorporating antibodies, in whole or part, including, to name just a few such entities: monoclonal antibodies, polyclonal antibodies, genetically engineered antibodies, hybrid antibodies, bi-specific antibodies, single chain antibodies, genetically altered antibodies, including antibodies with one or more amino acid substitutions, additions, and/or deletions (antibody muteins), chimeric antibodies, antibody derivatives, antibody fragments, which may be from any of the foregoing and also may be similarly engineered or modified derivatives thereof, fusion proteins comprising an antibody or a moiety derived from an antibody or from an antibody fragment, which may be any of the foregoing or a modification or derivative thereof, conjugates comprising an antibody or a moiety derived from an antibody, including any of the foregoing, or modifications or derivatives thereof, and chemically modified antibodies, antibody fragments, antibody fusion proteins, and the like, including all of the foregoing.

a. Antibodies, Antibody-Derived, and Antibody-Related Proteins and the Like

Among particularly preferred proteins in accordance with the invention are antibody polypeptides, such as heavy and light chain polypeptides that have the same amino acid sequence as those that occur in and make up naturally-occurring antibodies, such as those that occur in sera and antisera, including such polypeptides and proteins isolated from natural sources, as well as those that are made by hybridoma technologies, by activation of an endogenous gene (by homologous or non-homologous recombination, for instance), by expression of an exogenous gene under the control of an endogenous transcription control region, by expression of an exogenous expression construct, by semi-synthesis and by *de novo* synthesis, to name some techniques commonly employed for making antibodies and antibody-related polypeptides and

proteins that can be used to produce antibody polypeptides and proteins in accordance with the invention.

Included among these antibody-related polypeptides and proteins are those in whole or part having a *de novo* amino acid sequence, those that comprise all or one or more parts of an antibody (that is: a continuous chain of amino acids having the same sequence as any four or more residues in the amino acid sequence of a naturally occurring antibody polypeptide), those having an amino acid sequence that matches in some way that of a naturally occurring antibody, but differs from it in other ways, those that have the same but different amino acid sequences as a naturally occurring counterpart or sequence relating thereto, but differ from the counterpart in one or more post-translational modifications, and those comprised in part of any of the foregoing (in part or in whole) fused to one or more polypeptide regions that can be of or derived from or related to a second, different antibody polypeptide, and can be of or derived from any other polypeptide or protein, whether naturally occurring, resembling but differing therefrom, having a semi-*de novo* amino acid sequence and/or a *de novo* sequence, among others. Such hybrids are generally referred to herein as fusion polypeptides and/or fusion proteins.

Further among preferred proteins in accordance with the invention herein described are modified proteins in accordance with all of the foregoing. Included among such modified proteins are proteins modified chemically by a non-covalent bond, covalent bond, or both a covalent and non-covalent bond. Also included are all of the foregoing further comprising one or more post-translational modifications which may be made by cellular modification systems or modifications introduced *ex vivo* by enzymatic and/or chemical methods, or introduced in other ways.

Among preferred proteins of the invention in this regard are Fab fragment(s), such as those produced by cleaving a typical dimeric $(LH)_2$ antibody with certain protease that leave the light chain intact while cleaving the heavy chains between the variable region and the adjacent constant region, "above" the disulfide bonds that hold the heavy chains together. Such cleavage releases one Fc fragment comprising the remaining portions of the heavy chains linked together, and two dimeric Fab fragments each comprising an intact light chain and the variable region of the heavy chain. Fab fragments also can be produced by other techniques that do not require isolation of a naturally occurring antibody and/or cleavage with a protease.

Also preferred are Fab_2 fragment(s) such as those produced in much the same manner as Fab fragments using a protease that cleaves "between or below" the disulfide bonds. As a result, the two Fab fragments are held together by disulfide bonds and released as a single Fab_2 fragment. Fab_2 fragments can be produced by many other techniques including those that do not require isolation of an intact antibody or cleavage with a protease having the required specificity. Furthermore, both mono- and bi-specific Fab_2 fragments can now be made by a variety of routine techniques.

Also among preferred proteins in this regard are Fab_3 fragments, which are engineered antibody fragments in which three Fab fragments are linked together. Fab_3 fragments can be mono-, bi-, or tri-specific. They can be made in a variety of ways well-known to those of skill in the pertinent arts.

Among other preferred proteins in this regard are Fc fragments(s), such as those produced by cleavage with a protease in the same manner used for the production of either Fab fragments or Fab_2 fragments. However, for the production of Fc fragments, the dimeric heavy chain containing fragments are isolated rather than the light chain containing fragments. Fc fragments lack antigen combining sites, but comprise effector regions that play a role in physiological processes involving antibodies. Fc fragments can be made by a variety of techniques that are well-known and routinely employed by those of skill in the art for this purpose.

Among other preferred proteins in this regard are single-chain variable fragments ("scFv(s)"). scFv(s) are fusion proteins made by joining the variable regions of the heavy and light chains of an immunoglobulin. The heavy and light chains in an scFv typically are joined by a short serine, glycine linker. scFv(s) have the same specificity as the antibodies from which they were derived. Originally produced through phage display, scFv(s) now can be made by a variety of well-known methods.

Also preferred are Bis-scFv(s) which are fusions of two scFv(s). Bis-scFv(s) can be mono- or bi-specific. A variety of methods are well-known and can be applied in making Bis-scFv(s) in accordance with the invention.

Also preferred in accordance with the invention in this regard are minibodies; mono- and bi-specific diabodies; mono-, bi-, and tri-specific triabodies; mono-, bi-, tri-, and tetra-specific tetrabodies; VhH domains; V-NAR domains; V_H domains; V_L domains; camel Igs; Ig NARs; and others.

Also among preferred embodiments in accordance with various aspects and preferred embodiments of the invention in these and other regards are proteins

comprising one or more CDR and/or CDR-derived and/or CDR-related regions of an antibody or one or more FR and/or FR-derived and/or FR-related regions of an antibody. In this regard CDR means complementary determining region; that is, a hypervariable region of a light or heavy chain of an antibody, typically about 9 to 12 amino acids in length that usually is an important part of an antigen specific binding moiety of an antibody. FR in this regard means a framework region of an antibody; that is, a region of about 15 to 20 amino acids that separates CDRs in the antigen specific binding moiety of an antibody. The terms CDR-derived and CDR-related, and the terms FR-derived and FR-related have the same meanings as to CDR and FR, respectively, as set forth in the above Glossary for the terms antibody-derived and antibody-related as to the term antibody.

Regarding antibodies, antibody-derived, and antibody-related proteins in accordance with the foregoing and with other aspects of the invention herein disclosed, see, for instance, *Protein Engineering: Principles and Practice*, Jeffrey L. Cleland and Chares S. Craik, eds. Wiley-Liss, Inc., New York (1996), particularly therein Kelley, Robert F., "Engineering Therapeutic Antibodies," Chapter 15, pp. 399-434 and Hollinger, P. & Hudson, P., "Engineered antibody fragments and the rise of single domains," *Nature Biotechnology*, September 2005, 1126-1136, each of which is herein incorporated by reference in its entirety particularly in parts pertinent to the structure and engineering of antibodies, particularly biopharmaceutical antibodies, and antibody-derived and antibody-related proteins, particularly antibody-derived and antibody-related pharmaceutical proteins in accordance with the invention herein described.

As to all of the foregoing, particularly preferred in the invention are human, humanized, and other proteins that do not engender a significantly deleterious immune responses when administered to a human. Also preferred in the invention are proteins in accordance with all the foregoing that similarly do not cause a significantly deleterious immune responses on administration to non-humans.

Among very particularly preferred proteins in accordance with the invention in these regards are fusion proteins comprising antibodies and/or antibody-derived proteins, polypeptides, or fragments or the like, including all of those described above. Among very particularly preferred fusion proteins of the invention in this regard are fusion proteins comprising an antibody or antibody-derived protein or fragment such as those described above and a ligand-binding moiety, such as those illustratively described herein.

b. Target Binding Proteins

Also among preferred proteins of the invention in this regard are antibodies and other types of target binding proteins, and proteins relating thereto or derived therefrom, and protein ligands, and proteins derived therefrom or relating thereto. Among especially preferred ligand-binding proteins in this regard are proteins that bind signal and effector proteins, and proteins relating thereto or derived therefrom.

Among such binding proteins, including antibodies, including proteins derived therefrom and proteins related thereto, are those that bind to one or more of the following, alone or in any combination:

(i) CD proteins including but not limited to CD3, CD4, CD8, CD19, CD20, and CD34;

(ii) HER receptor family proteins, including, for instance, HER2, HER3, HER4, and the EGF receptor;

(iii) cell adhesion molecules, for example, LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM, and alpha v/beta 3 integrin;

(iv) growth factors, including but not limited to, for example, vascular endothelial growth factor ("VEGF"); growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-1-alpha), erythropoietin (EPO), nerve growth factor, such as NGF-beta, platelet-derived growth factor (PDGF), fibroblast growth factors, including, for instance, aFGF and bFGF, epidermal growth factor (EGF), transforming growth factors (TGF), including, among others, TGF-alpha and TGF-beta, including TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, or TGF-beta5, insulin-like growth factors-I and -II (IGF-I and IGF-II), des(1-3)-IGF-I (brain IGF-I), and osteoinductive factors;

(v) insulins and insulin-related proteins, including but not limited to insulin, insulin A-chain, insulin B-chain, proinsulin, and insulin-like growth factor binding proteins;

(vi) coagulation and coagulation-related proteins, such as, among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1–antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin;

(vii) colony stimulating factors (CSFs), including the following, among others, M-CSF, GM-CSF, and G-CSF;

(viii) other blood and serum proteins, including but not limited to albumin, IgE, and blood group antigens;

(ix) receptors and receptor-associated proteins, including, for example, flk2/flt3 receptor, obesity (OB) receptor, growth hormone receptors, and T-cell receptors;

(x) neurotrophic factors, including but not limited to, bone-derived neurotrophic factor (BDNF) and neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6);

(xi) relaxin A-chain, relaxin B-chain, and prorelaxin;

(xii) interferons, including for example, interferon-alpha, -beta, and -gamma;

(xiii) interleukins (ILs), e.g., IL-1 to IL-10;

(xiv) viral antigens, including but not limited to, an AIDS envelope viral antigen;

(xv) lipoproteins, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES (regulated on activation normally T-cell expressed and secreted), mouse gonadotropin-associated peptide, Dnase, inhibin, and activin;

(xvi) integrin, protein A or D, rheumatoid factors, immunotoxins, bone morphogenetic protein (BMP), superoxide dismutase, surface membrane proteins, decay accelerating factor (DAF), AIDS envelope, transport proteins, homing receptors, addressins, regulatory proteins, immunoadhesins, antibodies; and

(xvii) biologically active fragments or variants of any of the foregoing.

As to all of the foregoing, particularly preferred are those that are effective therapeutic agents, particularly those that exert a therapeutic effect by binding a target, particularly a target among those listed above, including targets derived therefrom, targets related thereto, and modifications thereof.

c. Particular Illustrative Proteins

Among particular illustrative proteins are certain antibody and antibody-related proteins, including peptibodies, such as, for instance, those listed immediately below and elsewhere herein:

OPGL specific antibodies and peptibodies and the like (also referred to as RANKL specific antibodies, peptibodies and the like), including fully humanized and human OPGL specific antibodies, particularly fully humanized monoclonal antibodies, including but not limited to the antibodies described in International Publication Number WO 03/002713, which is incorporated herein in its entirety as to OPGL specific antibodies and antibody related proteins, particularly those having the sequences set forth therein, particularly, but not limited to, those denoted therein: 9H7; 18B2; 2D8; 2E11; 16E1; and 22B3, including the OPGL specific antibodies having either the light chain of SEQ ID NO: 2 as set forth therein in Figure 2 and/or the heavy chain of SEQ ID NO:4, as set forth therein in Figure 4, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing publication. Acid and base titrations of an OPGL specific antibody ("Ab-hOPGL") over the pH ranges of 4.5 to 5.0 and 5.0 to 5.5 are described in the Examples below. The calculation of buffer capacity of Ab-hOPGL in these pH ranges also is described in the Examples below.

Myostatin binding agents or peptibodies, including myostatin specific peptibodies, particularly those described in US Application Publication Number 2004/0181033, which is incorporated by reference herein in its entirely particularly in parts pertinent to myostatin specific peptibodies, including but not limited to peptibodies of the mTN8-19 family, including those of SEQ ID NOS: 305-351, including TN8-19-1 through TN8-19-40, TN8-19 con1 and TN8-19 con2; peptibodies of the mL2 family of SEQ ID NOS: 357-383; the mL15 family of SEQ ID NOS: 384-409; the mL17 family of SEQ ID NOS: 410-438; the mL20 family of SEQ ID NOS: 439-446; the mL21 family of SEQ ID NOS: 447-452; the mL24 famuly of SEQ ID NOS: 453-454; and those of SEQ ID NOS: 615-631, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing publication.

IL-4 receptor specific antibodies, particularly those that inhibit activities mediated by binding of IL-4 and/or IL-13 to the receptor, including those described in International Publication No. WO 2005/047331 of International Application Number PCT/US2004/03742, which is incorporated herein by reference in its entirety particularly in parts pertinent to IL-4 receptor specific antibodies, particularly such antibodies as are described therein, particularly, and without limitation, those designated therein: L1H1; L1H2; L1H3; L1H4; L1H5; L1H6; L1H7; L1H8; L1H9;

L1H10; L1H11; L2H1; L2H2; L2H3; L2H4; L2H5; L2H6; L2H7; L2H8; L2H9; L2H10; L2H11; L2H12; L213; L2H14; L3H1; L4H1; L5H1; L6H1, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing publication. Acid and base titrations over the pH ranges of 4.5 to 5.0 and 5.0 to 5.5, and the calculation of buffer capacity in this range of an IL-4 receptor specific antibody ("Ab-hIL4R") are described in the Examples below.

Interleukin 1 - receptor 1 ("IL1-R1") specific antibodies, peptibodies and related proteins and the like, including but not limited to those described in U.S. Application Publication Number US2004/097712A1 which is incorporated herein by reference in its entirety in parts pertinent to IL1-R1 specific binding proteins, monoclonal antibodies in particular, especially, without limitation, those designated therein: 15CA, 26F5, 27F2, 24E12, and 10H7, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the aforementioned U.S. application publication.

Ang2 specific antibodies and peptibodies and related proteins and the like, including but not limited to those described in International Publication Number WO 03/057134 and U.S. Application Publication Number US2003/0229023, each of which is incorporated herein by reference in its entirety particularly in parts pertinent to Ang2 specific antibodies and peptibodies and the like, especially those of sequences described therein and including but not limited to: L1(N); L1(N) WT; L1(N) 1K WT; 2xL1(N); 2xL1(N) WT; Con4 (N), Con4 (N) 1K WT, 2xCon4 (N) 1K; L1(C); L1(C) 1K; 2xL1 (C); Con4 (C); Con4 (C) 1K; 2xCon4 (C) 1K; Con4-L1 (N); Con4-L1 (C); TN-12-9 (N); C17 (N); TN8-8(N); TN8-14 (N); Con 1 (N), also including anti-Ang 2 antibodies and formulations such as those described in International Publication Number WO 2003/030833 which is incorporated herein by reference in its entirety as to the same, particularly Ab526; Ab528; Ab531; Ab533; Ab535; Ab536; Ab537; Ab540; Ab543; Ab544; Ab545; Ab546; A551; Ab553; Ab555; Ab558; Ab559; Ab565; AbF1AbFD; AbFE; AbFJ; AbFK; AbG1D4; AbGC1E8; AbH1C12; AblA1; AblF; AblKAblP; and AblP, in their various permutations as described therein, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing publication.

NGF specific antibodies, including, in particular, but not limited to those described in US Application Publication Number US2005/0074821, which is incorporated herein by reference in its entirety particularly as to NGF-specific

antibodies and related proteins in this regard, including in particular, but not limited to, the NGF-specific antibodies therein designated 4D4, 4G6, 6H9, 7H2, 14D10 and 14D11, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing publication.

CD22 specific antibodies and related proteins, such as those described in US 5,789,554 which is incorporated herein by reference in its entirety as to CD22 specific antibodies and related proteins, particularly human CD22 specific antibodies, such as but not limited to humanized and fully human antibodies, including but not limited to humanized and fully human monoclonal antibodies, particularly including but not limited to human CD22 specific IgG antibodies, such as, for instance, a dimer of a human-mouse monoclonal hLL2 gamma-chain disulfide linked to a human-mouse monoclonal hLL2 kappa-chain, including, but limited to, for example, the human CD22 specific fully humanized antibody in Epratuzumab, CAS registry number 501423-23-0. Illustrative of the invention, acid and base titrations of a CD22-specific antibody ("Ab-hCD22") over the pH ranges of 4.5 to 5.0 and 5.0 to 5.5 are described in the Examples below. The calculation of buffer capacity of Ab-hCD22 in these pH ranges also is described in the Examples below.

IGF-1 receptor specific antibodies and related proteins such as those described in International Patent Application Number PCT/US2005/046493, which is incorporated herein by reference in its entirety as to IGF-1 receptor specific antibodies and related proteins, including but not limited to the IGF-1 specific antibodies therein designated L1H1, L2H2, L3H3, L4H4, L5H5, L6H6, L7H7, L8H8, L9H9, L10H10, L11H11, L12H12, L13H13, L14H14, L15H15, L16H16, L17H17, L18H18, L19H19, L20H20, L21H21, L22H22, L23H23, L24H24, L25H25, L26H26, L27H27, L28H28, L29H29, L30H30, L31H31, L32H32, L33H33, L34H34, L35H35, L36H36, L37H37, L38H38, L39H39, L40H40, L41H41, L42H42, L43H43, L44H44, L45H45, L46H46, L47H47, L48H48, L49H49, L50H50, L51H51, and L52H52, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing International Application.

B-7 related protein 1("B7RP-1") specific antibodies, (B7RP-1 also is referred to in the literature as B7H2, ICOSL, B7h, and CD275) particularly B7RP-specific fully human monoclonal IgG2 antibodies, particularly fully human IgG2 monoclonal antibody that binds an epitope in the first immunoglobulin-like domain of B7RP-1, especially those that inhibit the interaction of B7RP-1 with its natural receptor, ICOS,

on activated T cells in particular, especially, in all of the foregoing regards, those disclosed in U.S. Provisional Application Number 60/700,265, filed 18 July 2005, which is incorporated herein by reference in its entirety as to such antibodies and related proteins, including but not limited to antibodies designated therein as follow: 16H (having light chain variable and heavy chain variable sequences SEQ ID NO:1 and SEQ ID NO:7 respectively therein); 5D (having light chain variable and heavy chain variable sequences SEQ ID NO:2 and SEQ ID NO:9 respectively therein); 2H (having light chain variable and heavy chain variable sequences SEQ ID NO:3 and SEQ ID NO:10 respectively therein); 43H (having light chain variable and heavy chain variable sequences SEQ ID NO:6 and SEQ ID NO:14 respectively therein); 41H (having light chain variable and heavy chain variable sequences SEQ ID NO:5 and SEQ ID NO:13 respectively therein); and 15H (having light chain variable and heavy chain variable sequences SEQ ID NO:4 and SEQ ID NO:12 respectively therein), each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing U.S. Provisional Application. Acid and base titrations and determination of buffer capacity of a B7RP-1 specific antibody ("Ab-hB7RP1") are illustrated in the Examples below.

IL-15 specific antibodies, peptibodies and related proteins, such as, in particular, humanized monoclonal antibodies, particularly antibodies such as those disclosed in U.S. Application Publication Numbers: US2003/0138421; US2003/023586; US2004/0071702, each of which is incorporated herein by reference in its entirety as to IL-15 specific antibodies and related proteins, including peptibodies, including particularly, for instance, but not limited to, HuMax IL-15 antibodies and related proteins, such as, for instance, 146B7.

IFN gamma specific antibodies, especially human IFN gamma specific antibodies, particularly fully human anti-IFN gamma antibodies, such as, for instance, those described in US Application Publication Number US2005/0004353, which is incorporated herein by reference in its entirety as to IFN gamma specific antibodies, particularly, for example, the antibodies therein designated 1118; 1118*; 1119; 1121; and 1121* each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing US Application Publication.

TALL-1 specific antibodies and other TALL specific binding proteins such as those described in U.S. Application Publication Number 2003/0195156 which is incorporated herein by reference in its entirety as to TALL-1 binding proteins,

particularly the molecules of Tables 4 and 5B, each of which is individually and specifically incorporated by reference herein in its entirety fully as disclosed in the foregoing US Application Publication.

Stem Cell Factor (s) ("SCF") and related proteins such as those described in U.S. Patent Numbers 6,204,363 and 6,207,802, each of which is incorporated herein by reference in its entirety as to stem cell factors and related proteins, particularly, for example, the stem cells factor "STEMGEN ™."

Flt3-Ligands, ("Flt3L") and related proteins such as those described in U.S. Patent Number 6,632,424 which is incorporated herein by reference as to Flt3-ligands and related proteins in this regard.

IL-17 receptors and related proteins ("IL-17R"), such as those described in U.S. Patent Number 6,072,033 which is incorporated herein by reference as to Flt3-ligands and related proteins in this regard.

Etanercept, also referred to as Embrel, and related proteins.

Actimmune (Interferon-gamma-1b), Activase (Alteplase), Aldurazme (Laronidase), Amevive (Alefacept), Avonex (Interferon beta-1a), BeneFIX (Nonacog alfa), Beromun (Tasonermin), Beatseron (Interferon-beta-1b), BEXXAR (Tositumomab), Tev-Tropin (Somatropin), Bioclate or RECOMBINATE (Recombinant), CEREZME (Imiglucerase), ENBREL (Etanercept), Eprex (epoetin alpha), EPOGEN/Procit (Epoetin alfa), FABRAZYME (Agalsidase beta), Fasturtec/Elitek ELITEK (Rasburicase), FORTEO (Teriparatide), GENOTROPIN (Somatropin), GlucaGen (Glucagon), Glucagon (Glucagon, rDNA origin), GONAL-F (follitropin alfa), KOGENATE FS (Octocog alfa), HERCEPTIN (Trastuzumab), HUMATROPE (SOMATROPIN), HUMIRA (Adalimumab), Insulin in Solution, INFERGEN® (Interferon alfacon-1), KINERET® (anakinra), Kogenate FS (Antihemophilic Factor), LEUKIN (SARGRAMOSTIM Recombinant human granulocyte-macrophage colony stimulating factor (rhuGM-CSF)), CAMPATH (Alemtuzumab), RITUXAN® (Rituximab), TNKase (Tenecteplase), MYLOTARG (gemtuzumab ozogamicin), NATRECOR (nesiritide), ARANESP (darbepoetin alfa), NEULASTA (pegfilgrastim), NEUMEGA (oprelvekin), NEUPOGEN (Filgrastim), NORDITROPIN CARTRIDGES (Somatropin), NOVOSEVEN (Eptacog alfa), NUTROPIN AQ (somatropin), Oncaspar (pegaspargase), ONTAK (denileukin diftitox), ORTHOCLONE OKT (muromonab-CD3), OVIDREL (choriogonadotropin alfa), PEGASYS (peginterferon alfa-2a), PROLEUKIN (Aldesleukin), PULMOZYME

(dornase alfa), Retavase (Reteplase), REBETRON Combination Therapy containing REBETOL® (Ribavirin) and INTRON® A (Interferon alfa-2b), REBIF (interferon beta-1a), REFACTO (Antihemophilic Factor), REFLUDAN (lepirudin), REMICADE (infliximab), REOPRO (abciximab)ROFERON®-A (Interferon alfa-2a), SIMULECT (baasiliximab), SOMAVERT (Pegivisomant), SYNAGIS® (palivizumab), Stemben (Ancestim, Stem cell factor), THYROGEN, INTRON® A (Interferon alfa-2b), PEG-INTRON® (Peginterferon alfa-2b), XIGRIS® (Drotrecogin alfa activated), XOLAIR® (Omalizumab), ZENAPAX® (daclizumab), ZEVALIN® (Ibritumomab Tiuxetan).

d. Sequence Variation

Particularly preferred proteins in regard to all of the foregoing and the following, include those that comprise a region that is 70% or more, especially 80% or more, more especially 90% or more, yet more especially 95% or more, particularly 97% or more, more particularly 98% or more, yet more particularly 99% or more identical in amino acid sequence to a reference amino acid sequence of a binding protein, as illustrated above, particularly a pharmaceutical binding protein, such as a GenBank or other reference sequence of a reference protein.

Identity in this regard can be determined using a variety of well-known and readily available amino acid sequence analysis software. Preferred software includes those that implement the Smith-Waterman algorithms, considered a satisfactory solution to the problem of searching and aligning sequences. Other algorithms also may be employed, particularly where speed is an important consideration. Commonly employed programs for alignment and homology matching of DNAs, RNAs, and polypeptides that can be used in this regard include FASTA, TFASTA, BLASTN, BLASTP, BLASTX, TBLASTN, PROSRCH, BLAZE, and MPSRCH, the latter being an implementation of the Smith-Waterman algorithm for execution on massively parallel processors made by MasPar.

The BLASTN, BLASTX, and BLASTP programs are among preferred programs for such determinations, the former for polynucleotide sequence comparisons and the latter two for polypeptide sequence comparisons: BLASTX for comparison of the polypeptide sequences from all three reading frames of polynucleotide sequence and BLASTP for a single polypeptide sequence.

