[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY, COLLEGE OF PHYSICIANS AND SURGEONS, AND THE PRESBYTERIAN HOSPITAL]

# Immunological Specificities Involving Multiple Units of Galactose. II[1]

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Quantitative data are given on the precipitation of antibody in Type XIV antipneumococcal horse sera by a number of gums containing galactose such as gum arabic and its partial degradation product, the gum of *Acacia pycnantha*, the arabogalactans of Jeffrey pine and larch, the galactan of the seeds of *Strychnos nux vomica*, karaya gum and carob mucilage. The results are compared with earlier data on the cross reactivity in Type XIV serum of blood group substances, lung galactan, and tamarind seed polysaccharide. Multiple recurrences of non-reducing galactose end groups suffice for reactivity in Type XIV antipneumococcal sera, but gums with very few such end groups and a number of 1,3-, and/or 1,6-, and/or 1,3,6-linked galactose residues precipitate the antiserum as readily. The above galactose linkages are generally assumed to be β-.

It was recently shown that the galactan isolated from the residues of beef lungs used for the production of heparin precipitated more than one-quarter of the antibody in a Type XIV antipneumococcus serum.[2] This demonstrated a similar linkage or arrangement of some, at least, of the galactose units in the lung galactan to the as yet unknown linkages and arrangement of the galactose in the type specific capsular polysaccharide of Type XIV pneumococcus (S XIV). At that time S XIV was thought to be composed of three residues of galactose to one of N-acetylglucosamine[3] but it is now