BLAST provides a variety of user definable parameters that are set before implementing a comparison. Some of them are more readily apparent than others on

graphical user interfaces, such as those provided by NCBI BLAST and other sequence alignment programs that can be accessed on the internet. The settings and their values are set out and explained on the service web sites and are explained and set out in particular detail in a variety of readily available texts, including but not limited to BIOINFORMATICS: SEQUENCE AND GENOME ANALYSIS, 2nd Ed., David W. Mount, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2004), especially Chapters 3, 4, 5, and 6 as to comparison of protein and nucleic acid sequences in general and as to BLAST comparisons and searches in particular; SEQUENCE ANALYSIS IN A NUTSHELL: A GUIDE TO COMMON TOOLS AND DATABASES, Scott Markel and Darryl León, O'Reilly & Associates, Sebastopol, California (2003), especially Chapter 7 as to BLAST in particular, each of which is herein incorporated by reference in its entirety particularly in parts pertinent to comparison of nucleotide and polypeptide sequences and to determining their degree of identity, similarity, homology and/or the like, especially as to comparison of a test sequence and a reference sequence to calculate a degree (percent) of identity between them.

In preferred embodiments of the invention in this regard, relatedness of sequences is defined as the identity score in percent returned by any one or another of the aforementioned BLAST comparison searches with e =10 and all other parameters set to their default values on the NCBI web server as set forth in SEQUENCE ANALYSIS IN A NUTSHELL: A GUIDE TO COMMON TOOLS AND DATABASES, Scott Markel and Darryl León, O'Reilly & Associates, Sebastopol, California (2003), pages 47-51 which are incorporated herein by reference in their entireties and in all particulars of the preferred settings for parameters of the present invention for comparing sequences using BLAST, such as those on NCBI BLAST.

The following references provide additional information on sequence comparisons in this regard, and in others. GUIDE TO HUMAN GENOME COMPUTING, Ed. Martin J. Bishop, Academic Press, Harcourt Brace & Company Publishers, New York (1994), which is incorporated herein by reference in its entirety with regard to the foregoing, particularly in parts pertinent to determining identity and or homology of amino acid or polynucleotide sequences, especially Chapter 7. The BLAST programs are described in Altschul et al., "Basic Local Alignment Research Tool," *J Mol Biol* 215: 403-410 (1990), which is incorporated by reference herein in its entirety. Additional information concerning sequence analysis and homology and

identity determinations are provided in, among many other references well-known and readily available to those skilled in the art: NUCLEIC ACID AND PROTEIN SEQUENCE ANALYSIS: A PRACTICAL APPROACH, Eds. M. J. Bishop and C. J. Rawings, IRL Press, Oxford, UK (1987); PROTEIN STRUCTURE: A PRACTICAL APPROACH, Ed. T. E. Creighton, IRL Press, Oxford, UK (1989); Doolittle, R. F.: "Searching through sequence databases," *Met Enz.* 183: 99-110 (1990); Meyers and Miller: "Optimal alignments in linear space" *Comput. Applica. in Biosci* 4: 11-17 (1988); Needleman and Wunsch: "A general method applicable to the search for similarities in amino acid sequence of two proteins," *J Mol Biol* 48: 443-453 (1970) and Smith and Waterman "Identification of common molecular subsequences," *J Mol Biol* 147: 1950 et seq. (1981), each of which is incorporated herein by reference in its entirety with reference to the foregoing, particularly in parts pertinent to sequence comparison and identity and homology determinations.

Particularly preferred embodiments in this regard have 50% to 150% of the activity of the aforementioned reference protein, particularly highly preferred embodiments in this regard have 60% to 125% of the activity of the reference protein, yet more highly preferred embodiments have 75% to 110% of the activity of the reference protein, still more highly preferred embodiments have 85% to 125% the activity of the reference, still more highly preferred embodiments have 90% to 110% of the activity of the reference.

4. Formulations

Many reagents and methods conventionally employed for the formulation of protein pharmaceuticals can be used for the formulation of self-buffering protein compositions in accordance with various aspects and preferred embodiments of the invention. However, in self-buffering protein formulations in accordance with the invention, buffering is provided substantially entirely by the protein itself, not by a buffering agent, as is the case with conventional formulations. Moreover, self-buffering protein formulations in accordance with various aspects and preferred embodiments of the invention are substantially free of such buffering agents.

In many other respects, however, self-buffering protein compositions in accordance with various aspects and embodiments of the invention can be formulated using reagents and methods conventionally employed for the formulation of proteins, in particular, reagents and methods employed for the formulation of pharmaceuticals,

including pharmaceuticals for veterinary and human use, especially those reagents and methods suitable for formulating protein pharmaceuticals for veterinary and especially for human use.

In accordance therewith, many methods and ingredients for formulating and using pharmaceuticals that are well-known and routine in the pertinent arts can be used in designing, making, and using self-buffering protein formulations in accordance with various aspects and preferred embodiments of the invention relating thereto. Such methods and ingredients are described in, to name just a few readily available references in this regard, REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 21st Ed.; Beringer *et al.* Editors, Lippincott, Williams & Wilkins, Philadelphia, PA (2005); ANSEL'S PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 8th Ed., Allen *et al.*, Editors, Lippincott, Williams & Wilkins, Philadelphia, PA (2005); and PHARMACEUTICAL FORMULATION OF PEPTIDES AND PROTEINS, Sven Frokjaer and Lars Hovgaard, Editors, CRC Press, Boca Raton, Florida (2000), each of which is herein incorporated in its entirety particularly in parts pertinent to conventional ingredients and methods that may be used in self-buffering formulations of proteins in accordance with various aspects and preferred embodiments of the invention relating thereto.

Additional methods and ingredients that can be useful in this regard are disclosed in, among others, US 6,171,586; WO 2005/044854; US 6,288,030; US 6,267,958; WO 2004/055164; US 4,597,966; US 2003/0138417; US 6,252,055; US 5,608,038; US 6,875,432; US 2004/0197324; WO 02/096457; US 5,945,098; US 5,237,054; US 6,485,932; US 6,821,515; US 5,792,838; US 5,654,403; US 5,908,826; EP 0 804 163; and WO 2005/063291, each of which is incorporated herein by reference in its entirety particularly in parts pertinent to pharmaceutically acceptable self-buffering protein formulations in accordance with the invention.

Various specific aspects of the ingredients and specific types of formulations are further described below, by way of illustration. The description thus provided is not exhaustive of the methods and compositions possible for self-buffering protein formulations in accordance with the various aspects and embodiments of the invention, nor is it in any way exclusive.

In preferred embodiments of a variety of aspects of the invention, formulations of self-buffering proteins comprise a protein and a carrier, which also may be referred to herein variously, as the case may be, as one or more of: a vehicle, a primary vehicle,

a diluent, a primary diluent, a primary carrier, a solvent and/or a primary solvent. In the broadest sense the carrier may be a gas, a liquid, or a solid, as suits the phase of the composition and/or its use(s). In some embodiments of the invention in this regard, the carrier is a solid, such as a powder in which a protein may be dispersed. In preferred embodiments in this regard, the carrier is a liquid, particularly a liquid in which the self-buffering protein is highly soluble, particularly at concentrations that provide the desired buffer capacity. Liquid carriers may be organic or non-organic. Preferably they are aqueous, most preferably they are largely or entirely comprised of pure water.

It will be appreciated that formulations for pharmaceutical use in accordance with various aspects and embodiments of the invention must be compatible with the processes and conditions to which they will be subjected, such as, for instance, sterilization procedures (generally applied before mixing with an active agent), and conditions during storage.

Almost invariably, formulations in accordance with numerous aspects and embodiments of the invention will contain additional ingredients including but not limited in any way to excipients and other pharmaceutical agents. Nevertheless, it is to be understood that formulations in accordance with the invention are self-buffering formulations in which the buffer capacity is provided substantially or entirely by the primary protein itself, as described elsewhere herein.

Formulations in accordance with various aspects and embodiments of the invention may contain, among others, excipients, as described below, including but not limited to ingredients for modifying, maintaining, or preserving, for example, osmolality, osmolarity, viscosity, clarity, color, tonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the formulations and/or primary polypeptide and/or protein.

Formulations will, of course, depend upon, for example, the particular protein being formulated, the other active agents, such as other pharmaceuticals, that will be comprised in the formulation, the intended route of administration, the method of administration to be employed, the dosage, the dosing frequency, and the delivery format, among others.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide compositions comprising a protein preferably a pharmaceutical protein and a solvent, the protein having a buffer capacity per unit volume of at least that of approximately: 2.0 or 3.0 or 4.0 or 5.0 or 6.50 or 8.00 or 10.0

or 15.0 or 20.0 or 30.0 or 40.0 or 50.0 or 75.0 or 100 or 125 or 150 or 200 or 250 or 300 or 350 or 400 or 500 or 700 or 1,000 or 1,500 or 2,000 or 2,500 or 3,000 or 4,000 or 5,000 mM sodium acetate buffer as determined over the range of pH 5.0 to 4.0 pH or 5.0 to 5.5 as described in Example 1 or 2 and elsewhere herein.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, wherein, exclusive of the buffer capacity of the protein, the buffer capacity per unit volume of the composition is equal to or less than that of 1.0 or 1.5 or 2.0 or 3.0 or 4.0 or 5.0 mM sodium acetate buffer as determined over the range of pH 5.0 to 4.0 or pH 5.0 to 5.5 as described in Example 1 or 2 and elsewhere herein.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein at the pH of the composition the buffer capacity of the protein is at least approximately: 1.00 or 1.50 or 1.63 or 2.00 or 3.00 or 4.00 or 5.00 or 6.50 or 8.00 or 10.0 or 15.0 or 20.0 or 30.0 or 40.0 or 50.0 or 75.0 or 100 or 125 or 150 or 200 or 250 or 300 or 350 or 400 or 500 or 700 or 1,000 or 1,500 or 2,000 or 2,500 or 3,000 or 4,000 or 5,000 mEq per liter and per change in pH of one pH unit.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein at the pH of the composition, exclusive of the protein, the buffer capacity per unit volume of the composition is equal to or less than that of a 0.50 or 1.00 or 1.50 or 2.00 or 3.00 or 4.00 or 5.00 or 6.50 or 8.00 or 10.0 or 20.0 or 25.0 mM acetate buffer as determined over the range of pH 5.0 to 4.0 or pH 5.0 to 5.5 as described in Example 1 or 2 and elsewhere herein.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein at a desired pH, the protein provides at least approximately 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% of the buffer capacity of the composition.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, A, wherein the concentration of the protein is between approximately: 20 and 400, or 20 and 300, or 20 and 250, or 20 and 200, or 20 and 150 mg/ml.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein the pH maintained by the buffering action of the protein is a pH between approximately: 3.5 and 8.0, or 4.0 and 6.0, or 4.0 and 5.5, or 4.5 and 5.5.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein the salt concentration is less than: 150 mM or 125 mM or 100 mM or 75 mM or 50 mM or 25 mM.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, and further comprising one or more pharmaceutically acceptable salts; osmotic balancing agents (tonicity agents); anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; analgesics; or additional pharmaceutical agents.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, and further comprising one or more pharmaceutically acceptable polyols in an amount that is hypotonic, isotonic, or hypertonic, preferably approximately isotonic, particularly preferably isotonic, especially preferably any one or more of sorbitol, mannitol, sucrose, trehalose, or glycerol, particularly especially preferably approximately 5% sorbitol, 5% mannitol, 9% sucrose, 9% trehalose, or 2.5% glycerol, very especially in this regard 5% sorbitol, 5% mannitol, 9% sucrose, 9% trehalose, or 2.5% glycerol.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions,

particularly pharmaceutical protein compositions, comprising a protein and a solvent, and further comprising one or more pharmaceutically acceptable surfactants, preferably one or more of polysorbate 20, polysorbate 80, other fatty acid esters of sorbitan, polyethoxylates, and poloxamer 188, particularly preferably polysorbate 20 or polysorbate 80, preferably approximately 0.001 to 0.1% polysorbate 20 or polysorbate 80, very preferably approximately 0.002 to 0.02% polysorbate 20 or polysorbate 80, especially 0.002 to 0.02% polysorbate 20 or polysorbate 80.

Formulations in accordance with certain of the preferred embodiments in various aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, comprising a protein and a solvent, wherein the protein is a pharmaceutical agent and the composition is a sterile formulation thereof suitable for treatment of a veterinary or a human medical subject.

Also among formulations in accordance with various aspects and embodiments of the invention herein described are lyophilized compositions in accordance with the foregoing, particularly lyophilized compositions that when reconstituted provide a formulation as described above and elsewhere herein.

a. Excipients and Other Additional Ingredients

As discussed above, certain embodiments in accordance with aspects of the invention provide self-buffering protein compositions, particularly pharmaceutical protein compositions, that comprise, in addition to the protein, particularly a pharmaceutical protein, one or more excipients such as those illustratively described in this section and elsewhere herein. Excipients can be used in the invention in this regard for a wide variety of purposes, such as adjusting physical, chemical, or biological properties of formulations, such as adjustment of viscosity, and or processes of the invention to improve effectiveness and or to stabilize such formulations and processes against degradation and spoilage due to, for instance, stresses that occur during manufacturing, shipping, storage, pre-use preparation, administration, and thereafter.

A variety of expositions are available on protein stabilization and formulation materials and methods useful in this regard, such as Arakawa et al., "Solvent interactions in pharmaceutical formulations," *Pharm Res.* 8(3): 285-91 (1991); Kendrick *et al.*, "Physical stabilization of proteins in aqueous solution," in: RATIONAL DESIGN OF STABLE PROTEIN FORMULATIONS: THEORY AND PRACTICE, Carpenter and Manning, eds. *Pharmaceutical Biotechnology.* 13: 61-84

(2002), and Randolph *et al.*, "Surfactant-protein interactions," *Pharm Biotechnol.* 13: 159-75 (2002), each of which is herein incorporated by reference in its entirety, particularly in parts pertinent to excipients and processes of the same for self-buffering protein formulations in accordance with the current invention, especially as to protein pharmaceutical products and processes for veterinary and/or human medical uses.

Various excipients useful in the invention are listed in Table 1 and further described below.

Table 1: Types of Excipients and Their Functions

Type	Function	
	Liquids	Lyophilates
Tonicity Agents / Stabilizers	• Provides isotonicity to the formulation such that it is suitable for injection • Examples include polyols, salts, and amino acids • Help maintain the protein in a more compact state (polyols) • Minimize electrostatic, solution protein-protein interactions (salts)	• Stabilizers include cryo and lyoprotectants • Examples include polyols, sugars and polymers • Cryoprotectants protect proteins from freezing stresses • Lyoprotectants stabilize proteins in the freeze-dried state
Bulking Agents	• Not applicable	• Used to enhance product elegance and to prevent blowout • Provides structural strength to the lyo cake • Examples include mannitol and glycine
Surfactants	• Prevent/control aggregation, particle formation and surface adsorption of drug • Examples include polysorbate 20 and 80	• Employed if aggregation during the lyophilization process is an issue • May serve to reduce reconstitution times • Examples include polysorbate 20 and 80
Anti-oxidants	• Control protein oxidation	• Usually not employed, molecular reactions in the lyophilized cake are greatly retarded
Metal Ions / Chelating Agents	• A specific metal ion is included in a liquid formulation only as a co-factor • Divalent cations such as zinc and magnesium are utilized in suspension formulations • Chelating agents are used to inhibit heavy metal ion catalyzed reactions	• May be included if a specific metal ion is included only as a co-factor • Chelating agents are generally not needed in lyophilized formulations
Preservatives	• Important particularly for multi-dose formulations • Protects against microbial growth, • Example: benzyl alcohol	• For multi-dose formulations only • Provides protection against microbial growth in formulation • Is usually included in the reconstitution diluent (e.g. bWFI)

i. Salts

Salts may be used in accordance with certain of the preferred embodiments of the invention to, for example, adjust the ionic strength and/or the isotonicity of a self-buffering formulation and/or to improve the solubility and/or physical stability of a self-buffering protein or other ingredient of a self-buffering protein composition in accordance with the invention.

As is well known, ions can stabilize the native state of proteins by binding to charged residues on the protein's surface and by shielding charged and polar groups in the protein and reducing the strength of their electrostatic interactions, attractive, and repulsive interactions. Ions also can stabilize the denatured state of a protein by binding to, in particular, the denatured peptide linkages (-CONH) of the protein. Furthermore, ionic interaction with charged and polar groups in a protein also can reduce intermolecular electrostatic interactions and, thereby, prevent or reduce protein aggregation and insolubility.

Ionic species differ significantly in their effects on proteins. A number of categorical rankings of ions and their effects on proteins have been developed that can be used in formulating self-buffering protein compositions in accordance with the invention. One example is the Hofmeister series, which ranks ionic and polar non-ionic solutes by their effect on the conformational stability of proteins in solution. Stabilizing solutes are referred to as "kosmotropic." Destabilizing solutes are referred to as chaotropic. Kosmotropes commonly are used at high concentrations (e.g., >1 molar ammonium sulfate) to precipitate proteins from solution ("salting-out"). Chaotropes commonly are used to denture and/or to solubilize proteins ("salting-in"). The relative effectiveness of ions to "salt-in" and "salt-out" defines their position in the Hofmeister series.

In addition to their utilities and their drawbacks (as discussed above) salts also are effective for reducing the viscosity of protein formulations and can be used in the invention for that purpose.

In order to maintain isotonicity in a parenteral formulation in accordance with preferred embodiments of the invention, improve protein solubility and/or stability, improve viscosity characteristics, avoid deleterious salt effects on protein stability and aggregation, and prevent salt-mediated protein degradation, the salt concentration in self-buffering formulations in accordance with various preferred embodiments of the invention are less than 150 mM (as to monovalent ions) and 150 mEq/liter for

multivalent ions. In this regard, in certain particularly preferred embodiments of the invention, the total salt concentration is from about 75 mEq/L to about 140 mEq/L.

ii. Amino acids

Free amino acids can be used in protein formulations in accordance with various preferred embodiments of the invention as, to name a few, bulking agents, stabilizers and antioxidants. However, amino acids comprised in self-buffering protein formulations in accordance with the invention do not provide buffering action. For this reason, those with significant buffer capacity either are not employed, are not employed at any pH around which they have significant buffering activity, or are used at low concentration so that, as a result, their buffer capacity in the formulation is not significant. This is particularly the case for histidine and other amino acids that commonly are used as buffers in pharmaceutical formulations.

Subject to the foregoing consideration, lysine, proline, serine, and alanine can be used for stabilizing proteins in a formulation. Glycine is useful in lyophilization to ensure correct cake structure and properties. As a result it is a common ingredient in lyophilized formulations and reconstituted lyophilates, such as Neumega ®, Genotropin®, and Humatrope®. Arginine may be useful to inhibit protein aggregation, in both liquid and lyophilized formulations, such as Activase®, Avonex®, and Enbrel® liquid. Methionine is useful as an antioxidant.

iii. Polyols

Polyols include sugars, e.g., mannitol, sucrose, and sorbitol and polyhydric alcohols such as, for instance, glycerol and propylene glycol, and, for purposes of discussion herein, polyethylene glycol (PEG) and related substances. Polyols are kosmotropic. They are useful stabilizing agents in both liquid and lyophilized formulations to protect proteins from physical and chemical degradation processes. Polyols also are useful for adjusting the tonicity of formulations.

Among polyols useful in the invention in this regard, is mannitol, commonly used to ensure structural stability of the cake in lyophilized formulations, such as, for example Leukine®, Enbrel® – Lyo, and Betaseron®. It ensures structural stability to the cake. It is generally used with a lyoprotectant, e.g., sucrose. Sorbitol and sucrose are among preferred agents for adjusting tonicity and as stabilizers to protect against freeze-thaw stresses during transport or the preparation of bulks during the manufacturing process. Reducing sugars (which contain free aldehyde or ketone groups), such as glucose and lactose, can glycate surface lysine and arginine residues.

Therefore, they generally are not among preferred polyols for use in accordance with the invention. In addition, sugars that form such reactive species, such as sucrose, which is hydrolyzed to fructose and glucose under acidic conditions, and consequently engenders glycation, also is not among preferred amino acids of the invention in this regard. PEG is useful to stabilize proteins and as a cryoprotectant and can be used in the invention in this regard, such as it is in Recombinate®.

iv. Surfactants

Protein molecules are susceptible to adsorption on surfaces and to denaturation and consequent aggregation at air-liquid, solid-liquid, and liquid-liquid interfaces. These effects generally scale inversely with protein concentration. These deleterious interactions generally scale inversely with protein concentration and typically are exacerbated by physical agitation, such as that generated during the shipping and handling of a product.

Surfactants routinely are used to prevent, minimize, or reduce surface adsorption. Useful surfactants in the invention in this regard include polysorbate 20, polysorbate 80, other fatty acid esters of sorbitan polyethoxylates, and poloxamer 188.

Surfactants also are commonly used to control protein conformational stability. The use of surfactants in this regard is protein-specific since, any given surfactant typically will stabilize some proteins and destabilize others.

Polysorbates are susceptible to oxidative degradation and often, as supplied, contain sufficient quantities of peroxides to cause oxidation of protein residue side-chains, especially methionine. Consequently, polysorbates should be used carefully, and when used, should be employed at their lowest effective concentration. In this regard, polysorbates exemplify the general rule that excipients should be used in their lowest effective concentrations.

v. Antioxidants

A variety of processes can result in harmful oxidation of proteins in pharmaceutical formulations. To some extent deleterious oxidation of proteins can be prevented in pharmaceutical formulations by maintaining proper levels of ambient oxygen and temperature and by avoiding exposure to light. Antioxidant excipients can be used as well to prevent oxidative degradation of proteins. Among useful antioxidants in this regard are reducing agents, oxygen/free-radical scavengers, and chelating agents. Antioxidants for use in therapeutic protein formulations in accordance with the invention preferably are water-soluble and maintain their activity

throughout the shelf life of a product. EDTA is a preferred antioxidant in accordance with the invention in this regard and can be used in the invention in much the same way it has been used in formulations of acidic fibroblast growth factor and in products such as Kineret® and Ontak®.

Antioxidants can damage proteins. For instance, reducing agents, such as glutathione in particular, can disrupt intramolecular disulfide linkages. Thus, antioxidants for use in the invention are selected to, among other things, eliminate or sufficiently reduce the possibility of themselves damaging proteins in the formulation.

vi. Metal Ions

Formulations in accordance with the invention may include metal ions that are protein co-factors and that are necessary to form protein coordination complexes, such as zinc necessary to form certain insulin suspensions. Metal ions also can inhibit some processes that degrade proteins. However, metal ions also catalyze physical and chemical processes that degrade proteins.

Magnesium ions (10 –120 mM) can be used to inhibit isomerization of aspartic acid to isoaspartic acid. Ca^{+2} ions (up to 100 mM) can increase the stability of human deoxyribonuclease (rhDNase, Pulmozyme®). Mg^{+2}, Mn^{+2}, and Zn^{+2}, however, can destabilize rhDNase. Similarly, Ca^{+2} and Sr^{+2} can stabilize Factor VIII, it can be destabilized by Mg^{+2}, Mn^{+2} and Zn^{+2}, Cu^{+2} and $Fe^{+2,}$ and its aggregation can be increased by Al^{+3} ions.

vii. Preservatives

Preservatives are necessary when developing multi-dose parenteral formulations that involve more than one extraction from the same container. Their primary function is to inhibit microbial growth and ensure product sterility throughout the shelf-life or term of use of the drug product. Commonly used preservatives include benzyl alcohol, phenol and m-cresol. Although preservatives have a long history of use with small-molecule parenterals, the development of protein formulations that includes preservatives can be challenging. Preservatives almost always have a destabilizing effect (aggregation) on proteins, and this has become a major factor in limiting their use in multi-dose protein formulations. To date, most protein drugs have been formulated for single-use only. However, when multi-dose formulations are possible, they have the added advantage of enabling patient convenience, and increased marketability. A good example is that of human growth hormone (hGH) where the development of preserved formulations has led to commercialization of more convenient, multi-use

injection pen presentations. At least four such pen devices containing preserved formulations of hGH are currently available on the market. Norditropin® (liquid, Novo Nordisk), Nutropin AQ® (liquid, Genentech) & Genotropin (lyophilized – dual chamber cartridge, Pharmacia & Upjohn) contain phenol while Somatrope® (Eli Lilly) is formulated with m-cresol.

Several aspects need to be considered during the formulation and development of preserved dosage forms. The effective preservative concentration in the drug product must be optimized. This requires testing a given preservative in the dosage form with concentration ranges that confer anti-microbial effectiveness without compromising protein stability. For example, three preservatives were successfully screened in the development of a liquid formulation for interleukin-1 receptor (Type I) using differential scanning calorimetry (DSC). The preservatives were rank ordered based on their impact on stability at concentrations commonly used in marketed products.

As might be expected, development of liquid formulations containing preservatives are more challenging than lyophilized formulations. Freeze-dried products can be lyophilized without the preservative and reconstituted with a preservative containing diluent at the time of use. This shortens the time for which a preservative is in contact with the protein, significantly minimizing the associated stability risks. With liquid formulations, preservative effectiveness and stability have to be maintained over the entire product shelf-life (~ 18 to 24 months). An important point to note is that preservative effectiveness has to be demonstrated in the final formulation containing the active drug and all excipient components.

Self-buffering protein formulations in accordance with the invention, particularly self-buffering biopharmaceutical protein formulations, generally will be designed for specific routes and methods of administration, for specific administration dosages and frequencies of administration, for specific treatments of specific diseases, with ranges of bio-availability and persistence, among other things,

Formulations thus may be designed in accordance with the invention for delivery by any suitable route, including but not limited to orally, aurally, opthalmically, rectally, and vaginally, and by parenteral routes, including intravenous and intraarterial injection, intramuscular injection, and subcutaneous injection.

b. Formulations for Parenteral Administration

Formulations for parenteral administration may be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions may be prepared from sterile powders or granules using one or more of the carriers or diluents mentioned for use in the formulations for oral administration or by using other suitable dispersing or wetting agents and suspending agents.

When parenteral administration is contemplated, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the desired protein in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile pure water in which the protein is formulated as a sterile, isotonic self-buffering solution.

Such preparations may also involve the formulation of the desired protein in the form of, among other things, injectable microspheres, bio-erodible particles, polymeric compounds (polylactic acid, polyglycolic acid), beads, or liposomes, including those that provide for controlled or sustained release. Such formulations may be introduced by implantable drug delivery devices, among others.

Formulations for parenteral administration also may contain substances that adjust the viscosity. such as carboxymethyl cellulose, sorbitol, and dextran. Formulations may also contain ingredients that increase solubility of the desired protein or other ingredients and those that stabilize one or more such ingredients, including in some cases, the self-buffering protein.

c. Formulations for Pulmonary Administration

A pharmaceutical composition in accordance with certain embodiments of the invention may be suitable for inhalation. For pulmonary administration, the pharmaceutical composition may be administered in the form of an aerosol or with an inhaler including dry powder aerosol. For example, a binding agent may be formulated as a dry powder for inhalation. Inhalation solutions may also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions may be nebulized. Pulmonary administration is further described in PCT Application No. PCT/US94/001875, which describes pulmonary delivery of chemically modified proteins.

d. Formulations for Oral Administration

For oral administration, the pharmaceutical composition may be in the form of, for example, a tablet, capsule, suspension, or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a particular amount of the active ingredient. Examples of such dosage units are tablets or capsules. Formulations for oral administration in accordance with the invention in this regard can be made conventionally wherein buffering in the formulation is provided by the self-buffering protein as described elsewhere herein.

e. Controlled Release Formulations

Among additional formulations that can be useful in the invention as herein described are sustained- and controlled-delivery formulations. Techniques for making such sustained- and controlled-delivery formulations that may be used in accordance with various aspects and preferred embodiments of the invention are well-known to those skilled in the art. Among these are delivery methods that use liposome carriers, bio-erodible microparticles, porous beads, and semi-permeable polymer matrices, such as those described in PCT/US93/00829; U.S. 3,773,919; EP 58,481; Sidman et al., *Biopolymers*, 22:547-556 (1983); Langer et al., *J. Biomed. Mater. Res.*, 15:167-277, (1981); Langer et al., *Chem. Tech.*, 12:98-105(1982); EP 133,988; Eppstein et al., *Proc. Natl. Acad. Sci.* (USA), 82:3688-3692 (1985); EP 36,676; EP 88,046; and EP 143,949, each of which is hereby incorporated by reference in its entirety, particularly in parts pertinent to self-buffering sustained- and controlled-delivery pharmaceutical protein formulations in accordance with the invention herein described.

f. Sterilization

The pharmaceutical composition to be used for in vivo administration typically must be sterile. This may be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

g. Storage

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or a dehydrated or lyophilized powder. Such formulations may be stored either in a ready-to-use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration.

h. Additional Pharmaceutical Agents

Self-buffering protein compositions in accordance with the invention, particularly self-buffering pharmaceutical protein compositions, can comprise in addition to the self-buffering protein of the composition, one or more additional pharmaceutical agents. Such agents may be proteins as well, or they may be other types of agents. Included among such agents are those for prevention or treatment of any disorder or disease. Such agents include, for instance, antibiotics and antimycotics. They also include agents for treating human disorders, including but not limited to, agents for treating inflammatory diseases, cancers, metabolic disorders, neurological and renal disorders, to name just a few. Agents that may be used in the invention in this regard also include agents useful to augment the action of a self-buffering composition and or prevent, ameliorate, or treat any undesirable side effects of the administration thereof.

i. Methods for Making Self-Buffering Protein Formulations

Compositions in accordance with the invention may be produced using well-known, routine methods for making, formulating, and using proteins, particularly pharmaceutical proteins. In certain of the preferred embodiments of a number of aspects of the invention in this regard, methods for preparing the compositions comprise the use of counter ions to remove residual buffering agents. In this regard the term counter ion is any polar or charged constituent that acts to displace buffer from the composition during its preparation. Counter ions useful in this regard include, for instance, glycine, chloride, sulfate, and phosphate. The term counter ion in this regard is used to mean much the same thing as displacement ion.

Residual buffering agents can be removed using the counter ions in this regard, using a variety of well-known methods, including but not limited to, standard methods of dialysis and high performance membrane diffusion-based methods such as tangential

flow diafiltration. Methods for residual buffer removal employing a counter ion in this regard can also, in some cases, be carried out using size exclusion chromatography.

In certain related preferred embodiments in this regard, compositions in accordance with the invention are prepared by a process that involves dialysis against a bufferless solution at a pH below that of the preparation containing the self-buffering protein. In particularly preferred embodiments of the invention in this regard, the bufferless solution comprises counter ions, particularly those that facilitate removal of residual buffer and do not adversely affect the self-buffering protein or the formulation thereof. In further particularly preferred embodiments of the invention in this regard, following dialysis the pH of the preparation is adjusted to the desired pH using dilute acid or dilute base.

In certain related particularly preferred embodiments in this regard, compositions in accordance with the invention are prepared by a process that involves tangential flow diafiltration against a bufferless solution at a pH below that of the preparation containing the self-buffering protein. In particularly preferred embodiments of the invention in this regard, the bufferless solution comprises counter ions, particularly those that facilitate removal of residual buffer and do not adversely affect the self-buffering protein or the formulation thereof. In further particularly preferred embodiments of the invention in this regard, following diafiltration the pH of the preparation is adjusted to the desired pH using dilute acid or dilute base.

5. Routes of Administration

Formulations in accordance with the invention, in various embodiments, may be administered by a variety of suitable routes, well-known to those skilled in the art of administering therapeutics to a subject. In embodiments of the invention in this regard, one or more formulations, as described elsewhere herein, are administered via the alimentary canal. In other embodiments one or more formulations as described elsewhere herein are administered parenterally. In various embodiments one or more formulations may be administered via the alimentary canal in conjunction with one or more other formulations administered parenterally.

Such routes in a variety of embodiments include but are not limited to administration of the compositions orally, ocularly, mucosally, topically, rectally, pulmonarily, such as by inhalation spray, and epicutaneously. The following parenteral routes of administration also are useful in various embodiments of the invention:

administration by intravenous, intraarterial, intracardiac, intraspinal, intrathecal, intraosseous, intraarticular, intrasynovial, intracutaneous, intradermal, subcutaneous, peritoneal, and/or intramuscular injection. In some embodiments intravenous, intraarterial, intracutaneous, intradermal, subcutaneous and/or intramuscular injection are used. In some embodiments intravenous, intraarterial, intracutaneous, subcutaneous, and/or intramuscular injection are used.

In certain embodiments of the invention the compositions are administered locally, for instance by intraocular injection to treat ocular neovascularization, retinopathy, or age-related macular degeneration.

6. Doses

The amount of a self-buffering protein formulation administered and the dosage regimen for treating a disease condition with the formulation depends on a variety of factors, including the age, weight, sex, and medical condition of the subject, the type of disease, the severity of the disease, the route and frequency of administration, and the particular formulation employed. In particular the amount will depend on the protein therapeutic being administered and any other therapeutic agents being administered in conjunction therewith. Dosages can be determined for formulations in accordance with the invention using well-established routine pharmaceutical procedures for this purpose.

7. Dosing Regimens

Formulations of the invention can be administered in dosages and by techniques well-known to those skilled in the medical and veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the formulation that will be administered (e.g., solid vs. liquid). Doses for humans or other mammals can be determined without undue experimentation by the skilled artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

In accordance with various embodiments, proper dosages and dosing plans will depend on numerous factors, and may vary in different circumstances. The parameters that will determine the optimal dosage plans to be administered typically will include some or all of the following: the disease being treated and its stage; the species of the subject, their health, gender, age, weight, and metabolic rate; other therapies being

administered; and expected potential complications from the subject's history or genotype.

The optimal dosing plan in a given situation also will take into consideration the nature of the formulation, the way it is administered, the distribution route following administration, and the rate at which it will be cleared both from sites of action and from the subject's body. Finally, the determination of optimal dosing preferably will provide an effective dose that is neither below the threshold of maximal beneficial effect nor above the threshold where the deleterious effects associated with the dose of the active agents outweighs the advantages of the increased dose.

It will be appreciated that a "dose" may be delivered all at once, fractionally, or continuously over a period of time. The entire dose also may be delivered to a single location or spread fractionally over several locations. Furthermore, doses may remain the same over a treatment, or they may vary.

In various embodiments, formulations in accordance with the invention are administered in an initial dose, and thereafter maintained by further administrations. A formulation of the invention in some embodiments is administered by one method initially, and thereafter administered by the same method or by one or more different methods. The dosages of on-going administrations may be adjusted to maintain at certain values the levels of the active agents in the subject. In some embodiments the compositions are administered initially, and/or to maintain their level in the subject, by intravenous injection. In a variety of embodiments, other forms of administration are used.

Formulations of the invention may be administered in many frequencies over a wide range of times, including any suitable frequency and range of times that delivers a treatment-effective dose. Doses may be continuously delivered, administered every few hours, one or more times a day, every day, every other day or several times a week, or less frequently. In some embodiments they are administered over periods of one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more days. In some embodiments they are administered over periods of one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, or more months. In a variety of embodiments they are administered for a period of one, two, three, four, five, six, seven, eight, nine, ten, or more years. Suitable regimens for initial administration and further doses for sequential administrations may all be the same or may be variable. Appropriate regimens can be ascertained by the skilled artisan, from this disclosure, the

documents cited herein, and the knowledge in the art. Generally lengths of treatment will be proportional to the length of the disease process, the effectiveness of the therapies being applied, and the condition and response of the subject being treated.

8. Diseases and Treatments

Self-buffering pharmaceutical protein compositions in accordance with the invention, in preferred embodiments, are useful to treat subjects suffering from a wide variety of disorders and diseases. As noted elsewhere herein, the invention provides, among others, self-buffering compositions of pharmaceutical antibodies, antibody-derived pharmaceutical proteins, and antibody-related pharmaceutical proteins, that can comprise Fc effector functions and binding domains specific for a wide variety of disease-related targets and that are useful for treating disease. These proteins and self-buffering compositions thereof are described at length herein above, as well as their use in treating various disorders and diseases associated with their targets. Methods for using the compositions, including formulation methods, administration methods, doses, and dosing methods are all described illustratively above. The formulation and administration of any particular composition of the invention can be tailored to the treatment of a particular disease, using well-known and routine techniques in the arts for doing so, taken in light of the guidance provided by the present description of the invention. Among diseases usefully treated using self-buffering pharmaceutical protein formulations in accordance with various aspects and preferred embodiments of the invention are inflammatory diseases, cancers, metabolic disorders, neurological and renal disorders, to name just a few.

9. Packaging and Kits

The invention also provides kits comprising self-buffering protein formulations, particularly kits comprising in one more containers, a self-buffering pharmaceutical protein formulation and instructions regarding the use thereof, particularly such kits wherein the formulation is a pharmaceutically acceptable formulation for human use. Among preferred kits are those comprising one or more containers of a self-buffering protein formulation of the invention and one or more separate documents, information pertaining to the contents of the kit, and/or the use of its contents, particularly those wherein the protein is a biopharmaceutical protein, especially those wherein the protein is a biopharmaceutical protein formulated for the treatment of a disease in humans.

In certain aspects of the invention in this regard, preferred kits include kits as above further comprising one or more single or multi-chambered syringes (e.g., liquid syringes and lyosyringes) for administering one or more self-buffering protein formulations of the invention. In certain aspects of the invention in this regard, certain of the particularly preferred kits further comprise preloaded syringes. In further particularly preferred embodiments in this regard, the kits comprise a self-buffering pharmaceutical composition for parenteral administration, sealed in a vial under partial vacuum in a form ready for loading into a syringe and administration to a subject. In especially preferred embodiments in this regard, the composition is disposed therein under partial vacuum. In all of these regards and others, in certain further particularly preferred embodiments the kits contain one or more vials in accordance with any of the foregoing, wherein each vial contains a single unit dose for administration to a subject. In all these respects and others the invention further relates to kits comprising lyophilates, disposed as above, that upon reconstitution provide compositions in accordance therewith. In this regard, the invention further provides in certain of its preferred embodiments, kits that contain a lyophilate in accordance with the invention and a sterile diluent for reconstituting the lyophilate.

Examples

The present invention is additionally described by way of the following illustrative, non-limiting Examples.

EXAMPLE 1: Acid Titrations and Buffer Capacities of Sodium Acetate Buffers in the Range pH 5.0 to 4.0

A stock solution of known concentration of acetic acid was prepared by diluting ultrapure glacial acetic acid in HPLC grade water and then titrating the pH up to the desired value with NaOH. Stocks were equilibrated to the air and to 21° C. Volumetric standards were prepared at a concentration of 1 N and diluted as necessary with HPLC water.

One mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM, and 15 mM sodium acetate buffers were prepared by diluting the stock in HPLC water. The solutions were titrated with HCl. 0.2 N HCl was used for the 1, 2.5, and 5 mM solutions, 0.4 N HCl was used for the 7.5 mM solution, and 0.8 N HCl was used for the 10 and 15 mM solutions. The titrations were performed using standard analytical laboratory techniques.

Figure 1, Panel A shows the titration data and the least squares trend lines calculated from the data for each solution. The slope of the trend line calculated from each data set was taken as the buffer capacity of the corresponding acetate buffer. The linear dependence of buffer capacity on acetate buffer concentration is shown in Figure 1, Panel B.

EXAMPLE 2: Base Titrations and Buffer Capacities of Sodium Acetate Buffers in the Range pH 5.0 to 5.5

Acetate buffer stocks and solutions for titration were prepared as described in Example 1. The solutions were titrated as described in Example 1, except that the solutions were titrated from pH 5.0 to 5.5 and the titrations were done using NaOH instead of HCl. 0.2 N NaOH was used to titrate the 1, 2.5, and 5 mM solutions and 0.4 N NaOH was used for the 7.5, 10, and 15 mM solutions. The results of the titrations are shown in Figure 2A. The linear dependence of buffer capacity on concentration of acetate buffer is displayed in Figure 2B.

EXAMPLE 3: Determination of Acetate by HPLC

Acetate was determined in acetate buffer samples using analytical SE-HPLC. A standard curve for peak areas as a function of acetate concentration was established by analysis of acetate in buffers of known acetate concentration. The amount of acetate in test samples was interpolated from the standard curve. A standard curve is shown in Figure 3. Nominal and measured amount of acetate in test buffers are tabulated below the standard curve in the figure.

EXAMPLE 4: Acid Titrations of Ab-hOPGL Formulations Over the Range of pH 5.0 to pH 4.0

Bulk Ab-hOPGL in 10 mM acetate (nominal value), 5% sorbitol, pH 5.0 was diafiltered against 5.25% sorbitol, pH 3.2 (adjusted with HCl) in a LABSCALE TFF® system (Millipore) with a multi-manifold cassette, using 3 Millipore Pellicon XL 50 regenerated cellulose ultra-filtration membranes. The diafiltration solution was exchanged 8 to 10 times over the course of the diafiltration for each formulation. Following diafiltration, the pH of the resulting buffer-free solution was measured and adjusted to pH 5.0, using 0.05 N HCl or 0.05 N NaOH.

One, 10, 30, 60, 90, and 110 mg/ml solutions were prepared for titration by dilution. The pH of each dilution was adjusted to pH 5.0 with NaOH or HCl as necessary. Titrations were carried out as described in the foregoing Examples. 0.2 N HCl was used to titrate the 1, 10, and 30 mg/ml solutions. 0.4 N HCl was used to titrate the 60 mg/ml solution. 0.8 N HCl was used to titrate the 90 and 110 solutions.

The results of the titrations are depicted in Figure 4. The least squares regression line is shown for the dataset for each concentration. The buffer capacity was taken as the slope of the regression line for each concentration.

EXAMPLE 5: Base Titrations of Ab-hOPGL Formulations Over the Range of pH 5.0 to 6.0

One, 10, 30, 60, 90, and 110 mg/ml solutions of Ab-hOPGL were prepared for titration as described in Example 4. Base titrations were carried out using NaOH as described in preceding Examples. 0.2 N NaOH was used for the 1, 10, 30, and 60 mg/ml solutions and 0.4 N NaOH was used for the 90 and 110 mg/ml solutions. Results of the titrations are depicted in the graph in Figure 5. Linear regression lines are shown for the data for each concentration. The buffer capacity was taken as the slope of the regression line for each concentration.

EXAMPLE 6: Residual Acetate Levels in Self-Buffering Ab-hOPGL Formulations

The amount of residual acetate was determined in Ab-hOPGL formulations using the methods described in Example 3. The results are depicted graphically in Figure 6, which shows a standard curve relating HPLC measurements to acetate concentrations and, below the graph, a tabulation of the results of determinations made on Ab-hOPGL formulations at different concentrations. Ab-hOPGL concentrations are indicated on the left ("Nominal") and the measured concentration of acetate in each of the Ab-hOPGL concentration is indicated on the right.

EXAMPLE 7: Buffer Capacity of Ab-hOPGL Formulations Plus or Minus Residual Acetate in the Range of pH 5.0 to 4.0

Self-buffered Ab-hOPGL formulations were prepared and titrated with HCl as described in foregoing Examples. In addition, data was adjusted by subtracting the contribution of residual acetate buffer based on the determination of acetate content by

SE-HPLC as described in, for instance, Example 3. Buffer capacities were determined as described above. The same analysis was carried out on both sets of data. The results, depicted in Figure 7, show the effect of residual acetate on the buffer capacity of the Ab-hOPGL preparations. The results make it clear that the buffer capacity of residual acetate is a minor factor in the buffer capacity of the self-buffering Ab-hOPGL formulations that were analyzed.

EXAMPLE 8: Buffer Capacity of Ab-hOPGL Plus or Minus Residual Acetate in the Range of pH 5.0 to 6.0

Self-buffered Ab-hOPGL formulations were prepared and titrated with NaOH as described in foregoing Examples. In addition, data was adjusted by subtracting the contribution of residual acetate buffer based on the determination of acetate content by SE-HPLC as described in, for instance, Example 3. Buffer capacities were determined as described above. The same analysis was carried out on both sets of data. The results, depicted in Figure 8, show the effect of residual acetate on the buffer capacity of the Ab-hOPGL preparations. The results make it clear that the buffer capacity of residual acetate is a minor factor in the buffer capacity of the self-buffering Ab-hOPGL formulations that were analyzed.

EXAMPLE 9: pH and Ab-hOPGL Stability in Self-Buffered and Conventionally Buffered Formulations

Self-buffering formulations of Ab-hOPGL were prepared as described in the foregoing Examples. In addition, formulations were made containing a conventional buffering agent, either acetate or glutamate. All formulations contained 60 mg/ml Ab-hOPGL. The stability of pH and Ab-hOPGL in the formulations was monitored for six months of storage at 4° C. Stability was monitored by determining monomeric Ab-hOPGL in the formulations over the time course of storage. The determination was made using SE-HPLC as described above. The results for all three formulations are shown in Figure 9. Panel A shows the stability of Ab-hOPGL in the three formulations. Stability in the self-buffered formulation is as good as in the conventionally buffered formulations. Panel B shows the pH stability of the three formulations. Again, pH stability in the self-buffered formulation is as good as in the conventionally buffered formulations.

EXAMPLE 10: Titration and Buffer Capacities of Ab-hB7RP1 – pH 5.0 to 4.0

Self-buffering formulations of Ab-hB7RP1 were prepared in concentrations of 1, 10, 30, and 60 mg/ml, as described for Ab-hOPGL in the foregoing Examples. Titrations were carried out using HCl as described above. In addition, data was adjusted by subtracting the contribution of residual acetate buffer based on the determination of acetate content by SE-HPLC as described in, for instance, Example 3. Figure 10, Panel A shows the titration results. Figure 10, Panel B shows the dependence of buffer capacity on the concentration of Ab-hB7RP1 formulations before and after subtracting the contribution of residual acetate buffer. The results clearly show the self-buffering capacity of Ab-hB7RP1 in this pH range. At 40 mg/ml it provides approximately as much buffer capacity in this pH range as 10 mM sodium acetate buffer. At 60 mg/ml it provides approximately as much buffer capacity as 15 mM sodium acetate buffer.

EXAMPLE 11: Titration and Buffer Capacities for Ab-hB7RP1 – pH 5.0 to 6.0

Self-buffering formulations of Ab-hB7RP1 were prepared in concentrations of 1, 10, 30, and 60 mg/ml, as described for Ab-hOPGL in the foregoing Examples. Titrations were carried out using NaOH as described above. In addition, data was adjusted by subtracting the contribution of residual acetate buffer based on the determination of acetate content by SE-HPLC as described in, for instance, Example 3. Figure 11, Panel A shows the titration results. Figure 11, Panel B shows the dependence of buffer capacity on the concentration of Ab-hB7RP1 formulations before and after subtracting the contribution of residual acetate buffer. The results clearly show the self-buffering capacity of Ab-hB7RP1 in this pH range. At 60 mg/ml it provides approximately as much buffer capacity in this pH range as 10 mM sodium acetate buffer.

EXAMPLE 12: Ab-hB7RP1 Stability in Self-Buffering and Conventionally Buffered Formulations at 4° C and 29° C

Ab-hB7RP1 was prepared as described in the foregoing Examples and formulated as described above, in self-buffering formulations and in formulations using a conventional buffering agent, either acetate or glutamate. All formulations contained 60 mg/ml Ab-hB7RP1. The stability of the solution's pH and of the Ab-hB7RP1 in the solution was monitored for twenty-six weeks of storage at 4° C or at 29° C. Stability

was monitored by determining monomeric Ab-hB7RP1 in the formulations over the time course of storage. The determination was made using SE-HPLC as described above. The results are shown in Figure 12. Panel A shows the results for storage at 4° C. Panel B shows the results for storage at 29° C. Ab-hB7RP1 was at least as stable in the self-buffered formulation at 4° C as the conventionally buffered formulations. At 29° C the self-buffered formulation was at least as stable as the conventionally buffered formulations, and may have been slightly better from 10 weeks through the last time point.

EXAMPLE 13: pH Stability of Self-Buffered Ab-hB7RP1 at 4° C and 29° C

Self-buffered Ab-hB7RP1 at 60 mg/ml was prepared as described in the foregoing Example. pH was monitored over the time course and at the same temperatures as described therein. The results are shown in Figure 13.

EXAMPLE 14: Buffer Capacity of Ab-hCD22 Formulations – pH 4.0 to 6.0

Self-buffering formulations of Ab-hCD22 were prepared and titrated over the range of pH 5.0 to 4.0 and the range of 5.0 to 6.0, as described for Ab-hOPGL and Ab-hB7RP1 in the foregoing Examples. Buffer capacities were calculated from the titration data, also as described above. Buffer capacity as a function of concentration is shown in Figure 14 for both pH ranges. Panel A shows the buffer capacity of the Ab-hCD22 formulations over the range of pH 5.0 to 4.0. Buffer capacity is linearally dependent on concentration, and an approximately 21 mg/ml formulation of Ab-hCD22 has a buffer capacity equal to that of 10 mM sodium acetate buffer pH 5.0, measured in the same way. Panel B shows the buffer capacity as a function of concentration over the pH range 5.0 to 6.0. In this range of pH an approximately 30 mg/ml formulation of Ab-hCD22 has a buffer capacity equal to that of 10 mM sodium acetate buffer pH 5.0, measured in the same way.

EXAMPLE 15: Titrations and Buffer Capacities of Ab-hIL4R Formulations – pH 5.0 to 4.0

Self-buffering formulations of Ab-hIL4R were prepared in concentrations of 1, 10, 25, and 90 mg/ml, as described for Ab-hOPGL in the foregoing Examples. Titrations were carried out using HCl as described above. Figure 15, Panel A shows the titration results. Figure 15, Panel B shows the dependence of buffer capacity on the

concentration of Ab-hIL4R. The results clearly show the self-buffering capacity of Ab-hIL4R in this pH range. At approximately 75 mg/ml it provides as much buffer capacity in this pH range as 10 mM sodium acetate pH 5.0, measured in the same way.

EXAMPLE 16: Titrations and Buffer Capacities of Ab-hIL4R Formulations – pH 5.0 to 6.0

Self-buffering formulations of Ab-hIL4R were prepared in concentrations of 1, 10, 25, and 90 mg/ml, as described for Ab-hOPGL in the foregoing Examples. Titrations were carried out using NaOH as described above. Figure 16, Panel A shows the titration results. Figure 16, Panel B shows the dependence of buffer capacity on the concentration of Ab-hIL4R in this pH range. The results clearly show the self-buffering capacity of Ab-hIL4R in this pH range. At approximately 90 mg/ml it provides as much buffer capacity in this pH range as 10 mM sodium acetate pH 5.0, measured in the same way.

EXAMPLE 17: Ab-hIL4R and pH Stability in Acetate and Self-Buffered Ab-hIL4R Formulations at 37° C

Self-buffered and acetate buffered formulations of Ab-hIL4R at pH 5.0 and 70 mg/ml were prepared as described above. pH and Ab-hIL4R stability were monitored in the formulations for 4 weeks at 37° C. Ab-hIL4R stability was monitored by SE-HPLC as described above. The results are shown in Figure 17. Panel A shows that Ab-hIL4R is at least as stable in the self-buffered formulation as in the sodium acetate buffer formulation. Panel B shows that pH in the self-buffered formulation is as stable as in the sodium acetate buffer formulation.

What is claimed:

1. A composition comprising a pharmaceutical protein, wherein at the pH of the composition, 21° C, one atmosphere, and equilibrium with ambient atmosphere, the protein has a buffer capacity per unit volume of at least that of approximately 4.0 mM sodium acetate buffer in pure water in the range of pH 5.0 to 4.0 or pH 5.0 to 5.5 under the same conditions, wherein further, exclusive of the buffer capacity of said protein, the buffer capacity per unit volume of the composition under the same conditions is no more than that of 2.0 mM sodium acetate buffer in pure water in the range of pH 5.0 to 4.0 or pH 5.0 to 5.5 under the same conditions, wherein the composition has been approved for pharmaceutical use by an authority legally empowered to grant such approval.

2. A composition comprising a pharmaceutical protein, wherein at the pH of the composition, 21° C, one atmosphere, and equilibrium with ambient atmosphere, the protein has a buffer capacity per unit volume of at least 1.50 mEq/liter-pH unit, wherein further, exclusive thereof, the buffer capacity per unit volume of the composition is less than 0.5 mEq/liter-pH unit, wherein the composition has been approved for pharmaceutical use by an authority legally empowered to grant such approval.

3. A composition according to claim 1, wherein the protein provides at least 80% of the buffer capacity of the composition.

4. A composition according to claim 3, wherein the concentration of the protein is between approximately 20 and 400 mg/ml.

5. A composition according to claim 4, wherein the pH maintained by the buffering action of the protein is between approximately 3.5 and 8.0.

6. A composition according to claim 5, wherein the pH maintained by the buffering action of the protein is between approximately 4 and 6.

7. A composition according to claim 5, further comprising one or more pharmaceutically acceptable salts, wherein the total salt concentration is less than 150 mM.

8. A composition according to claim 7, further comprising one or more pharmaceutically acceptable salts, wherein the total salt concentration is less than 100 mM.

9. A composition according to claim 5, further comprising one or more pharmaceutically acceptable polyols.

10. A composition according to claim 9, wherein the polyol is one or more of sorbitol, mannitol, sucrose, trehalose, or glycerol.

11. A composition according to claim 5, further comprising one or more pharmaceutically acceptable surfactants.

12. A composition according to claim 11, wherein the surfactant is one or more of polysorbate 20, polysorbate 80, other fatty acid esters of sorbitan, polyethoxylates, and poloxamer 188.

13. A composition according to claim 9, further comprising one or more pharmaceutically acceptable surfactants.

14. A composition according to claim 1, further comprising one or more pharmaceutically acceptable: osmotic balancing agents; anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; analgesics; or additional pharmaceutical agents.

15. A composition according to claim 5, further comprising one or more pharmaceutically acceptable: osmotic balancing agents; anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; analgesics; or additional pharmaceutical agents.

16. A composition according to claim 7, further comprising one or more pharmaceutically acceptable: osmotic balancing agents; anti-oxidants; antibiotics; antimycotics; bulking agents; lyoprotectants; anti-foaming agents; chelating agents; preservatives; colorants; analgesics; or additional pharmaceutical agents.

17. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is or comprises: an antibody, Fab fragment, Fab_2 fragment, Fab_3 fragment, Fc fragment, scFv fragment, bis-scFv(s) fragment, minibody, diabody, triabody tetrabody, VhH domain, V-NAR domain, V_H domain, V_L domain, camel Ig, Ig NAR, receptibody, peptibody, or a variant or a derivative thereof or a protein related thereto, or a modification thereof.

18. A composition according to claim 17, wherein the protein comprises an Fc fragment or a part thereof, or a variant or a derivative of an Fc fragment or a part thereof or a protein related to an Fc fragment or part thereof, or a modification of any thereof.

19. A composition according to claim 18, wherein the protein further comprises a first binding moiety of a pair of cognate binding moieties.

20. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is selected from the group consisting of proteins that bind specifically to one or more CD proteins, HER receptor family proteins, cell adhesion molecules, growth factors, nerve growth factors, fibroblast growth factors, transforming growth factors (TGF), insulin-like growth factors, osteoinductive factors, insulins and insulin-related proteins, coagulation and coagulation-related proteins, colony stimulating factors (CSFs), other blood and serum proteins blood group antigens; receptors, receptor-associated proteins, growth hormone receptors, T-cell receptors; neurotrophic factors. neurotrophins, relaxins, interferons, interleukins, viral antigens, lipoproteins, integrins, rheumatoid factors, immunotoxins, surface membrane proteins, transport proteins, homing receptors, addressins, regulatory proteins, and immunoadhesins,

21. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is selected from the group consisting of: OPGL specific binding proteins, myostatin specific binding proteins, IL-4 receptor specific binding proteins, IL1-R1 specific binding proteins, Ang2 specific binding proteins, NGF-specific binding proteins, CD22 specific binding proteins, IGF-1 receptor specific binding proteins, B7RP-1 specific binding proteins, IFN gamma specific binding proteins, TALL-1 specific binding proteins, stem cell factors, Flt-3 ligands, and IL-17 receptors.

22. A composition according to any of claims 1, 5, 7, 9, 11, 13 or 14, wherein the protein is selected from the group consisting of proteins that bind specifically to one ormore of: CD3, CD4, CD8, CD19, CD20, CD34; HER2, HER3, HER4, the EGF receptor; LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM, alpha v/beta 3 integrin; vascular endothelial growth factor ("VEGF"); growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-1-alpha), erythropoietin (EPO), NGF-beta, platelet-derived growth factor (PDGF), aFGF, bFGF, epidermal growth factor (EGF), TGF-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, TGF-beta5, IGF-I, IGF-II, des(1-3)-IGF-I (brain IGF-I), insulin, insulin A-chain, insulin B-chain, proinsulin, insulin-like growth factor binding proteins;, such as, among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1–antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin; M-CSF, GM-CSF, G-CSF, albumin, IgE, flk2/flt3

receptor, obesity (OB) receptor, bone-derived neurotrophic factor (BDNF), NT-3, NT-4, NT-5, NT-6); relaxin A-chain, relaxin B-chain, prorelaxin; interferon-alpha, -beta, and -gamma;IL-1 to IL-10; AIDS envelope viral antigen; calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES, mouse gonadotropin-associated peptide, Dnase, inhibin, and activin; protein A or D, bone morphogenetic protein (BMP), superoxide dismutase, decay accelerating factor (DAF).

23. A composition according to any of claims 1, 5, 7, 9, 11, 13 or 14, wherein the protein is selected from the group consisting of:Actimmune (Interferon-gamma-1b), Activase (Alteplase), Aldurazme (Laronidase), Amevive (Alefacept), Avonex (Interferon beta-1a), BeneFIX (Nonacog alfa), Beromun (Tasonermin), Beatseron (Interferon-beta-1b), BEXXAR (Tositumomab), Tev-Tropin (Somatropin), Bioclate or RECOMBINATE (Recombinant), CEREZME (Imiglucerase), ENBREL (Etanercept), Eprex (epoetin alpha), EPOGEN/Procit (Epoetin alfa), FABRAZYME (Agalsidase beta), Fasturtec/Elitek ELITEK (Rasburicase), FORTEO (Teriparatide), GENOTROPIN (Somatropin), GlucaGen (Glucagon), Glucagon (Glucagon, rDNA origin), GONAL-F (follitropin alfa), KOGENATE FS (Octocog alfa), HERCEPTIN (Trastuzumab), HUMATROPE (SOMATROPIN), HUMIRA (Adalimumab), Insulin in Solution, INFERGEN® (Interferon alfacon-1), KINERET® (anakinra), Kogenate FS (Antihemophilic Factor), LEUKIN (SARGRAMOSTIM Recombinant human granulocyte-macrophage colony stimulating factor (rhuGM-CSF)), CAMPATH (Alemtuzumab), RITUXAN® (Rituximab), TNKase (Tenecteplase), MYLOTARG (gemtuzumab ozogamicin), NATRECOR (nesiritide), ARANESP (darbepoetin alfa), NEULASTA (pegfilgrastim), NEUMEGA (oprelvekin), NEUPOGEN (Filgrastim), NORDITROPIN CARTRIDGES (Somatropin), NOVOSEVEN (Eptacog alfa), NUTROPIN AQ (somatropin), Oncaspar (pegaspargase), ONTAK (denileukin diftitox), ORTHOCLONE OKT (muromonab-CD3), OVIDREL (choriogonadotropin alfa), PEGASYS (peginterferon alfa-2a), PROLEUKIN (Aldesleukin), PULMOZYME (dornase alfa), Retavase (Reteplase), REBETRON Combination Therapy containing REBETOL® (Ribavirin) and INTRON® A (Interferon alfa-2b), REBIF (interferon beta-1a), REFACTO (Antihemophilic Factor), REFLUDAN (lepirudin), REMICADE (infliximab), REOPRO (abciximab)ROFERON®-A (Interferon alfa-2a), SIMULECT (baasiliximab), SOMAVERT (Pegivisomant), SYNAGIS® (palivizumab), Stemben (Ancestim, Stem cell factor), THYROGEN, INTRON® A (Interferon alfa-2b), PEG-

INTRON® (Peginterferon alfa-2b), XIGRIS® (Drotrecogin alfa activated), XOLAIR® (Omalizumab), ZENAPAX® (daclizumab), and ZEVALIN® (Ibritumomab Tiuxetan).

24. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hOPGL or a fragment thereof, or a variant or derivative of Ab-hOPGL or of a fragment thereof, or an Ab-hOPGL related protein or fragment thereof, or a modification of any thereof.

25. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hOPGL.

26. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hIL4R or a fragment thereof, or a variant or derivative of Ab-hIL4R or of a fragment thereof, or an Ab-hIL4R related protein or fragment thereof, or a modification of any thereof.

27. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hIL4R.

28. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hB7RP1 or a fragment thereof, or a variant or derivative of Ab-hB7RP1 or of a fragment thereof, or an Ab-hB7RP1 related protein or fragment thereof, or a modification of any thereof.

29. A composition according to any of claims 1, 5, 7, 9, 11, 13, or 14, wherein the protein is Ab-hB7RP1.

30. A lyophilate which upon reconstitution provides a composition according to any of claim 1, 5, 7, 9, 11, 13 or 14.

31. A kit comprising in one or more containers a composition according to any of claim 1, 5, 7, 9, 11, 13 or 14 and instructions regarding the use thereof.

32. A kit comprising in one or more containers a lyophilate according to claim 31, and instructions regarding the use thereof.

33. A method for treating a subject, comprising administering to a subject in an amount and by a route effective for treatment, a composition according to any of claim 1, 5, 7, 9, 11, 13 or 14.

34. A process for preparing a composition according to any of claim 1, 5, 7, 9, 11, 13 or 14, comprising removing residual buffer using a counter ion.

35. A process for preparing a composition according to claim 34, comprising removing residual buffer using any one or more of the following in the

presence of a counter ion: size exclusion chromatography, dialysis, and/or tangential flow filtration.

36. A process for preparing a composition according to claim 35, comprising removing residual buffer using ion exchange chromatography.

37. A process for preparing a composition according to any of claim 1, 5, 7, 9, 11, 13 or 14, comprising removing residual buffer by diafiltration against a bufferless solution having a pH below the desired pH.

38. A process for preparing a composition according to claim 37, wherein following diafiltration the pH is adjusted to a desired pH by addition of dilute acid and/or dilute base.

39. A lyophilate which upon reconstitution provides a composition according to claim 20.

40. A kit comprising in one or more containers a composition according to claim 20 and instructions regarding the use thereof.

41. A kit comprising in one or more containers a lyophilate according to claim 40, and instructions regarding the use thereof.

42. A method for treating a subject, comprising administering to a subject in an amount and by a route effective for treatment, a composition according to claim 20.

43. A process for preparing a composition according to claim 20, comprising removing residual buffer using a counter ion.

44. A process for preparing a composition according to claim 43, comprising removing residual buffer using any one or more of the following in the presence of a counter ion: size exclusion chromatography, dialysis, and/or tangential flow filtration.

45. A process for preparing a composition according to claim 43, comprising removing residual buffer using ion exchange chromatography.

46. A process for preparing a composition according to claim 20, comprising removing residual buffer by diafiltration against a bufferless solution having a pH below the desired pH.

47. A process for preparing a composition according to claim 46, wherein following diafiltration the pH is adjusted to a desired pH by addition of dilute acid and/or dilute base.

48. A lyophilate which upon reconstitution provides a composition according to claim 21.

49. A kit comprising in one or more containers a composition according claim 21 and instructions regarding the use thereof.

50. A kit comprising in one or more containers a lyophilate according to claim 49, and instructions regarding the use thereof.

51. A method for treating a subject, comprising administering to a subject in an amount and by a route effective for treatment, a composition according to claim 21.

52. A process for preparing a composition according to claim 21, comprising removing residual buffer using a counter ion.

53. A process for preparing a composition according to claim 52, comprising removing residual buffer using any one or more of the following in the presence of a counter ion: size exclusion chromatography, dialysis, and/or tangential flow filtration.

54. A process for preparing a composition according to claim 52, comprising removing residual buffer using ion exchange chromatography.

55. A process for preparing a composition according to claim 21, comprising removing residual buffer by diafiltration against a bufferless solution having a pH below the desired pH.

56. A process for preparing a composition according to claim 55, wherein following diafiltration the pH is adjusted to a desired pH by addition of dilute acid and/or dilute base.

57. A lyophilate which upon reconstitution provides a composition according to claim 23.

58. A kit comprising in one or more containers a composition according claim 23 and instructions regarding the use thereof.

59. A kit comprising in one or more containers a lyophilate according to claim 57, and instructions regarding the use thereof.

60. A method for treating a subject, comprising administering to a subject in an amount and by a route effective for treatment, a composition according to claim 23.

61. A process for preparing a composition according to claim 23, comprising removing residual buffer using a counter ion.

62. A process for preparing a composition according to claim 61, comprising removing residual buffer using any one or more of the following in the

presence of a counter ion: size exclusion chromatography, dialysis, and/or tangential flow filtration.

63. A process for preparing a composition according to claim 61, comprising removing residual buffer using ion exchange chromatography.

64. A process for preparing a composition according to claim 21, comprising removing residual buffer by diafiltration against a bufferless solution having a pH below the desired pH.

65. A process for preparing a composition according to claim 62, wherein following diafiltration the pH is adjusted to a desired pH by addition of dilute acid and/or dilute base.

Figure 1. Buffer Capacity of Acetate in the pH 4.0 - 5.0 Range

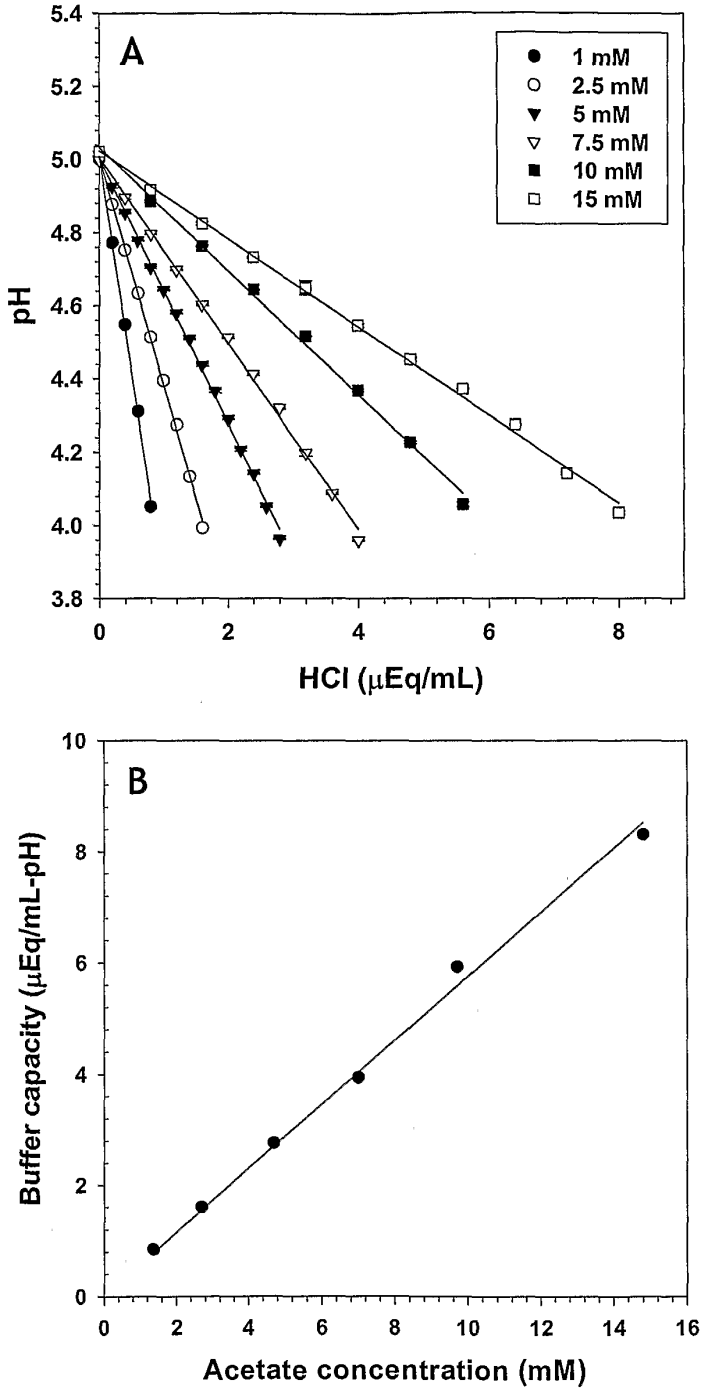
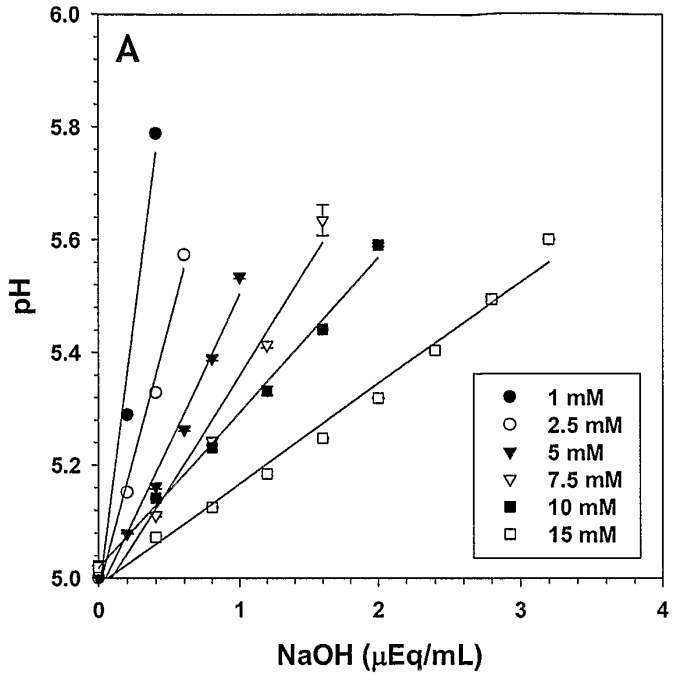


Figure 2. Buffer Capacity of Acetate in the pH 5.0 – 5.5 Range



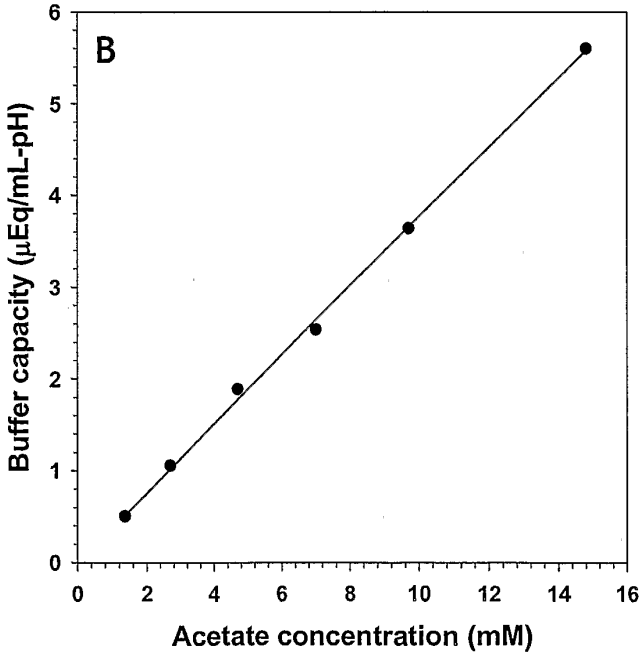
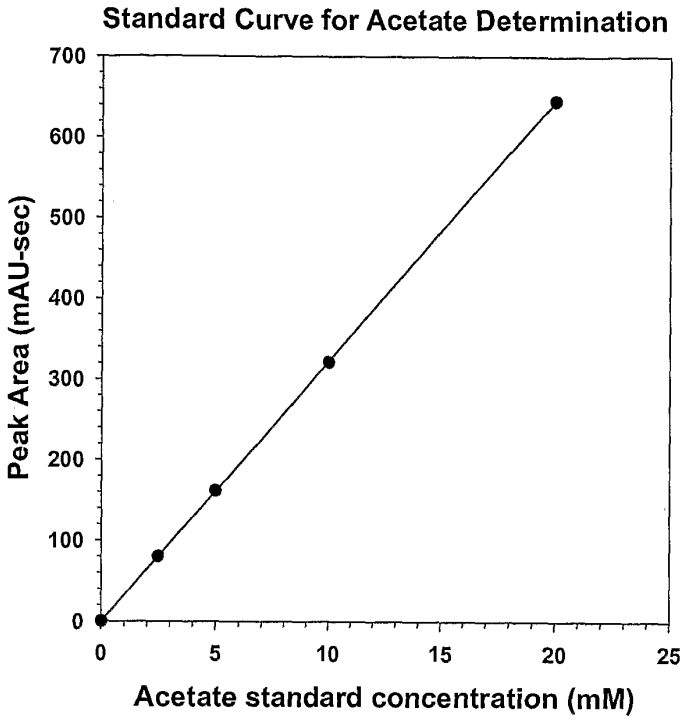


Figure 3. Determination of acetate concentration in acetate standards used for buffer capacity measurements



Experimentally determined acetate values for solutions used to determine acetate buffer capacity

Acetate concentration (mM)	
Nominal	Experimental
1	1.37
2.5	2.7
5	4.7
7.5	7.0
10	9.7
15	14.8

Figure 4. Acid titration of Ab-hOPGL Solutions

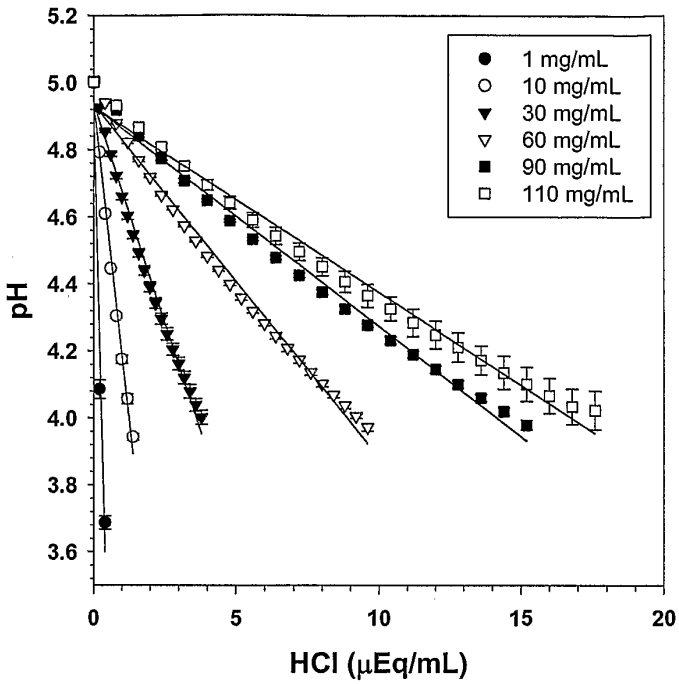


Figure 5. Base titration of Ab-hOPGL Solutions

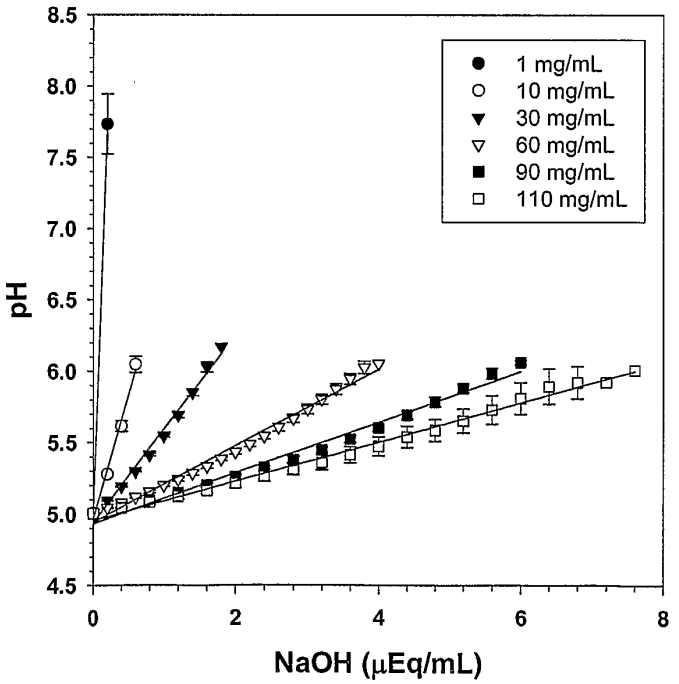
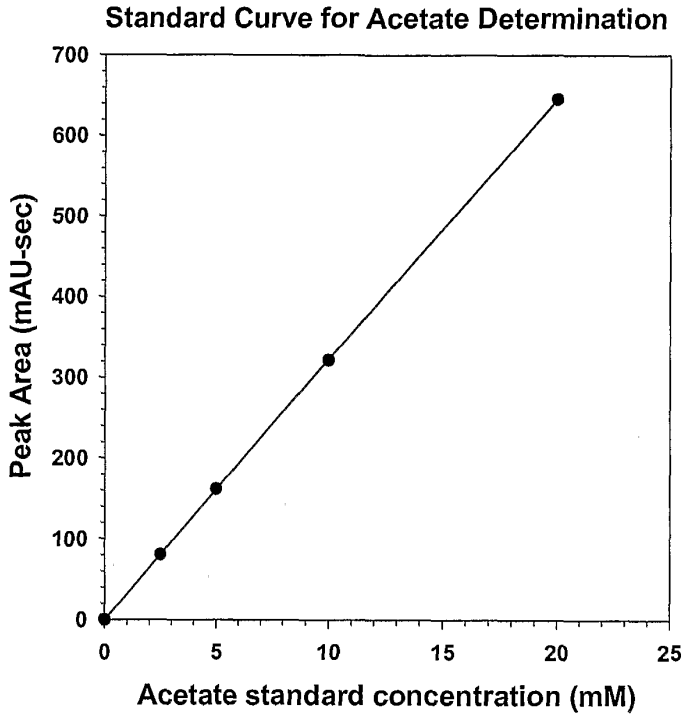


Figure 6. Determination of residual acetate levels in Ab-hOPGL Solutions for buffer capacity measurements



Residual acetate levels in Ab-hOPGL solutions used to determine buffer capacity

Acetate concentration (mM)	
Nominal	**Experimental**
1	0.05
10	0.20
30	0.51
60	1.16
90	1.71
110	1.82

Figure 7. Buffer capacity of Ab-hOPGL +/- residual acetate as a function of its concentration in the pH 4.0 – pH 5.0 range

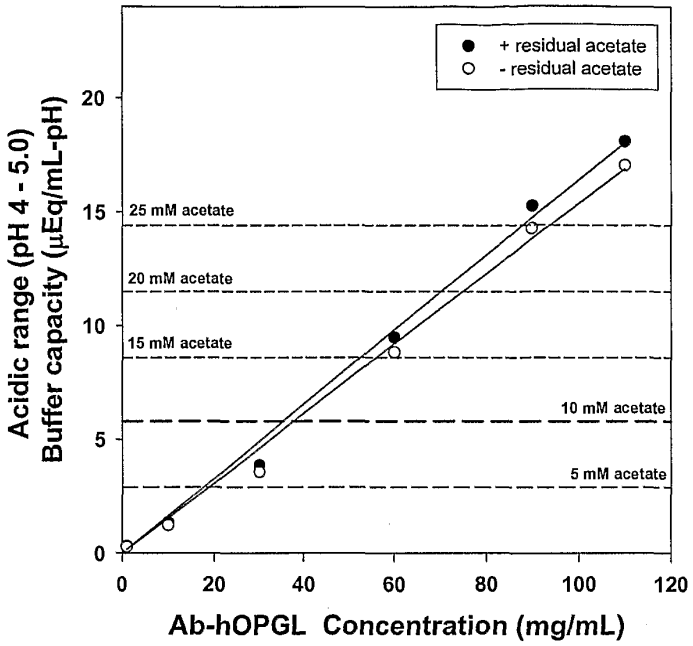


Figure 8. Buffer capacity of Ab-hOPGL +/- residual acetate as a function of its concentration in the pH 5.0 – pH 6.0 range

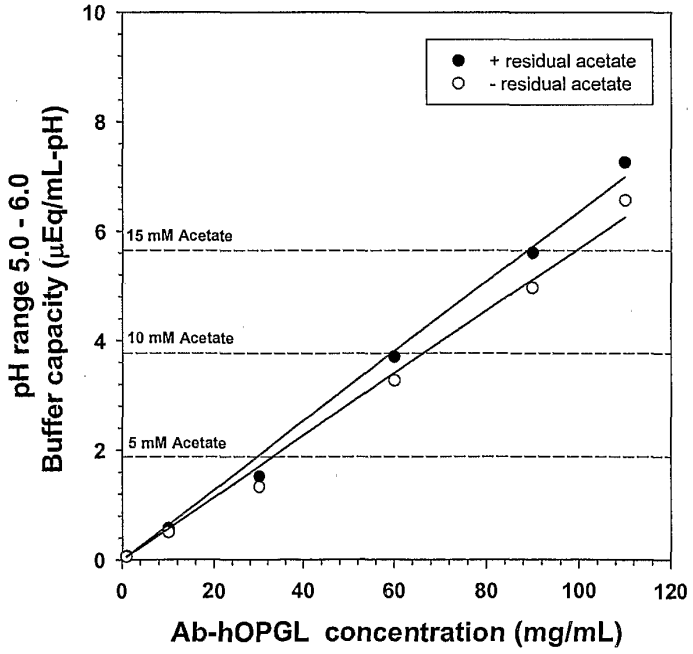
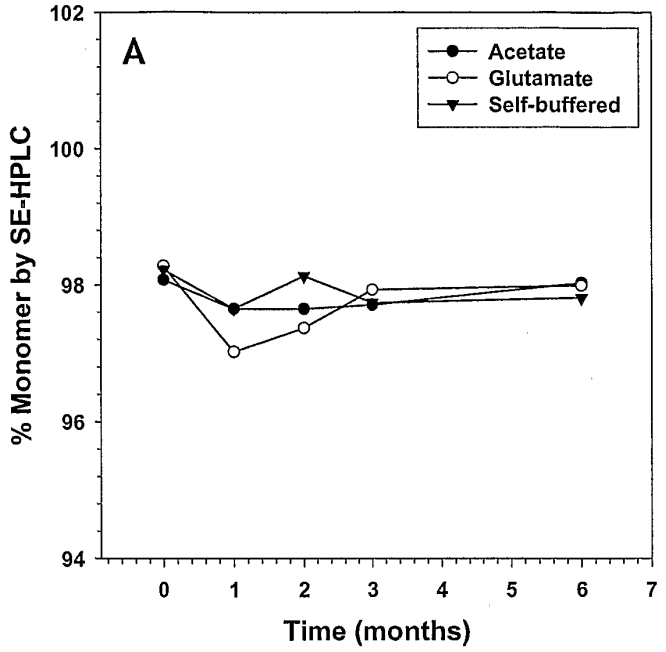


Figure 9. Stability of the Self-Buffered, 60 mg/mL, Ab-hOPGL Formulation Compared to Conventionally Buffered Formulations at 4 C



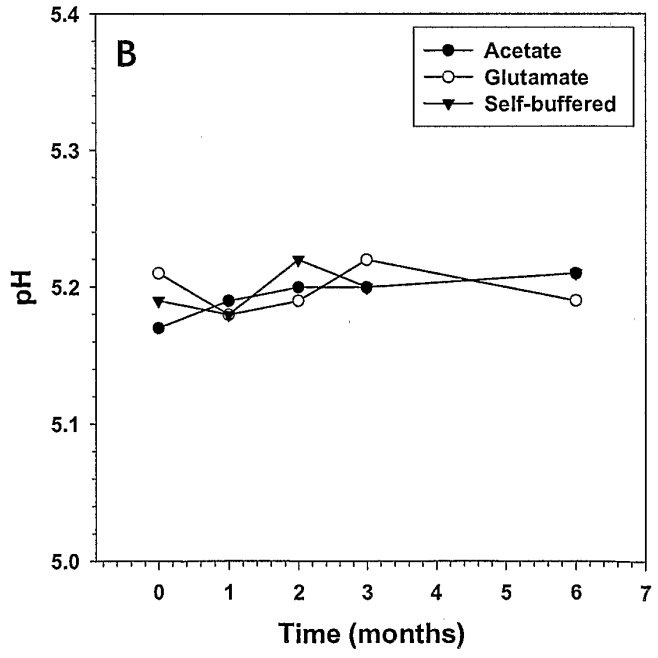
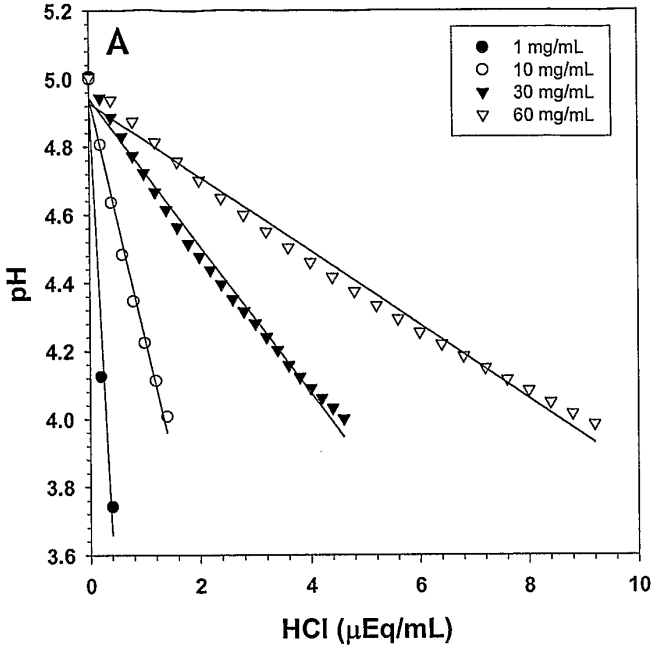


Figure 10. Buffer capacity of Ab-hB7RP1 Solutions in the pH 4.0 – 5.0 range



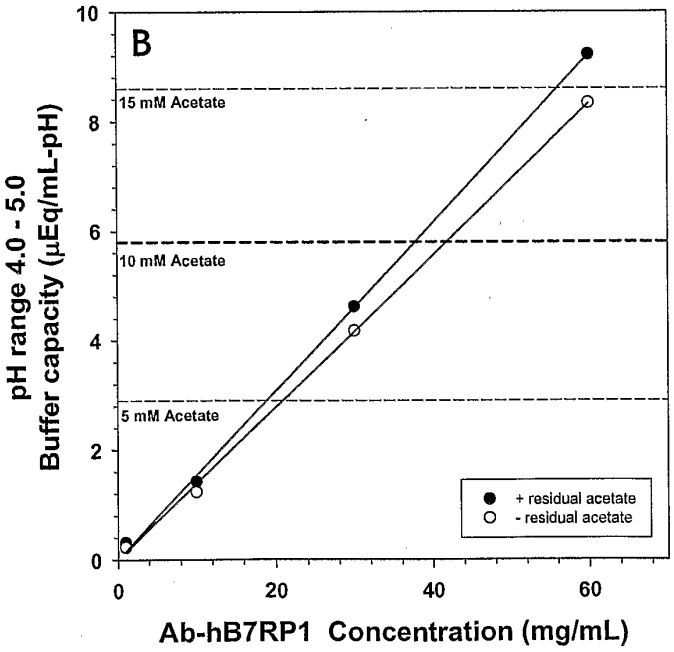


Figure 11. Buffer capacity of Ab-hB7RP1 Solutions in the pH 5.0-6.0 range

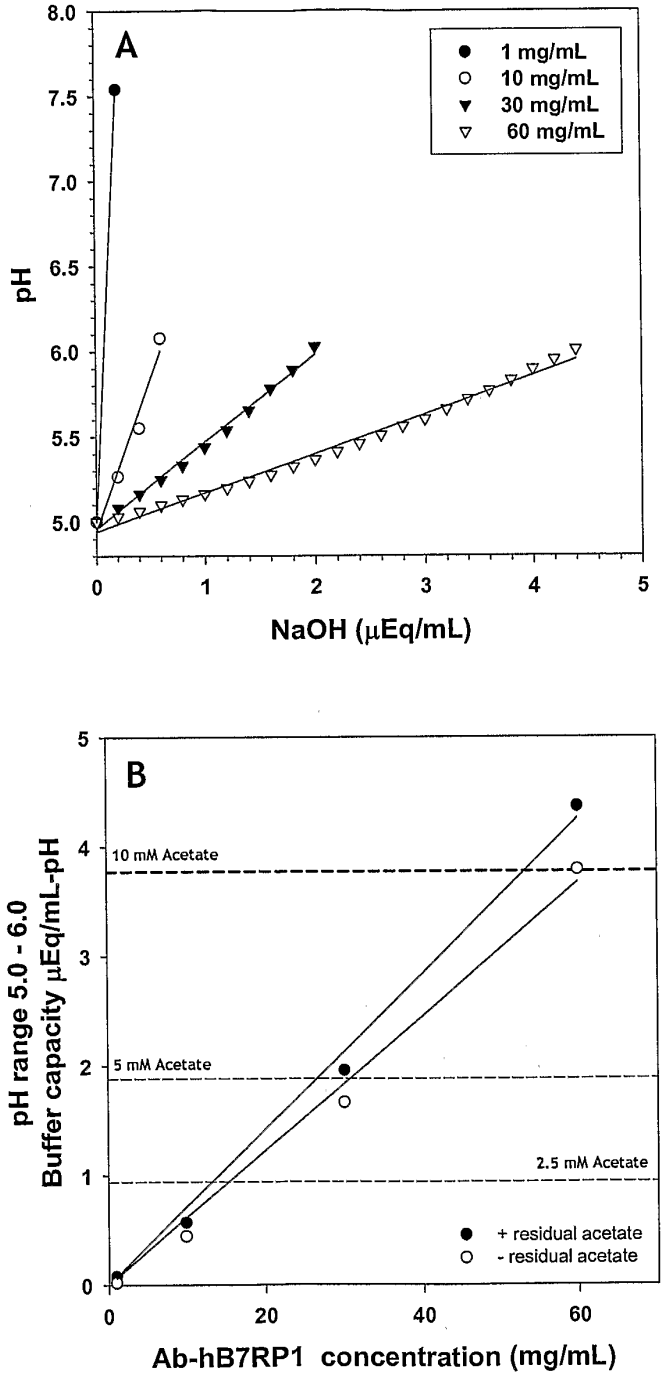
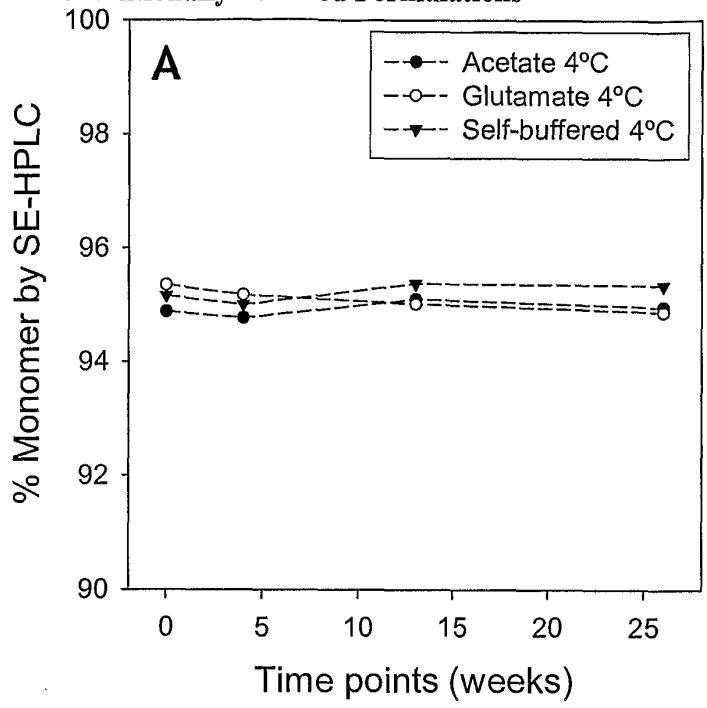


Figure 12. Stability of the Self-Buffered, 60 mg/mL, Ab-hB7RP1 Formulation Compared to Conventionally Buffered Formulations



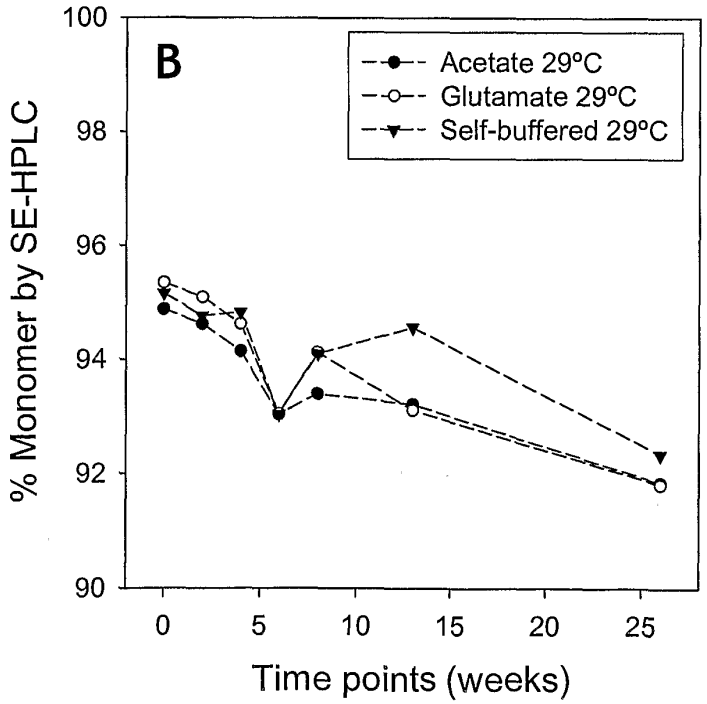


Figure 13. pH control of the Self-Buffered, 60 mg/mL, Ab-hB7RP1 Formulation

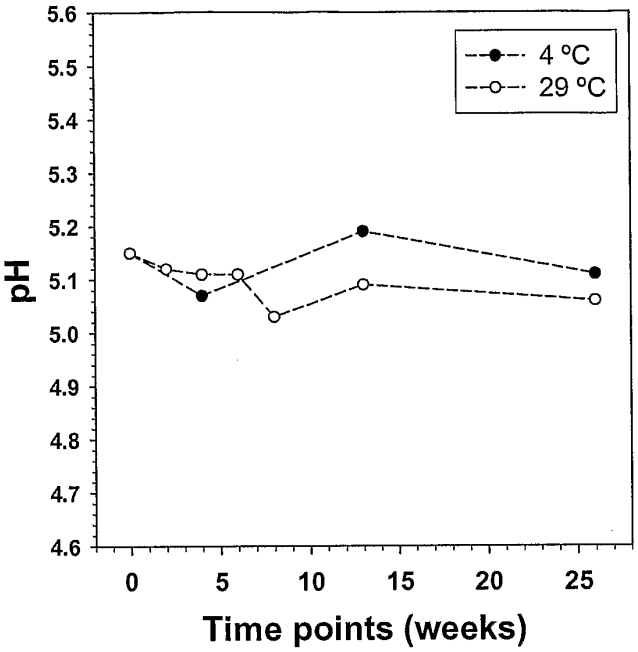


Figure 14. Buffer capacity of Ab-hCD22 solutions in the pH 4.0- 6.0 range

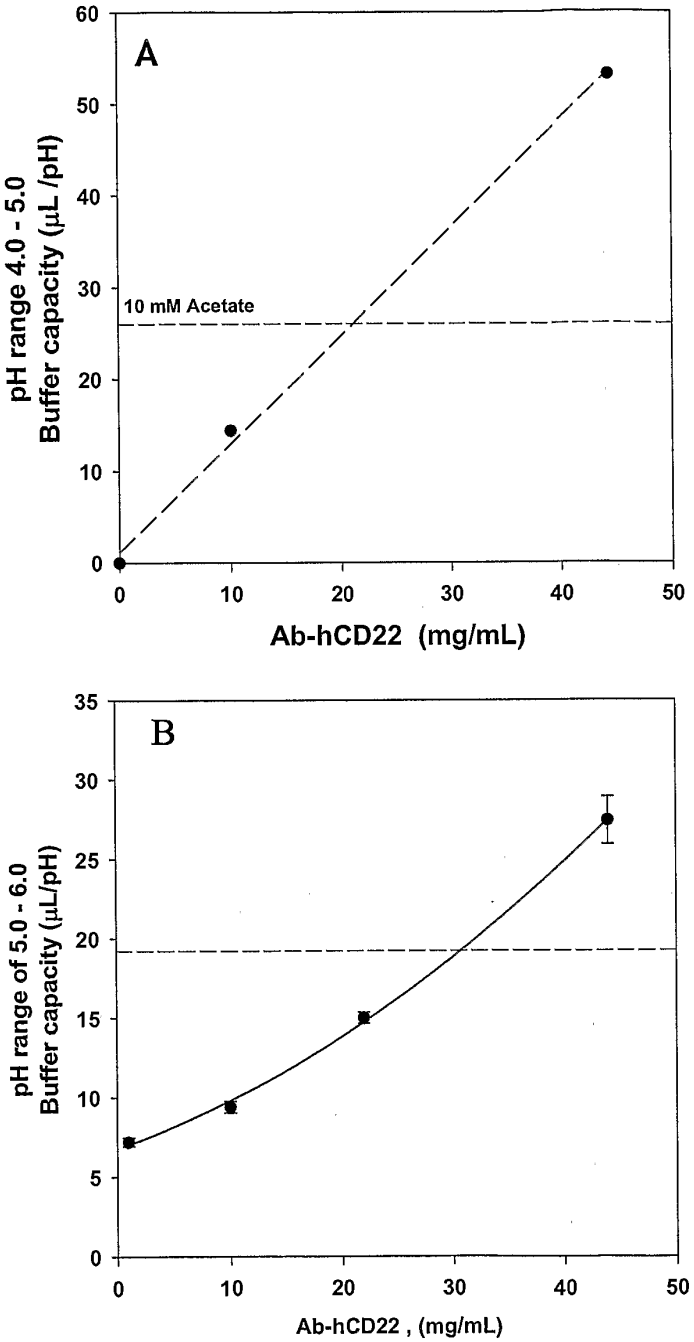


Figure 15. Buffer capacity of Ab-hIL4R Solutions in the pH 4.0 - 5.0 Range

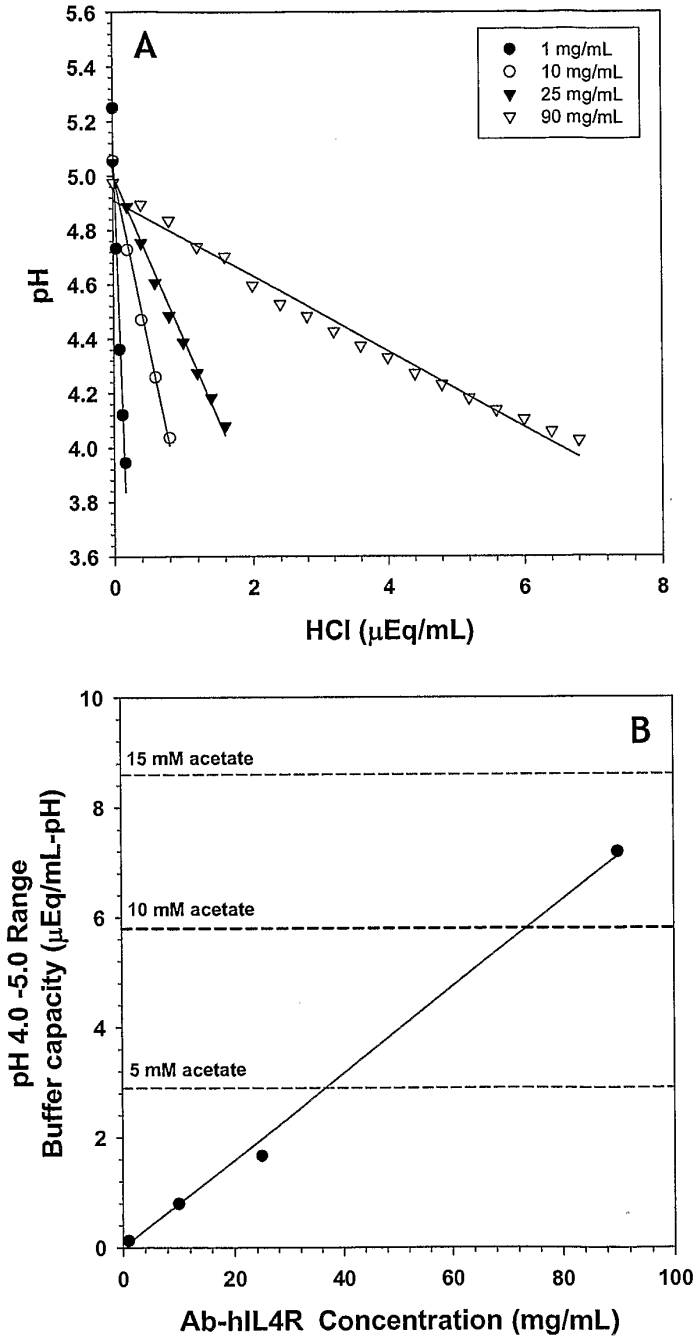


Figure 16. Buffer capacity of Ab-hIL4R Solutions in the pH 5.0 - 6.0 Range

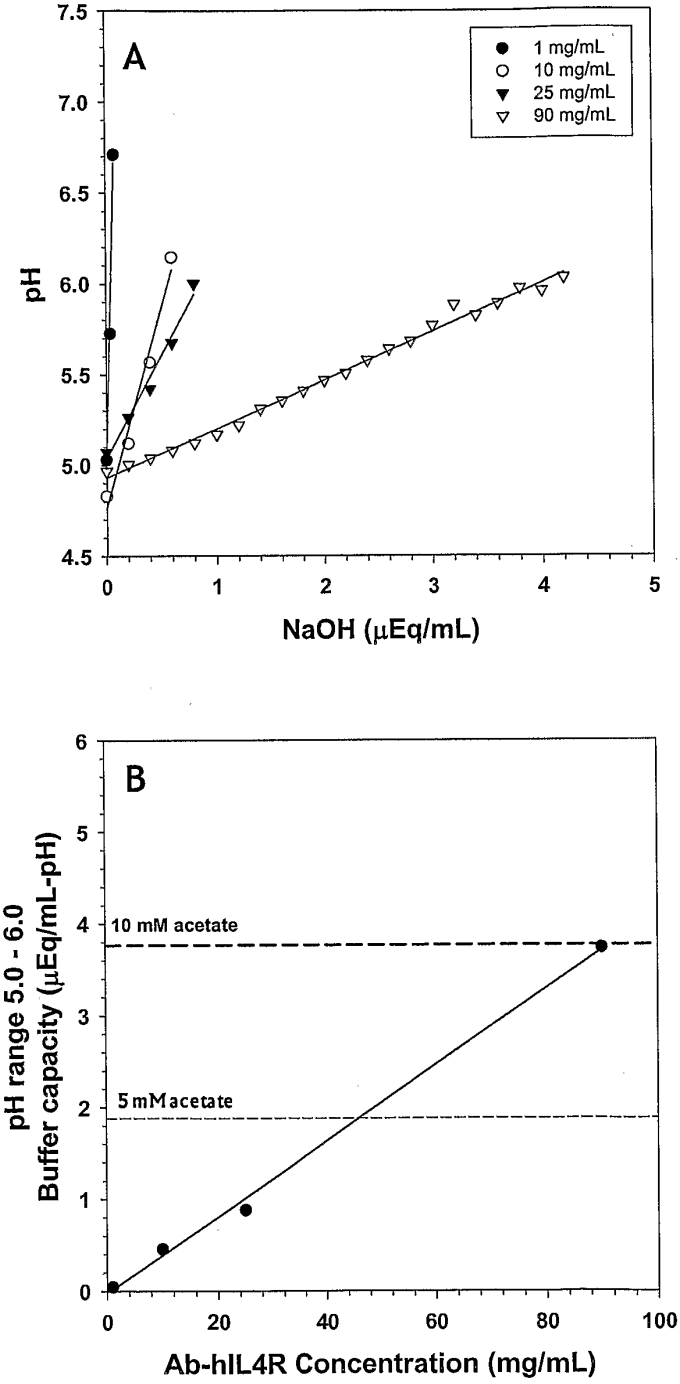
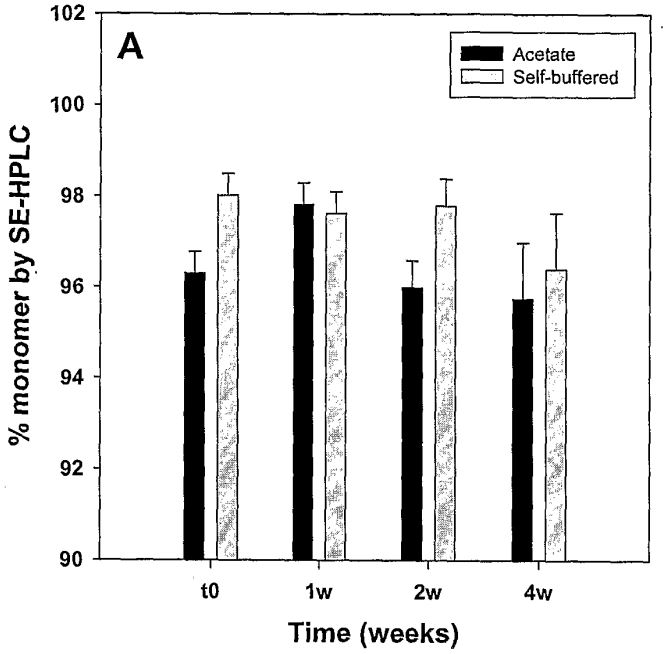
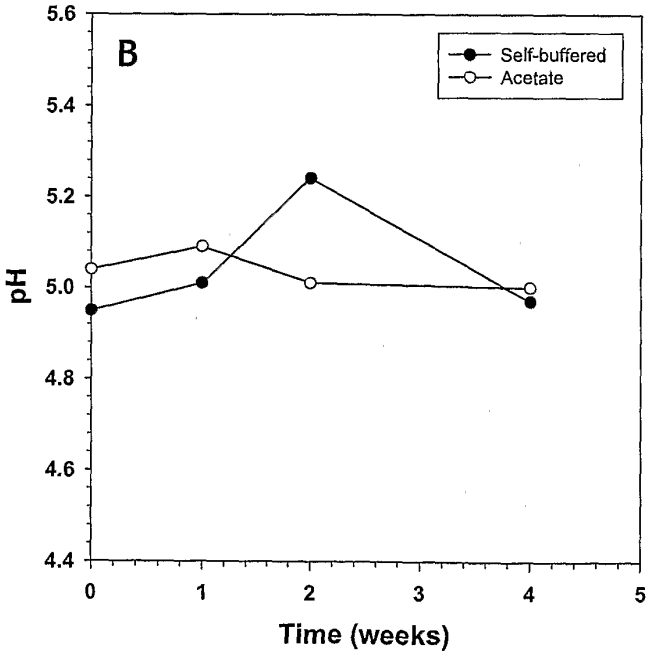


Figure 17. Accelerated Stability & pH control of the Self-Buffered, 70 mg/mL, Ab-hIL4R Formulation Compared to the Acetate Buffered Formulation at 37 C





Trout Ex. 138

AN ANALYSIS OF THE TITRATION DATA OF OXYHEMOGLOBIN OF THE HORSE BY A THERMAL METHOD

By JEFFRIES WYMAN, Jr.

(*From the Biological Laboratories, Harvard University, Cambridge*)

(Received for publication, July 16, 1938)

There is a great amount of data on the titration of proteins with acids and bases. Most of this has received some degree of interpretation in terms of the composition of the molecules. In a number of cases the total acid- and base-combining power has been shown to be in very close agreement with that calculated on the basis of the analytical figures for the amino acid content of the proteins in question. In at least one case, moreover, it has been possible to fit the data in a satisfactory way over the whole range by dividing the total number of acid- and base-combining groups into a relatively small number of classes and assigning to each class a suitably chosen acidity constant, and the required choice of classes and constants has not been inconsistent with the known composition of the protein.

There is another method of approach available for the analysis of the acid- and base-combining power of the proteins which so far has not been tried, but which may be expected to shed new light on the problem and to supplement the information derived in other ways. This, which we may call the thermal method, consists in determining the effect of temperature on the titration curve. Since the different acid- and base-binding groups of these polyvalent molecules are for the most part characterized by different heats of dissociation, we should expect, in accordance with the van't Hoff equation, that the effect of temperature would be different in different ranges of the titration curve, and this should make it possible to see directly in which ranges different groups are active. In this paper we describe the application of this method to the case of a well known protein, oxyhemoglobin of the horse. First we shall give the results of the titration of oxyhemoglobin in solution at three temperatures and the apparent heat of

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dissociation as a function of pH calculated from these data; we shall then develop the theory underlying the method, and make use of it in the analysis of the data on oxyhemoglobin.[1]

The horse hemoglobin was crystallized by the method of Ferry and Green, as described by German and Wyman (2), from concentrated cells kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory. The final crystals were dissolved in 0.3 M aqueous sodium chloride to provide the stock solution which was used for all the measurements. This solution contained, according to a Kjeldahl analysis, 12.42 gm. of nitrogen per liter, or 73.8 gm. of hemoglobin per liter, on the basis of a nitrogen factor of 5.93 (Vickery and Leavenworth (3)). At the end of the measurements the solution was found to have an oxygen-combining capacity of 9.71 volumes per cent. Assuming the molecular weight of hemoglobin to be 66,800, this indicates that only 1.9 per cent of the protein was inactive. This amount is so small that any corrections based on it are less than the experimental error, and it has been ignored in calculating the results.

Measured amounts of approximately 0.1 N HCl or NaOH were added to aliquots of stock solution, and the resulting mixture diluted with water to give the same final concentration of protein in all cases. This involved a dilution of the stock solution to 132 per cent of its original volume. The titrated aliquots were equilibrated with oxygen at a pressure of about 90 to 100 cm. of mercury in a tonometer before injection into the electrode for measurement.

The pH was determined with a glass electrode in connection with a high resistance vacuum tube bridge (1). The measurements were made at three temperatures, 6.5°, 25°, and 37.7°, controlled by water pumped from a constant temperature bath through a glass jacket surrounding the electrode. The electrode was calibrated constantly throughout the course of the measurements with various standard buffers. The composition of these buffers and the pH assigned to each for each of the three temperatures are given in Table I. The values of the pH are based on interpolations or extrapolations from data given by Clark (4) wherever the values are not given directly by him.

[1] It should be noted that in 1924 Stadie and Martin (1) made an estimate of the heat of ionization of hemoglobin as a base from the shift of the isoelectric point with temperature.

TABLE I

pH of Standard Buffers

								6.5°	25°	37.7°
10	cc.	citrate*						4.92	4.98	5.03
9.0	"	"	+	1.0	cc.	NaOH*		5.07	5.13	5.18
6.0	"	"	+	4.0	"	"	*	5.93	6.00	6.03
0.25	"	Na_2HPO_4	+	9.75	"	KH_2PO_4	†	5.31	5.28	5.26
1.0	"	"	+	9.0	"	"	†	5.92	5.90	5.87
49.6	"	"	+	50.4	"	"	‡	6.83	6.80	6.78
84.1	"	"	+	15.9	"	"	‡	7.52	7.50	7.48
87.0	"	"	+	13.0	"	"	‡	7.64	7.60	7.58
89.4	"	"	+	10.6	"	"	‡	7.73	7.70	7.67
94.7	"	"	+	5.3	"	"	‡	8.05	8.01	7.98
10	"	borate§						9.33	9.19	9.10

* Clark (4), Table 42.
† Clark (4), Tables 41 and 43.
‡ Clark (4), Table 43.
§ Clark (4), Table 38.

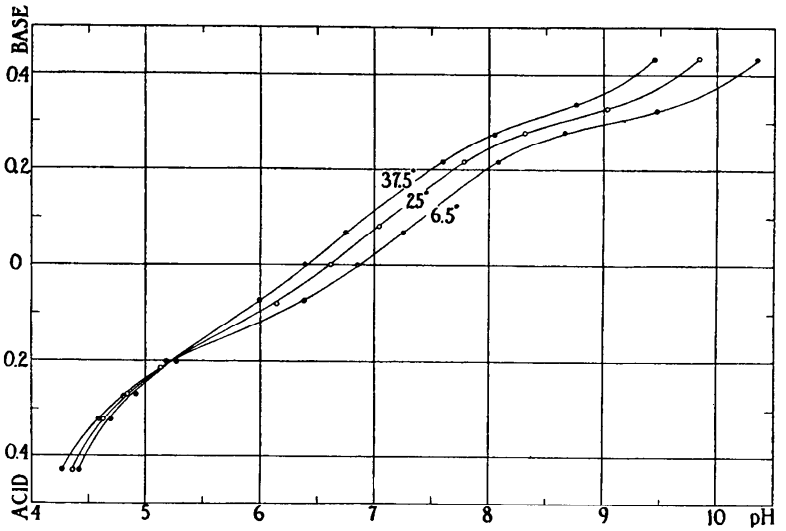

FIG. 1. Titration curves of oxyhemoglobin at three temperatures. Acid and base are expressed in milliequivalents per gm. of hemoglobin.

The titration curves for each of the three temperatures are shown graphically in Fig. 1. In Fig. 1 the ordinates give milli-equivalents of acid or base per gm. of hemoglobin, which, if desired, may be converted into equivalents per mole simply by multiplying by 66.8 on the assumption of a molecular weight of 66,800. The results at 25° are in good agreement with other data and it is unnecessary to tabulate them numerically.

The point in which we are primarily interested here is a second order effect; namely, the difference of pH between the three curves for given values of the ordinates. It is this quantity which determines what we shall call the apparent heat of dissociation, Q', and define, by analogy with the case of a weak monobasic acid, by the equation

$$Q' = -2.303 RT^2 \left(\frac{\partial \text{ pH}}{\partial T}\right)_B \tag{1}$$

In this equation and subsequently, R denotes the gas constant, T the absolute temperature, and B the total amount of base present. The justification of this definition is given below. In Fig. 2 we have plotted values of this quantity expressed in calories as a function of pH over the entire range covered by the data. These values were actually calculated from the pH values taken from the smooth curves of Fig. 1 at various convenient values of the ordinate by the expression

$$-4.579\, T_1 T_2 \frac{\text{pH}_2 - \text{pH}_1}{T_2 - T_1} \tag{2}$$

in which subscripts 1 and 2 refer to the two different temperatures in question. In Fig. 2 each value so calculated is plotted against the corresponding mean pH; *i.e.*, $(\text{pH}_2 + \text{pH}_1)/2$.

Let us now consider first very briefly the case of a simple acid SH which dissociates to give the products S^- and H^+ in accordance with the equation

$$\frac{a_S\, a_{H^+}}{a_{SH}} = k \tag{3}$$

where a denotes activity and k is the thermodynamic equilibrium constant. Q, the heat of dissociation per mole,[2] is given by the

[2] This is the increase of total heat; *i.e.*, the heat absorbed as a result of the dissociation.

van't Hoff equation

$$Q = RT^2 \frac{\partial \ln k}{\partial T} \tag{4}$$

Actually, what is more readily determined from the titration data is not the thermodynamic constant k but the acidity constant k' defined as

$$\frac{C_{S^-}}{C_{SH}} a_{H^+} = k' = k \frac{f_{SH}}{f_{S^-}} \tag{5}$$

in which C denotes concentration, and f activity coefficient. The van't Hoff equation, expressed in terms of k', is

$$Q = RT^2 \frac{\partial}{\partial T} \left(\ln k' + \ln \frac{f_{S^-}}{f_{SH}} \right) \tag{6}$$

The expression $RT^2(\partial \ln k'/\partial T)$ gives what we call the apparent heat of dissociation Q'. This may be calculated from the titra-

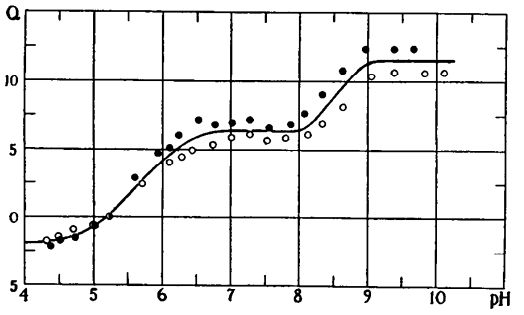

FIG. 2. Apparent heat of dissociation of oxyhemoglobin as a function of pH. ● = values for the 25–37.7° interval; ○ = values for the 6.5–25° interval.

tion data at once without any knowledge of activity coefficients from the equation

$$Q' = RT^2 \frac{\partial \ln k'}{\partial T} \tag{7}$$

Q' is of course not the same as the true heat of dissociation Q, but in general it is very close to it, since ln (f_{S^-}/f_{SH}) varies but little

with temperature regardless of its absolute magnitude. The fact that Q' involves only the temperature derivative of f_{S^-}/f_{SH} and not this quantity itself is of considerable importance for the more complex case about to be considered, since it shows that even very great modifications in the residue attached to a given dissociating group can have little or no effect on the apparent heat of dissociation in so far as they lead only to changes in f_{SH} and f_{S^-}, however great. Provided the acid is sufficiently weak in relation to its concentration and the pH of the solution to allow us to make the usual approximation of setting C_{S^-} equal to the amount of base added,[3] the apparent heat of dissociation may be simply reckoned from the change of pH with temperature of a partially titrated solution containing a fixed amount of base, by the following equation, which results from Equations 5 and 7

$$Q' = -RT^2\left(\frac{\partial\ \mathrm{pH}}{\partial T}\right)_B \tag{8}$$

This method of reckoning Q' is the only one practicable in the more complex case which we will now consider, that of a polyvalent molecule, like a protein, containing n acid or basic groups. We shall treat all these groups as acid groups and express their dissociation in terms of n acidity constants k. For convenience of notation in what follows we shall omit the prime marks when referring to the acidity constants and apparent heats of dissociation and shall use H to refer to the activity of the hydrogen ions, omitting the symbols a and $+$. We shall assume that the n groups are independent, so that the dissociation of each is unaffected by that of the others. As a result of the independent dissociation of these n groups there will be $n + 1$ classes of ions, to which we shall refer by subscripts 0, 1,. . . n. Each class is characterized by the total number of hydrogen ions dissociated, or, what amounts to the same thing, by the number of equivalents

[3] The error involved in this approximation is

$$\frac{C_{S^-} - C_B}{C_{S^-}} = \frac{C_{H^+}(C_{H^+} + k')}{k'(C_{SH} + C_{S^-})}$$

provided we assume the base to be completely dissociated, neglect the dissociation of water, and take $C_{H^+} = a_{H^+}$.

of base bound, per mole, and this number is the same as the subscript. These $n + 1$ classes are all in equilibrium with one another, and the concentration C_{S_i} of ions in the i'th class may be expressed in terms of the concentration C_{S_0} of ions of the 0'th class as follows:

$$\frac{C_{S_i}}{C_{S_0}} = \frac{K_i}{H^i} \tag{9}$$

Each of the n constants K depends on the n individual k's and K_i is the sum of all the $n!/(n-i)!i!$ different products of i k's chosen from the total number of n k's. The total concentration of all the ions of all classes is

$$C_S\left(1 + \frac{K_1}{H} + \cdots \frac{K_n}{H^n}\right) \equiv C_S A \tag{10}$$

and the total amount of base bound is

$$C_S\left(\frac{K_1}{H} + \frac{2K_2}{H^2} + \cdots \frac{nK_n}{H^n}\right) \equiv C_S G \tag{11}$$

We have introduced the symbols A and G for convenience to refer to the two expressions in parentheses. The amount of base bound per mole of material is

$$b = G/A \tag{12}$$

A formal differentiation of b in terms of $\ln H$ and $\ln K_1, \ldots \ln K_n$ gives

$$db = \left(\frac{\partial b}{\partial \ln H}\right)_{K_i} d \ln H + \sum_{i=1}^{n} \left(\frac{\partial b}{\partial \ln K_i}\right)_{H,K_j} d \ln K_i \tag{13}$$

If b is constant and $\ln H$ and $\ln K_i \ldots$ are expressed as functions of the temperature, this becomes, after multiplication by RT^2,

$$-RT^2\left(\frac{\partial \ln H}{\partial T}\right)_b = \sum_{i=1}^{n} \left(\frac{\partial b}{\partial \ln K_i}\right)_H \frac{Q_i}{(\partial b/\partial \ln H)_{K_i}} \tag{14}$$

in which

$$Q_i = RT^2 \frac{\partial \ln K_i}{\partial T} \tag{15}$$

If we make use of Equations 10, 11, and 12 and remember that $\partial \ln x/\partial x = 1/x$, Equation 14 becomes

$$RT^2\left(\frac{\partial \ln \mathrm{H}}{\partial T}\right)_b = \frac{A\sum_{i=1}^{n}\frac{iK_iQ_i}{\mathrm{H}^i} - G\sum_{i=1}^{n}\frac{K_i}{\mathrm{H}^i}Q_i}{A\sum_{i=1}^{n}\frac{i^2K_i}{\mathrm{H}^i} - G^2} \tag{16}$$

It follows from the structure of K_i that Q_i consists of $n!/i!(n-i)!$ terms all constructed in the same way, of which the first is

$$k_1k_2\cdots k_i(q_1 + q_2 + \cdots q_i)/K_i$$

Each q is the apparent heat of dissociation of an individual group; *e.g.*, $q_1 = RT^2\,\partial \ln k_1/\partial T$. Q_i is the apparent heat of dissociation for the total step from class 0 to class i: $\mathrm{S}_0 \rightarrow \mathrm{S}_i + i$ hydrogen ions. $Q' = RT^2\,(\partial \ln \mathrm{H}/\partial T)_b$ is the apparent heat of dissociation of the molecule as a whole and may be determined from the experimental data by Equation 1, as was done in constructing Fig. 2.

When the q's are the same for all the groups and equal to q, Q' calculated from Equation 16 reduces to q, as we know it must. When the q's are different, but the k's are all the same, it follows from the same equation that Q' is independent of pH and equal to the arithmetic mean of all the q's. If the groups fall into two equal classes, each characterized by a given q and k, then Q' is the same as for a simple dibasic acid with one group having one k and q, the other group having the other k and q. Equation 16 shows how in any given case Q' varies as the pH is changed. The sharpness of the transition of Q' from one level to the next and the separation of the levels depend on the spacing of the successive pK's as well as on the corresponding q's and increase as the spacing increases.

The behavior of Q' in a number of special cases is illustrated in Figs. 3 and 4. Fig. 3 is for a molecule containing two groups having the constants pk_1 and $\mathrm{pk}_2 > \mathrm{pk}_1$ and heats q_1 and $q_2 > q_1$. The ordinate gives $(Q' - q_1)/(q_2 - q_1)$; the abscissa gives $\mathrm{pH} - (\mathrm{pk}_1 + \mathrm{pk}_2)/2$. The curves are all symmetrical and in each case the value of the upper asymptote is given. The positions of the pk's are shown by the circles.[4] It may be seen that when the

[4] *I.e.*, values of the abscissa for which $\mathrm{pH} = \mathrm{pk}_1$ or $\mathrm{pH} = \mathrm{pk}_2$.

separation of the pk's is about 1.5 units or more the case is not very different from that in which the pk's are infinitely separated.

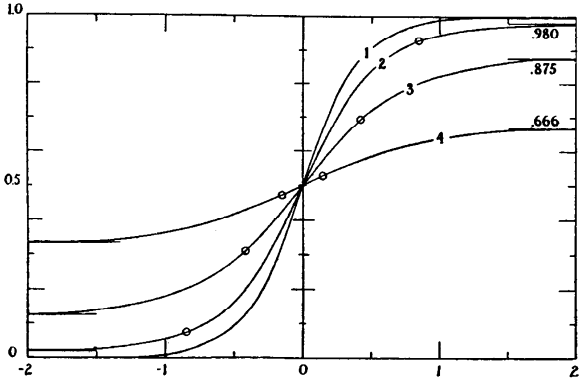

FIG. 3. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas pH $-$ $(pk_1 + pk_2)/2$. Curve 1, $pk_2 - pk_3 \rightarrow \infty$; Curve 2, $pk_2 - pk_1 = 1.690$; Curve 3, $pk_2 - pk_1 = 0.845$; Curve 4, $pk_2 - pk_1 = 0.301$.

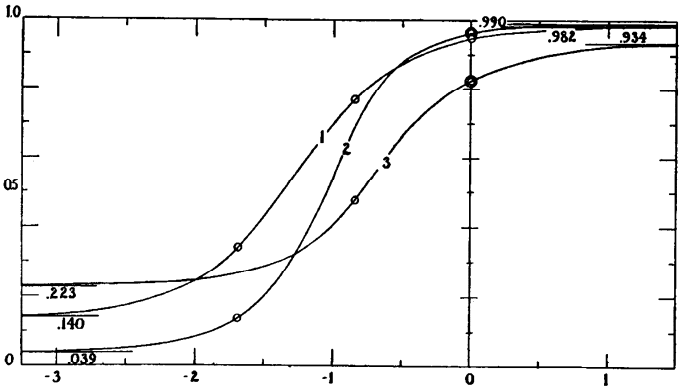

FIG. 4. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas pH $-$ pk_3. Curve 1, $pk_3 - pk_2 = pk_2 - pk_1 = 0.845$; Curve 2, $pk_3 = pk_2$, $pk_2 - pk_1 = 1.690$; Curve 3, $pk_3 = pk_2$, $pk_2 - pk_1 = 0.845$.

Fig. 4 illustrates three cases of a molecule containing three groups characterized by pk_1 and q_1, $pk_2 > pk_1$ and $q_2 > q_1$, and $pk_3 \geqq pk_2$ and $q_3 = q_2$. The ordinates give $(Q' - q_1)/(q_2 - q_1)$ and

the abscissas pH $-$ pk_3. Here again the positions of the pk's are indicated with circles, and the values of the upper and lower asymptotes are given.

In the light of this analysis it appears from Fig. 2 that over the range from pH 4 to pH 10 the dissociating groups of oxyhemoglobin fall into three classes on the basis of the heat of dissociation. Members of Class 1 have an average apparent heat of dissociation of -2000 or -3000 calories, and are active in the acid range of the titration. Members of Class 2 have an average apparent heat of dissociation of about $+6200$ calories and are active in the middle range. Members of Class 3 have an average apparent heat of dissociation of $+11{,}500$ calories and are active in the alkaline range of the titration. These three classes correspond to the three plateaus of Fig. 2, separated by two transition regions whose mid-points lie close to pH 5.5 and 8.5 respectively. The right-hand transition is complete in about 1 pH unit and this rather sudden transition suggests a wide separation, 2 units or more, between the pk of the most alkaline group of Class 2 and that of the most acid group of Class 3, which must be, respectively, not more than about 7.5 and not less than about 9.5. With a separation of the nearest groups of the two classes as great as this the effect of other groups further to the right and left is negligible.

In contrast to this, the left-hand transition in Fig. 2 occupies at least 2 pH units. This suggests that the separation of the pk of the last group of Class 1 and that of the first groups of Class 2 is much less than 2 units and that other groups to the right and left have an effect. This situation when the groups are closely spaced is more complicated. Fig. 4 gives some idea of what may be expected. Curve 1, which represents the case where the three pk's are equally spaced and separated by 0.85 unit, is the one in which the transition is most flattened and prolonged. The addition of other groups to the right and left would serve to extend the transition slightly more and to shift the asymptotes toward $+1$ and -1, and with this modification Curve 1 is probably fairly representative of the acid transition of Fig. 2. We may conclude, therefore, that the pk of the most alkaline group of Class 1 is about 5 and that of the most acid groups of Class 2 about 6.

Let us consider the amount of base bound by the groups of Class 2, having a heat of dissociation of 6200 calories. If we recall that more than 80 per cent of the titration of any acid group is accomplished within a space of 2 pH units centered about the pk value, we see that there can be very little overlapping as regards the base bound by these groups and those of Class 3. The latter do not begin to bind any considerable amount of base up to about pH 8.5 or more. In the range of the left-hand transition of Fig. 2 we may expect an appreciable amount of overlapping as regards base bound by groups belonging to Classes 1 and 2. Consideration shows, however, that owing to the roughly symmetrical way in which the overlapping occurs we obtain approximately the correct result by attributing all the base bound on the alkaline side of the mid-point of the left-hand transition region of Fig. 2, *i.e.* pH 5.5 or 5.6, to the groups of Class 2 and all the base bound on the acid side of this point to groups of Class 1. The difference between the total amount of base bound per gm. of protein at 25° between pH 8.5 and 5.5 read from the middle curve of Fig. 1 is 0.46 equivalent. This corresponds to 31 equivalents per mole. This is almost the same as the analytical figure for the number of histidine units per mole; namely, 33 (3, 5). Now the characteristic heat of dissociation of the imidazole group of histidine is known to be 6200 or 6900 calories (6, 7). There can be little doubt therefore that Class 2 consists of the imidazole groups of the histidine units. Since the pH range covered by groups of Class 2 includes the range where the base-binding power of hemoglobin is affected by oxygenation (2), it must be certain of these groups which lie close to the oxygen-combining centers and are affected by the introduction of oxygen into the molecule.

On the other hand, the groups of Class 1, the acid class, are certainly carboxyl groups. These are known to have a very small heat of dissociation, sometimes positive, sometimes negative, but never more than ± 2000 or 3000 calories (6) and this agrees well with the observed heats.

The remaining groups to be considered are the guanido groups of the 13 to 16 arginine units (4, 5), the amino groups of the 37 to 44 lysine units (4, 5), the hydroxyl groups of the 12 tyrosine units (8), and the sulfhydryl groups of the somewhat uncertain number of cysteine units, given by chemical analysis of the hemoglobin

molecule. Data are lacking on the heat of dissociation of the sulfhydryl group, but that of the hydroxyl group in dihalogenated tyrosines is 800 to 900 (7), that of the guanido group of arginine is 12,400 (7), and that of the amino group is between 9000 and 12,000 (7). All these groups dissociate at strongly alkaline reactions, but our results indicate that the strongly alkaline groups of Class 3 must be either the amino groups of lysine or the guanido groups of arginine.

This analysis is in good accord with the recent results of Cohn and others (9) involving the titration of carboxyhemoglobin, which is believed in this respect to be like oxyhemoglobin, in the absence of salt. They have shown that between pH 1.5 and 13 the curve involves in all the dissociation of 261×10^{-5} equivalent of hydrogen ions per gm. or 174 equivalents per mole of hemoglobin, and they show that the data may be fitted within the accuracy of the experiments by assuming the presence in each molecule of fourteen groups of $pk' = 11.6$, forty of pk' 10.8, twenty of pk' 7.5, thirteen of pk' 5.7, four of pk' 4.8, and 83 of pk' 3.7 or 4.0. They suggest, as our results confirm, that the thirty-three groups of pk' 5.7 or 7.5 are the imidazole groups of histidine. They also suggest that the forty groups of $pk' = 10.8$ are the amino groups of lysine, which accords with the heats that we have observed.

SUMMARY

The apparent heat of dissociation of a polyvalent substance like a protein is defined in terms of the effect of temperature on the titration curve. An analysis is given showing how this apparent heat of dissociation is related to the heats of dissociation of the individual groups of the molecule. From the observed variation of the apparent heat of dissociation with pH and a knowledge of the characteristic heats of dissociation of the individual groups it is possible to decide which groups are active in different ranges of the titration curve.

The apparent heat of dissociation of oxyhemoglobin of the horse has been determined between pH 4 and 10. The data indicate that the groups active acid to pH $\sim$ 5.5 are carboxyl groups, that those active between pH $\sim$ 5.5 and pH $\sim$ 8.5 are the imidazole groups of histidine residues, and that those active alkaline

to pH $\sim$ 8.5 are either amino groups or the guanido groups of arginine residues. From this it follows that the groups affected by oxygenation of hemoglobin are the imidazole groups of a certain number of histidine residues.

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IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF WEST VIRGINIA
CLARKSBURG DIVISION

IN RE: AFLIBERCEPT PATENT LITIGATION	MDL No.: 1:24-md-3103-TSK
THIS DOCUMENT RELATES TO: Civil Action No. 1:24-cv-39-TSK	

PLAINTIFF REGENERON PHARMACEUTICALS, INC.'S NOTICE OF APPEAL

Pursuant to Federal Rules of Appellate Procedure 3 and 4, and 28 U.S.C. § 1292(a)(1), notice is hereby given that Plaintiff Regeneron Pharmaceuticals, Inc. ("Regeneron") appeals to the United States Court of Appeals for the Federal Circuit from the Order entered in this action on September 23, 2024 (Docket No. 343) denying Regeneron's motion for a preliminary injunction, and from any and all other orders, decisions, rulings, findings, and conclusions underlying or relating to that Order.

DATE: September 23, 2024

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CERTIFICATE OF SERVICE

I certify that on September 23, 2024, a true and correct copy of the foregoing was served on all counsel of record via the Court's CM/ECF system.

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(12) **United States Patent**
Furfine et al.

(10) **Patent No.: US 11,084,865 B2**
(45) **Date of Patent: *Aug. 10, 2021**

(54) **VEGF ANTAGONIST FORMULATIONS SUITABLE FOR INTRAVITREAL ADMINISTRATION**

(71) Applicant: **REGENERON PHARMACEUTICALS, INC.**, Tarrytown, NY (US)

(72) Inventors: **Eric Furfine**, Concord, MA (US); **Daniel Dix**, LaGrangeville, NY (US); **Kenneth Graham**, Pleasant Valley, NY (US); **Kelly Frye**, Mendham, NJ (US)

(73) Assignee: **REGENERON PHARMACEUTICALS, INC.**, Tarrytown, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **16/739,559**

(22) Filed: **Jan. 10, 2020**

(65) **Prior Publication Data**

US 2020/0131246 A1 Apr. 30, 2020

Related U.S. Application Data

(60) Continuation of application No. 16/582,486, filed on Sep. 25, 2019, which is a continuation of application No. 16/159,269, filed on Oct. 12, 2018, now Pat. No. 10,464,992, which is a continuation of application No. 15/879,294, filed on Jan. 24, 2018, now Pat. No. 10,400,025, which is a continuation of application No. 15/095,606, filed on Apr. 11, 2016, now Pat. No. 9,914,763, which is a continuation of application No. 14/330,096, filed on Jul. 14, 2014, now Pat. No. 9,340,594, which is a continuation of application No. 13/914,996, filed on Jun. 11, 2013, now Pat. No. 8,802,107, which is a continuation of application No. 13/329,770, filed on Dec. 19, 2011, now Pat. No. 8,481,046, which is a continuation of application No. 12/833,417, filed on Jul. 9, 2010, now Pat. No. 8,092,803, which is a continuation of application No. 12/560,885, filed on Sep. 16, 2009, now Pat. No. 7,807,164, which is a division of application No. 11/818,463, filed on Jun. 14, 2007, now Pat. No. 7,608,261.

(60) Provisional application No. 60/814,484, filed on Jun. 16, 2006.

(51) **Int. Cl.**

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A61K 38/18	(2006.01)
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C07K 14/71	(2006.01)
A61K 9/00	(2006.01)
A61K 9/19	(2006.01)
C07K 14/47	(2006.01)
A61M 5/178	(2006.01)
A61K 47/26	(2006.01)
A61K 47/02	(2006.01)
A61K 47/10	(2017.01)

(52) **U.S. Cl.**

CPC ***C07K 14/71*** (2013.01); ***A61K 9/0019*** (2013.01); ***A61K 9/0048*** (2013.01); ***A61K 9/19*** (2013.01); ***A61K 38/179*** (2013.01); ***A61K 38/1793*** (2013.01); ***A61K 47/02*** (2013.01); ***A61K 47/10*** (2013.01); ***A61K 47/26*** (2013.01); ***A61M 5/178*** (2013.01); ***C07K 14/47*** (2013.01); ***C07K 14/4705*** (2013.01); *C07K 2319/00* (2013.01); *C07K 2319/30* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Ophthalmic formulations of a vascular endothelial growth factor (VEGF)-specific fusion protein antagonist are provided suitable for intravitreal administration to the eye. The ophthalmic formulations include a stable liquid formulation and a lyophilizable formulation. Preferably, the protein antagonist has an amino acid sequence of SEQ ID NO:4.

64 Claims, No Drawings

Specification includes a Sequence Listing.

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VEGF ANTAGONIST FORMULATIONS SUITABLE FOR INTRAVITREAL ADMINISTRATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation application of U.S. patent application Ser. No. 16/582,486, filed on Sep. 25, 2019, which is a continuation application of U.S. patent application Ser. No. 16/159,269, filed on Oct. 12, 2018, which issued as U.S. Pat. No. 10,464,992 on Nov. 5, 2019, which is a continuation application of U.S. patent application Ser. No. 15/879,294, which issued as U.S. Pat. No. 10,400,025 on Sep. 2, 2013, filed on Jan. 24, 2018, which is a continuation application of U.S. patent application Ser. No. 15/095,606, filed on Apr. 11, 2016, which issued as U.S. Pat. No. 9,914,763 on Mar. 13, 2018, which is a continuation application of U.S. patent application Ser. No. 14/330,096, filed Jul. 14, 2014, which issued as U.S. Pat. No. 9,340,594 on May 17, 2016, which is a continuation of U.S. patent application Ser. No. 13/914,996, filed Jun. 11, 2013, which issued as U.S. Pat. No. 8,802,107 on Aug. 12, 2014, which is a continuation application of U.S. patent application Ser. No. 13/329,770, filed Dec. 19, 2011, which issued as U.S. Pat. No. 8,481,046 on Jul. 9, 2013, which is a continuation application of U.S. patent application Ser. No. 12/833,417, filed Jul. 9, 2010, which issued as U.S. Pat. No. 8,092,803 on Jan. 10, 2012, which is a continuation application of U.S. patent application Ser. No. 12/560,885, filed Sep. 16, 2009, which issued as U.S. Pat. No. 7,807,164 on Oct. 5, 2010, which is a divisional application of U.S. patent application Ser. No. 11/818,463, filed Jun. 14, 2007, which issued as U.S. Pat. No. 7,608,261 on Oct. 27, 2009, which claims the benefit under 35 U.S.C. .§ 119(e) of U.S. Provisional Application No. 60/814,484, filed Jun. 16, 2006, which applications are each hereby incorporated by reference.

BACKGROUND OF INVENTION

Field of the Invention

The present invention is directed to pharmaceutical formulations suitable for intravitreal administration comprising agents capable of inhibiting vascular endothelial growth factor (VEGF), and to methods for making and using such formulations. The invention includes liquid pharmaceutical formulations having increased stability, as well as formulations that may be lyophilize and reconstituted for intravitreal administration.

Statement of Related Art

Vascular endothelial growth factor (VEGF) expression is nearly ubiquitous in human cancer, consistent with its role as a key mediator of tumor neoangiogenesis. Blockade of VEGF function, by binding to the molecule or its VEGFR-2 receptor, inhibits growth of implanted tumor cells in multiple different xenograft models (see, for example, Gerber et al. (2000) Cancer Res. 60:6253-6258). A soluble VEGF-specific fusion protein antagonist, termed a "VEGF trap" has been described (Kim et al. (2002) Proc. Natl. Acad. Sci. USA 99:11399-404; Holash et al. (2002) Proc. Natl. Acad. Sci. USA 99:11393-8), which applications are specifically incorporated by reference in their entirety.

Ophthalmic formulations are known, see for example, U.S. Pat. Nos. 7,033,604 and 6,777,429. An ophthalmic formulation of a VEGF antibody is described in U.S. Pat. No. 6,676,941.

Lyophilization (freeze drying under controlled conditions) is commonly used for long-term storage of proteins. The lyophilized protein is substantially resistant to degradation, aggregation, oxidation, and other degenerative processes while in the freeze-dried state (see, for example, U.S. Pat. No. 6,436,897).

BRIEF SUMMARY OF THE INVENTION

Stable formulations of a VEGF-specific fusion protein antagonist are provided. Pharmaceutically acceptable formulations are provided that comprise a VEGF "trap" antagonist with a pharmaceutically acceptable carrier. In specific embodiments, liquid and lyophilized formulations are provided.

In a first aspect, a stable liquid ophthalmic formulation of a VEGF-specific fusion protein antagonist is provided, comprising a fusion protein that comprises a receptor component consisting essentially of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor, and a multimerizing component (also termed a "VEGF trap"). In a specific embodiment of the VEGF-specific fusion protein antagonist, the first VEGF receptor is Flt1 and the second VEGF receptor is Flk1 or Flt4. In a more specific embodiment the fusion protein has the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4. Preferably, the VEGF antagonist is a dimer comprising two fusion proteins of SEQ ID NO:4.

In one aspect, a stable liquid ophthalmic formulation is provided that comprises 1-100 mg/ml VEGF-specific fusion protein antagonist, 0.01-5% of one or more organic co-solvent(s), 30-150 mM of one or more tonicity agent(s), 5-40 mM of a buffering agent, and optionally, 1.0-7.5% of a stabilizing agent, pH between about 5.8-7.0.

In one or more specific embodiments, the organic co-solvent may be polysorbate, for example, polysorbate 20 or polysorbate 80, polyethylene glycol (PEG), for example, PEG 3350, or propylene glycol, or a combination thereof; the tonicity agent may be, for example, sodium chloride or potassium chloride; the stabilizing agent may be sucrose, sorbitol, glycerol, trehalose, or mannitol; and the buffering agent may be, for example, phosphate buffer. In a specific embodiment, the phosphate buffer is a sodium phosphate buffer.

In various embodiments, the organic co-solvent is polysorbate and/or PEG, the stabilizing agent is sucrose, the buffering agent is phosphate buffer, and the tonicity agent is sodium chloride.

More specifically, the stable liquid ophthalmic formulation comprises about 40-50 mg/ml of the VEGF antagonist (SEQ ID NO:4), about 10 mM phosphate buffer, 0.01-3% polysorbate and/or PEG, 40-135 mM sodium chloride, and optionally 5.0% sucrose, pH about 6.2-6.3.

In a specific preferred embodiment, the stable liquid ophthalmic formulation comprises about 50 mg/ml of the VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 50 mM sodium chloride, 0.1% polysorbate, and 5% sucrose, pH about 6.2-6.3.

In a specific preferred embodiment, the stable liquid ophthalmic formulation comprises about 50 mg/ml of the VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 50 mM sodium chloride, 3% PEG, and 5% sucrose, pH about 6.2-6.3.

In a specific preferred embodiment, the stable liquid ophthalmic formulation comprises about 40 mg/ml of the VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 40 mM sodium chloride, 0.03% polysorbate, and 5% sucrose, pH about 6.2-6.3.

In a specific preferred embodiment, the stable liquid ophthalmic formulation comprises about 40 mg/ml of the VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 135 mM sodium chloride, and 0.03% polysorbate, pH about 6.2-6.3.

In another aspect, a stable liquid ophthalmic formulation is provided that comprises 1-100 mg/ml VEGF-specific fusion protein antagonist; 0.01-5% of one or more organic co-solvent(s); 5-40 mM of a buffering agent; and optionally 30-150 mM of one or more tonicity agent(s) and/or 1.0-7.5% of a stabilizing agent; having a pH between about 5.8-7.0.

In various embodiments, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 10 to about 80 mg/ml. In various embodiments, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 10, about 20, about 30, about 40, about 50, about 60, about 70, or about 80 mg/ml. In a preferred embodiment, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 40 mg/ml.

In another embodiment, the stabilizing agent is selected from one or more of sucrose, sorbitol, glycerol, trehalose, and mannitol.

In another embodiment, the organic co-solvent is selected from one or more of polysorbate, for example, polysorbate 20 or polysorbate 80, polyethylene glycol (PEG), for example, PEG 3350, and propylene glycol.

In another embodiment, the buffer is a phosphate buffer, for example, sodium phosphate.

In another embodiment, the tonicity agent is a salt, for example, sodium chloride.

In one embodiment, the stable liquid ophthalmic formulation comprises 10 mM sodium phosphate buffer, about 0.03 to about 0.1% polysorbate and/or about 3% PEG or propylene glycol, about 40 mM sodium chloride, and about 5% sucrose. In a specific embodiment, the stable liquid ophthalmic formulation comprises 10 mM sodium phosphate buffer, about 0.03% polysorbate, about 40 mM sodium chloride, and about 5% sucrose. In another specific embodiment, the pH of the formulation is about 6.2 to about 6.3. In another specific embodiment, the pH is achieved by mixing mono- and dibasic sodium phosphate to the desired pH without acid/base titration.

In a specific embodiment, the stable liquid ophthalmic formulation consists essentially of a VEGF antagonist (SEQ ID NO:4) at 40 mg/ml, 10 mM sodium phosphate buffer, polysorbate at 0.03%, sodium chloride at 40 mM, and sucrose at 5%, pH 6.2-6.3.

In another aspect, a stable liquid ophthalmic formulation is provided that comprises about 10 to about 80 mg/ml VEGF antagonist, about 10 mM sodium phosphate buffer, about 0.03% polysorbate, and about 135 mM sodium chloride, pH 6.2 to 6.3.

In various embodiments, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 10 to about 80 mg/ml. In various embodiments, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 10, about 20, about 30, about 40, about 50, about 60, about 70, or about 80 mg/ml. In a specific embodiment, the VEGF antagonist (SEQ ID NO:4) is present at a concentration of about 40 mg/ml.

In one embodiment, the stable liquid ophthalmic formulation comprises 40 mg/ml of VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 0.03% polysorbate, and 135 mM sodium chloride at pH 6.2-6.3. In a specific embodiment, the stable liquid ophthalmic formulation consists essentially of 40 mg/ml of VEGF antagonist (SEQ ID NO:4), 10 mM sodium phosphate buffer, 0.03% polysorbate, and 135 mM sodium chloride at pH 6.2-6.3.

In another aspect, a lyophilizable formulation of a VEGF antagonist is provided, wherein upon lyophilization followed by reconstitution, a stable liquid ophthalmic formulation as described herein is obtained.

In another aspect, a lyophilizable formulation of a vascular endothelial growth factor (VEGF)-specific fusion protein antagonist is provided, comprising 5-50 mg/ml of the VEGF antagonist, 5-25 mM buffer, such as phosphate buffer, 0.01 to 0.15% of one or more of an organic co-solvent, such as polysorbate, propylene glycol and/or PEG, and optionally 1-10% of a stabilizing agent such as sucrose, sorbitol, trehalose, glycerol, or mannitol, pH about 5.8-7.0. In various embodiments, the VEGF antagonist (SEQ ID NO:4) is present at about 5, about 10, about 20, about 30, or about 40 mg/ml. In a specific embodiment, the lyophilizable ophthalmic formulation of the invention comprises 20 mg/ml of the VEGF antagonist, 10 mM sodium phosphate buffer, 0.03% polysorbate, 0.1% PEG, and 2.5% sucrose, pH about 6.2-6.3. In further embodiments, the lyophilizable formulation further comprises sodium chloride. In a specific embodiment, the sodium chloride is present at a concentration of about 20 mM. In another specific embodiment, the sodium chloride is present at a concentration of about 67.5 mM.

In another specific embodiment, the lyophilizable ophthalmic formulation of the invention comprises 20 mg/ml of the VEGF antagonist, 5 mM sodium phosphate buffer, 0.015% polysorbate, 20 mM sodium chloride, and 2.5% sucrose, pH about 6.2-6.3.

In another embodiment, the lyophilizable ophthalmic formulation comprises 5 mg/ml, 10 mg/ml, or 40 mg/ml VEGF antagonist, 5 mM sodium phosphate buffer, 0.015% polysorbate, 20 mM sodium chloride, and 2.5% sucrose, at pH 6.2-6.3. In a specific embodiment, the lyophilizable ophthalmic formulation consists essentially of 5 mg/ml, 10 mg/ml, or 40 mg/ml VEGF antagonist (SEQ ID NO:4), 5 mM sodium phosphate buffer, 0.015% polysorbate, 20 mM sodium chloride, and 2.5% sucrose, at pH 6.2-6.3.

In another specific embodiment, the lyophilizable ophthalmic formulation comprises 20 mg/ml of the VEGF antagonist, 5 mM sodium phosphate buffer, 0.015% polysorbate, and 67.5 mM sodium chloride, pH about 6.2-6.3. In a more specific embodiment, the lyophilizable ophthalmic formulation consists essentially of 20 mg/ml of the VEGF antagonist (SEQ ID NO:4), 5 mM sodium phosphate buffer, 0.015% polysorbate, and 67.5 mM sodium chloride, pH 6.2-6.3.

In another specific embodiment, the lyophilizable ophthalmic formulation comprises 5 mg/ml, 10 mg/ml, or 40 mg/ml VEGF antagonist, 5 mM sodium phosphate buffer, 0.015% polysorbate, and 67.5 mM sodium chloride, pH about 6.2-6.3. In a more specific embodiment, the lyophilizable ophthalmic formulation consists essentially of 5 mg/ml, 10 mg/ml, or 40 mg/ml VEGF antagonist (SEQ ID NO:4), 5 mM sodium phosphate buffer, 0.015% polysorbate, and 67.5 mM sodium chloride, pH about 6.2-6.3.

Generally, the reconstituted formulation is about 2 times the concentration of the pre-lyophilized formulation, e.g., a 20 mg fusion protein/ml pre-lyophilized formulation is reconstituted to a final formulation of 40 mg fusion protein/ml.

Generally, the lyophilized formulation is reconstituted with sterile water suitable for injection. In one embodiment, the reconstitution liquid is bacteriostatic water.

In another aspect, the invention features a method of producing a lyophilized formulation of a VEGF-specific fusion protein antagonist, comprising subjecting the lyophilizable formulation of the invention to lyophilization to generate a lyophilized formulation. The lyophilized formulation may be lyophilized by any method known in the art for lyophilizing a liquid.

In another related aspect, the invention features a method of producing a reconstituted lyophilized formulation of a VEGF antagonist, comprising reconstituting the lyophilized formulation of the invention to a reconstituted formulation. In one embodiment, the reconstituted formulation is twice the concentration of the pre-lyophilized formulation, e.g., the method of the invention comprises: (a) producing a pre-lyophilized formulation of a VEGF-specific fusion protein antagonist, (b) subjecting the pre-lyophilized formulation of step (a) to lyophilization; and (c) reconstituting the lyophilized formulation of step (b).

The invention further features ophthalmic formulations provided in a pre-filled syringe or vial, particularly suitable for intravitreal administration.

Other objects and advantages will become apparent from a review of the ensuing detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting unless indicated, since the scope of the present invention will be limited only by the appended claims.

Unless stated otherwise, all technical and scientific terms and phrases used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference.

General Description

Safe handling and administration of formulations comprising proteins represent significant challenges to pharmaceutical formulators. Proteins possess unique chemical and physical properties that present stability problems: a variety of degradation pathways exist for proteins, implicating both chemical and physical instability. Chemical instability includes deamination, aggregation, clipping of the peptide backbone, and oxidation of methionine residues. Physical instability encompasses many phenomena, including, for example, aggregation and/or precipitation.

Chemical and physical stability can be promoted by removing water from the protein. Lyophilization (freeze-drying under controlled conditions) is commonly used for long-term storage of proteins. The lyophilized protein is substantially resistant to degradation, aggregation, oxidation, and other degenerative processes while in the freeze-dried state. The lyophilized protein may be reconstituted with water optionally containing a bacteriostatic preservative (e.g., benzyl alcohol) prior to administration.

Definitions

The term "carrier" includes a diluent, adjuvant, excipient, or vehicle with which a composition is administered. Carriers can include sterile liquids, such as, for example, water and oils, including oils of petroleum, animal, vegetable or synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil, sesame oil and the like.

The term "excipient" includes a non-therapeutic agent added to a pharmaceutical composition to provide a desired consistency or stabilizing effect. Suitable pharmaceutical excipients include, for example, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like.

The term "lyophilized" or "freeze-dried" includes a state of a substance that has been subjected to a drying procedure such as lyophilization, where at least 90% of moisture has been removed.

VEGF Antagonists

A VEGF antagonist is a compound capable of blocking or inhibiting the biological action of vascular endothelial growth factor (VEGF), and includes fusion proteins capable of trapping VEGF. In a preferred embodiment, the VEGF antagonist is the fusion protein of SEQ ID NO:2 or 4; more preferably, SEQ ID NO:4. In specific embodiments, the VEGF antagonist is expressed in a mammalian cell line such as a CHO cell and may be modified post-translationally. In a specific embodiment, the fusion protein comprises amino acids 27-457 of SEQ ID NO:4 and is glycosylated at Asn residues 62, 94, 149, 222 and 308. Preferably, the VEGF antagonist is a dimer composed of two fusion proteins of SEQ ID NO:4.

The VEGF antagonist of the methods and formulations of the invention can be prepared by any suitable method known in the art, or that comes to be known. The VEGF antagonist is preferably substantially free of protein contaminants at the time it is used to prepare the pharmaceutically acceptable formulation. By "substantially free of protein contaminants" is meant, preferably, that at least 90% of the weight of protein of the VEGF-specific fusion protein antagonist preparation used for making a formulation is VEGF fusion protein antagonist protein, more preferably at least 95%, most preferably at least 99%. The fusion protein is preferably substantially free of aggregates. "Substantially free of aggregates" means that at least 90% of the weight of fusion protein is not present in an aggregate at the time the fusion protein is used to prepare the pharmaceutically effective formulation. Unless stated otherwise, the phosphates employed are sodium phosphates and a desired buffering pH is achieved by mixing appropriate amounts of mono- and dibasic sodium phosphate.

Stable Liquid Ophthalmic Formulations

In one aspect, the invention provides a stable pharmaceutically acceptable formulation comprising a VEGF antagonist, wherein the formulation is a liquid formulation suitable for ophthalmic use. Preferably, the liquid formulation comprises a pharmaceutically effective amount of the VEGF antagonist. The formulation can also comprise one or more

pharmaceutically acceptable carriers, buffers, tonicity agents, stabilizers, and/or excipients. An example of a pharmaceutically acceptable liquid formulation comprises a VEGF antagonist in a pharmaceutically effective amount, a buffer, an organic co-solvent such as polysorbate, a tonicity agent such as NaCl, and optionally, a stabilizer such as sucrose or trehalose.

Stability is determined in a number of ways at specified time points, including determination of pH, visual inspection of color and appearance, determination of total protein content by methods known in the art, e.g., UV spectroscopy, and purity is determined by, for example, SDS-PAGE, size-exclusion HPLC, bioassay determination of activity, isoelectric focusing, and isoaspartate quantification. In one example of a bioassay useful for determining VEGF antagonist activity, a BAF/3 VEGFR1/EPOR cell line is used to determine VEGF165 binding by the VEGF antagonist of the invention.

Liquid formulations can be stored in an oxygen-deprived environment. Oxygen-deprived environments can be generated by storing the formulations under an inert gas such as, for example, nitrogen or argon. Liquid formulations are preferably stored at about 5° C.

Ophthalmic Lyophilized Formulations

In one aspect of the invention, an ophthalmically acceptable formulation comprising a VEGF antagonist is provided, wherein the formulation is a lyophilizable formulation. Lyophilizable formulations can be reconstituted into solutions, suspensions, emulsions, or any other suitable form for administration or use. Lyophilizable formulations are typically first prepared as liquids, then frozen and lyophilized. The total liquid volume before lyophilization can be less, equal to, or more than, the final reconstituted volume of the lyophilized formulation. The lyophilization process is well known to those of ordinary skill in the art, and typically includes sublimation of water from a frozen formulation under controlled conditions.

Lyophilized formulations can be stored at a wide range of temperatures. Lyophilized formulations may be stored below 25° C., for example, refrigerated at 2-8° C., or at room temperature (e.g., approximately 25° C.). Preferably, lyophilized formulations are stored below about 25° C., more preferably, at about 4-20° C.; below about 4° C.; below about −20° C.; about −40° C.; about −70° C., or about −80° C. Stability of the lyophilized formulation may be determined in a number of ways known to the art, for example, by visual appearance of the cake and/or by moisture content.

Lyophilized formulations are typically reconstituted for use by addition of an aqueous solution to dissolve the lyophilized formulation. A wide variety of aqueous solutions can be used to reconstitute a lyophilized formulation. Preferably, lyophilized formulations are reconstituted using water. Lyophilized formulations are preferably reconstituted with a solution consisting essentially of water (e.g., USP WFI, or water for injection) or bacteriostatic water (e.g., USP WFI with 0.9% benzyl alcohol). However, solutions comprising buffers and/or excipients and/or one or more pharmaceutically acceptable carries can also be used.

Freeze-dried or lyophilized formulations are typically prepared from liquids, that is, from solutions, suspensions, emulsions, and the like. Thus, the liquid that is to undergo freeze-drying or lyophilization preferably comprises all components desired in a final reconstituted liquid formulation. As a result, when reconstituted, the freeze-dried or lyophilized formulation will render a desired liquid formulation upon reconstitution.

EXAMPLES

Before the present methods are described, it is to be understood that this invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only to the appended claims.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus for example, a reference to "a method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference in their entirety.

Example 1. Stability of 50 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in 3 ml Glass Vials

An ophthalmic liquid formulation containing 50 mg/ml VEGF Trap (SEQ ID NO:4), 10 mM phosphate, 50 mM NaCl, 0.1% polysorbate 20, 5% sucrose, and pH 6.25, was stored at 5° C. in 3 ml glass vials and samples tested at 3, 6, 9, 12, 18 and 24 months. Stability was determined by SE-HPLC The results are shown in Table 1. Turbidity was measured at OD_{405} nm; and percent recovered protein and purity by size exclusion HPLC.

TABLE 1

Stability of 50 mg/ml VEGF Trap Protein (VGFT-SS065)

Months	Visual Appearance	Turbidity (OD_{405} nm)	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.2	100	98.8
3	Pass	0.00	6.2	101	98.7
6	Pass	0.01	6.3	100	98.3
9	Pass	0.01	6.3	101	98.3
12	Pass	0.01	6.3	104	98.4
18	Pass	0.01	6.3	96	98.1
24	Pass	0.01	6.3	105	98.1

Example 2. Stability of 50 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in 3 ml Glass Vials

A liquid formulation containing 50 mg/ml VEGF Trap (SEQ ID NO:4), 10 mM phosphate, 50 mM NaC1, 3% polyethylene glycol 3350, 5% sucrose, and pH 6.25, was stored at 5° C. in 3 nil glass vials and samples tested at 3, 6,

9, 12, 18 and 24 months. Stability results are shown in Table 2. Turbidity, percent recovered protein and purity was determined as described above.

TABLE 2

Stability of 50 mg/ml VEGF Trap Protein (VGFT-SS065)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.2	100	98.9
3	Pass	0.00	6.1	104	98.5
6	Pass	0.01	6.3	99	98.3
9	Pass	0.00	6.3	102	97.6
12	Pass	0.01	6.3	103	98.0
18	Pass	0.00	6.3	113	97.7
24	Pass	0.00	6.2	106	97.6

Example 3. Stability of 40 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in 3 ml Glass Vials

A liquid formulation containing 40 mg/ml VEGF Trap (SEQ ID NO:4), 10 mM phosphate, 40 mM NaCl, 0.03% polysorbate 20, 5% sucrose, and pH 6.3, was stored at 5° C. in 3 ml glass vials and samples tested at 0.5, 1, 2, 3, and 4 months. Stability results are shown in Table 3. Turbidity, percent recovered protein and purity was determined as described above.

TABLE 3

Stability of 40 mg/ml VEGF Trap Protein (VGFT-SS207)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.3	100	99.5
0.5	Pass	0.00	6.3	99	99.4
1	Pass	0.00	6.2	98	99.5
2	Pass	0.00	6.2	95	99.2
3	Pass	0.01	6.4		
4	Pass	0.01	6.3		

Example 4. Stability of 40 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in Pre-Filled Glass Syringe

A liquid formulation containing 40 mg/ml VEGF trap (SEQ ID NO:4), 10 mM phosphate, 40 mM NaCl, 0.03% polysorbate 20, 5% sucrose, and pH 6.3, was stored at 5° C. in 1 ml prefilled luer glass syringe with 4023/50 FluroTec coated plunger and samples tested at 0.5, 1, 2, 3, and 4 months. Stability results are shown in Table 4. Turbidity, percent recovered protein and purity was determined as described above.

TABLE 4

Stability of 40 mg/ml VEGF Trap Protein (VGFT-SS207)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.3	100	99.4
0.5	Pass	0.00	6.3	100	99.3

TABLE 4-continued

Stability of 40 mg/ml VEGF Trap Protein (VGFT-SS207)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
1	Pass	0.00	6.3	100	99.4
2	Pass	0.00	6.3	97	99.1
3	Pass	0.01	6.4		
4	Pass	0.01	6.3		

Example 5. Stability of 40 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in 3 ml Glass Vials

A liquid formulation containing 40 mg/ml VEGF trap (SEQ ID NO:4), 10 mM phosphate, 135 mM NaCl, 0.03% polysorbate 20, and pH 6.3, was stored at 5° C. in 3 ml glass vials and samples tested at 0.5, 1, 2, 3, and 4 months. Stability results are shown in Table 5. Turbidity, percent recovered protein and purity was determined as described above.

TABLE 5

Stability of 40 mg/ml VEGF Trap Protein (VGFT-SS203)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.3	100	99.3
0.5	Pass	0.00	6.2	87	99.2
1	Pass	0.00	6.2	88	99.1
2	Pass	0.00	6.3	103	99.2
3	Pass	0.00	6.3	88	99.0
4	Pass	0.00	6.2	85	98.9
5	Pass	0.00	6.3	84	99.0

Example 6. Stability of 40 mg/ml VEGF Trap Liquid Formulation Stored at 5° C. in 1 ml Pre-Filled Glass Syringe

A liquid formulation containing 40 mg/ml VEGF trap (SEQ ID NO:4), 10 mM phosphate, 135 mM NaCl, 0.03% polysorbate 20, and pH 6.3, was stored at 5° C. in 1 ml prefilled glass luer syringe with 4023/50 FluroTec coated plunger and samples tested at 0.5, 1, 2, 3, 4, and 5 months. Stability results are shown in Table 6. Turbidity, percent recovered protein and purity was determined as described above.

TABLE 6

Stability of 40 mg/ml VEGF Trap Protein (VGFT-SS203)

Months	Visual Appearance	Turbidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Configuration
0	Pass	0.00	6.3	100	99.2
0.5	Pass	0.01	6.3	101	99.2
1	Pass	0.00	6.3	101	99.2
2	Pass	0.00	6.3	—	—
3	Pass	0.01	6.3	102	99.1
4	Pass	0.01	6.3	103	98.8
5	Pass	0.00	6.3	99	98.9

Example 7. Stability of Lyophilized 20 mg/ml VEGF Trap Formulation Stored at 5° C. in 3 ml Glass Vials and Reconstituted to 40 mg/ml

0.8 ml of a liquid formulation containing 20 mg/ml VEGF trap (SEQ ID NO:4), 5 mM phosphate, 20 mM NaCl, 0.015% polysorbate 20, 2.5% sucrose, and pH 6.3, were lyophilized in 3 ml glass vials. Samples were stored at 5° C. and tested at 1, and 2 months. VEGF trap was reconstituted to a final concentration of 40 mg/ml VEGF Trap (final volume of 0.4 ml). Stability results are shown in Table 7 (t=time in months; *=visual appearance; **=reconstitution time). Turbidity, percent recovered protein and purity was determined as described above.

TABLE 7

Stability of Lyophilized 20 mg/ml VEGF Trap Protein (VGFT-SS216)

t	Vis. App.*	Recon. Time** (min)	Vis. App.* Reconst'd Liquid	Tur-bidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Config.
0	Pass	0.6	Pass	0.00	6.3	100	99.5
1	Pass	0.6	Pass	0.01	6.3	106	99.4
2	Pass	0.4	Pass	0.01	6.2	103	99.3

Example 8. Stability of Lyophilized 20 mg/ml VEGF Trap Formulation Stored at 5° C. in 3 ml Glass Vials

0.8 ml of a liquid formulation containing 20 mg/ml VEGF trap (SEQ ID NO:4), 5 mM phosphate, 67.5 mM NaCl, 0.015% polysorbate 20, and pH 6.3, were lyophilized in 3 ml glass vials. Samples were stored at 5° C. and tested at 1, 2, and 3 months. VEGF trap was reconstituted to a final concentration of 40 mg/ml VEGF trap (final volume of 0.4 ml). Stability results are shown in Table 8 (t=time in months; *=visual appearance; **=reconstitution time).

TABLE 8

Stability of Lyophilized 20 mg/ml VEGF Trap Protein (VGFT-SS216)

t	Vis. App.*	Recon. Time** (min)	Vis. App. Reconst'd Liquid	Tur-bidity	pH	% VEGF Trap Recovered	% VEGF Trap Native Config.
0	Pass	0.7	Pass	0.00	6.3	100	99.0
1	Pass	0.7	Pass	0.01	6.2	105	98.9
2	Pass	0.4	Pass	0.01	6.2	103	98.9

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ctgttggaga aaagcttgtc ttaaattgta cagcaagaac tgaactaaat gtggggattg 540

acttcaactg ggaataccct tcttcgaagc atcagcataa gaaacttgta aaccgagacc 600

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taacccggag tgaccaagga ttgtacacct gtgcagcatc cagtgggctg atgaccaaga 720

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caccgtgccc agcacctgaa ctcctggggg gaccgtcagt cttcctcttc cccccaaaac 840

ccaaggacac cctcatgatc tcccggaccc ctgaggtcac atgcgtggtg gtggacgtga 900

gccacgaaga ccctgaggtc aagttcaact ggtacgtgga cggcgtggag gtgcataatg 960

ccaagacaaa gccgcgggag gagcagtaca acagcacgta ccgtgtggtc agcgtcctca 1020

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ccgtcctgca ccaggactgg ctgaatggca aggagtacaa gtgcaaggtc tccaacaaag   1080

ccctcccagc ccccatcgag aaaaccatct ccaaagccaa agggcagccc cgagaaccac   1140

aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc agcctgacct   1200

gcctggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc aatgggcagc   1260

cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc ttcttcctct   1320

atagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc tcatgctccg   1380

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aatgagcggc cgc                                                      1453


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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45

Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60

Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80

Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95

Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110

Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Val
        115                 120                 125

Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val
    130                 135                 140

Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn
145                 150                 155                 160

Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val Asn Arg
                165                 170                 175

Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu Ser Thr
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Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr Thr Cys
        195                 200                 205

Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg
    210                 215                 220

Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys
225                 230                 235                 240

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                245                 250                 255

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
            260                 265                 270

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
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Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
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His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
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Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
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Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                 360                 365

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
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Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
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<223> OTHER INFORMATION: Synthetic

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cccgaaatta tacacatgac tgaaggaagg gagctcgtca ttccctgccg ggttacgtca    180

cctaacatca ctgttacttt aaaaaagttt ccacttgaca ctttgatccc tgatggaaaa    240

cgcataatct gggacagtag aaagggcttc atcatatcaa atgcaacgta caaagaaata    300

gggcttctga cctgtgaagc aacagtcaat gggcatttgt ataagacaaa ctatctcaca    360

catcgacaaa ccaatacaat catagatgtg gttctgagtc cgtctcatgg aattgaacta    420

tctgttggag aaaagcttgt cttaaattgt acagcaagaa ctgaactaaa tgtggggatt    480

gacttcaact gggaataccc ttcttcgaag catcagcata agaaacttgt aaaccgagac    540

ctaaaaaccc agtctgggag tgagatgaag aaatttttga gcaccttaac tatagatggt    600

gtaacccgga gtgaccaagg attgtacacc tgtgcagcat ccagtgggct gatgaccaag    660

aagaacagca catttgtcag ggtccatgaa aaggacaaaa ctcacacatg cccaccgtgc    720

ccagcacctg aactcctggg gggaccgtca gtcttcctct tccccccaaa acccaaggac    780

accctcatga tctcccggac ccctgaggtc acatgcgtgg tggtggacgt gagccacgaa    840

gaccctgagg tcaagttcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca    900

aagccgcggg aggagcagta caacagcacg taccgtgtgg tcagcgtcct caccgtcctg    960

caccaggact ggctgaatgg caaggagtac aagtgcaagg tctccaacaa agccctccca   1020

gcccccatcg agaaaaccat ctccaaagcc aaagggcagc cccgagaacc acaggtgtac   1080
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aactacaaga ccacgcctcc cgtgctggac tccgacggct ccttcttcct ctacagcaag   1260

ctcaccgtgg acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat   1320

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<210> SEQ ID NO 4
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Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu
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Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr
    50                  55                  60

Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys
65                  70                  75                  80

Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr
                85                  90                  95

Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His
            100                 105                 110

Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile
        115                 120                 125

Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
    130                 135                 140

Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
145                 150                 155                 160

Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
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Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
            180                 185                 190

Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
        195                 200                 205

Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
    210                 215                 220

Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys
225                 230                 235                 240

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                245                 250                 255

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
            260                 265                 270

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
        275                 280                 285

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
    290                 295                 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
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305                 310                 315                 320

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
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Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340                 345                 350

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                 360                 365

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                 375                 380

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                 425                 430

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                 440                 445

Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455
```

We claim:

1. A vial comprising an ophthalmic formulation suitable for intravitreal administration that comprises:

a vascular endothelial growth factor (VEGF) antagonist

an organic co-solvent,

a buffer, and

a stabilizing agent,

wherein said VEGF antagonist fusion protein is glycosylated and comprises amino acids 27-457 of SEQ ID NO:4; and

wherein at least 98% of the VEGF antagonist is present in native conformation following storage at 5° C. for two months as measured by size exclusion chromatography.

2. The vial of claim **1**, wherein the concentration of said VEGF antagonist fusion protein is 40 mg/ml, and wherein said organic co-solvent comprises polysorbate.

3. The vial of claim **2**, wherein said organic co-solvent comprises 0.01% to 3% polysorbate.

4. The vial of claim **2**, wherein said organic co-solvent comprises about 0.03% to about 0.1% polysorbate 20.

5. The vial of claim **2**, wherein said organic co-solvent comprises 0.01% to 3% polysorbate 20.

6. The vial of claim **5**, wherein said buffer comprises a phosphate buffer.

7. The vial of claim **5**, wherein said buffer comprises 5-25 mM buffer.

8. The vial of claim **5**, wherein said buffer comprises a pH between about 5.8-7.0.

9. The vial of claim **5**, wherein said buffer comprises a pH about 6.2-6.3.

10. The vial of claim **5**, wherein said stabilizing agent comprises a sugar.

11. The vial of claim **10**, wherein said sugar is selected from the group consisting of sucrose, sorbitol, glycerol, trehalose, and mannitol.

12. The vial of claim **5**, wherein said stabilizing agent comprises 1.0-7.5% of sucrose.

13. The vial of claim **5**, wherein said formulation further comprises a tonicity agent.

14. The vial of claim **5**, wherein said VEGF antagonist fusion protein is glycosylated at asparagine residues corresponding to asparagine residues 62, 94, 149, 222 and 308 of SEQ ID NO: 4.

15. The vial of claim **5**, wherein said formulation is capable of providing a turbidity of 0.01 or lower at OD_{405} after 2 month storage at 5° C.

16. The vial of claim **5**, wherein at least 99% of said VEGF antagonist fusion protein is present in native conformation after 2 month storage at 5° C. as measured by size exclusion chromatography.

17. The vial of claim **5**, wherein at least 98% of said VEGF antagonist fusion protein is present in native conformation following storage at 5° C. for 24 months as measured by size exclusion chromatography.

18. The vial of claim **5**, wherein said formulation does not contain phosphate.

19. The vial of claim **5**, wherein said formulation does not contain trehalose.

20. The vial of claim **5**, wherein said stabilizing agent comprises 1.0-10% of sucrose.

21. The vial of claim **20**, wherein said formulation further comprises a tonicity agent.

22. The vial of claim **20**, wherein said VEGF antagonist fusion protein is glycosylated at asparagine residues corresponding to asparagine residues 62, 94, 149, 222 and 308 of SEQ ID NO: 4.

23. The vial of claim **20**, wherein said formulation is capable of providing a turbidity of 0.01 or lower at OD_{405} after 2 month storage at 5° C.

24. The vial of claim **20**, wherein at least 99% of said VEGF antagonist fusion protein is present in native conformation after 2 month storage at 5° C. as measured by size exclusion chromatography.

25. The vial of claim **20**, wherein at least 98% of said VEGF antagonist fusion protein is present in native conformation following storage at 5° C. for 24 months as measured by size exclusion chromatography.

26. A pre-filled syringe comprising an ophthalmic formulation suitable for intravitreal administration comprising:

a vascular endothelial growth factor (VEGF) antagonist fusion protein,

an organic co-solvent,

a buffer, and

a stabilizing agent;

wherein said VEGF antagonist fusion protein is glycosylated and comprises amino acids 27-457 of SEQ ID NO:4; and

wherein at least 98% of said VEGF antagonist fusion protein is present in native conformation following storage at 5° C. for two months as measured by size exclusion chromatography.

27. The pre-filled syringe of claim **26**, wherein the concentration of said VEGF antagonist fusion protein is 40 mg/ml, and wherein said organic co-solvent comprises polysorbate.

28. The pre-filled syringe of claim **27**, wherein said organic co-solvent comprises 0.01% to 3% polysorbate.

29. The pre-filled syringe of claim **27**, wherein said organic co-solvent comprises about 0.03% to about 0.1% polysorbate 20.

30. The pre-filled syringe of claim **27**, wherein said organic co-solvent comprises 0.01% to 3% polysorbate 20.

31. The pre-filled syringe of claim **30**, wherein said buffer comprises a phosphate buffer.

32. The pre-filled syringe of claim **30**, wherein said buffer comprises 5-25 mM buffer.

33. The pre-filled syringe of claim **30**, wherein said buffer comprises a pH between about 5.8-7.0.

34. The pre-filled syringe of claim **30**, wherein said buffer comprises a pH about 6.2-6.3.

35. The pre-filled syringe of claim **30**, wherein said stabilizing agent comprises a sugar.

36. The pre-filled syringe of claim **35**, wherein said sugar is selected from the group consisting of sucrose, sorbitol, glycerol, trehalose, and mannitol.

37. The pre-filled syringe of claim **30**, wherein said stabilizing agent comprises 1.0-7.5% of sucrose.

38. The pre-filled syringe of claim **30**, wherein said formulation further comprises a tonicity agent.

39. The pre-filled syringe of claim **30**, wherein said VEGF antagonist fusion protein is glycosylated at asparagine residues corresponding to asparagine residues 62, 94, 149, 222 and 308 of SEQ ID NO: 4.

40. The pre-filled syringe of claim **30**, wherein said formulation is capable of providing a turbidity of 0.01 or lower at OD_{405} after 2 month storage at 5° C.

41. The pre-filled syringe of claim **30**, wherein at least 99% of said VEGF antagonist fusion protein is present in native conformation after 2 month storage at 5° C. as measured by size exclusion chromatography.

42. The pre-filled syringe of claim **30**, wherein at least 98% of said VEGF antagonist fusion protein is present in native conformation following storage at 5° C. for 24 months as measured by size exclusion chromatography.

43. The pre-filled syringe of claim **30**, wherein said formulation does not contain phosphate.

44. The pre-filled syringe of claim **30**, wherein said formulation does not contain trehalose.

45. The pre-filled syringe of claim **30**, wherein said stabilizing agent comprises 1.0-10% of sucrose.

46. The pre-filled syringe of claim **45**, wherein said formulation further comprises a tonicity agent.

47. The pre-filled syringe of claim **45**, wherein said VEGF antagonist fusion protein is glycosylated at asparagine residues corresponding to asparagine residues 62, 94, 149, 222 and 308 of SEQ ID NO: 4.

48. The pre-filled syringe of claim **45**, wherein said formulation is capable of providing a turbidity of 0.01 or lower at OD_{405} after 2 month storage at 5° C.

49. The pre-filled syringe of claim **45**, wherein at least 99% of said VEGF antagonist fusion protein is present in native conformation after 2 month storage at 5° C. as measured by size exclusion chromatography.

50. The pre-filled syringe of claim **45**, wherein at least 98% of said VEGF antagonist fusion protein is present in native conformation following storage at 5° C. for 24 months as measured by size exclusion chromatography.

51. An ophthalmic formulation comprising:

(a) 40 mg/ml of a glycosylated VEGF antagonist fusion protein comprising amino acids 27-457 of SEQ ID NO:4;

(b) 0.03% to 0.1% polysorbate;

(c) 5-40 mM of sodium phosphate buffer, pH between 5.8-7.0; and

(d) sucrose;

wherein the ophthalmic formulation is suitable for intravitreal administration; and

wherein at least 98% of the VEGF antagonist is present in native conformation following storage at 5° C. for 2 months as measured by size exclusion chromatography.

52. The formulation of claim **51**, wherein said formulation comprises at least 5% sucrose.

53. The formulation of claim **51**, wherein said formulation comprises 1-10% sucrose.

54. A pre-filled syringe suitable for intravitreal administration comprising the formulation of claim **51**.

55. A vial suitable for intravitreal administration comprising the formulation of claim **51**.

56. The formulation of claim **51**, wherein said formulation comprises 10 mM sodium phosphate buffer, 0.03% polysorbate, 5% sucrose, and a pH between 6.2-6.3.

57. A pre-filled syringe suitable for intravitreal administration comprising the formulation of claim **56**.

58. A vial suitable for intravitreal administration comprising the formulation of claim **56**.

59. The formulation of claim **56**, wherein said formulation further comprises 40 mM NaCl.

60. A pre-filled syringe suitable for intravitreal administration comprising the formulation of claim **59**.

61. A vial suitable for intravitreal administration comprising the formulation of claim **59**.

62. The formulation of claim **59**, wherein said VEGF antagonist fusion protein is glycosylated at asparagine residues corresponding to asparagine residues 62, 94, 149, 222 and 308 of SEQ ID NO: 4.

63. A pre-filled syringe suitable for intravitreal administration comprising the formulation of claim **62**.

64. A vial suitable for intravitreal administration comprising the formulation of claim **62**.

* * * * *

Confidential Material from
Add916-Add926
Omitted

CERTIFICATE OF SERVICE

I certify that today, September 23, 2024, I electronically filed the foregoing Motion and accompanying documents with the Clerk of the Court for the U.S. Court of Appeals for the Federal Circuit using the appellate CM/ECF system. Copies of the foregoing Motion and accompanying documents were served by e-mail on counsel of record and by Federal Express at the following address:

Marshall, Gerstein & Borun LLP
233 South Wacker Drive
Suite 6300
Chicago IL 60606-6357

I further certify that all parties required to be served have been served.

SEPTEMBER 23, 2024

/s/ David I. Berl
DAVID I. BERL

Attorney for Plaintiff-Appellant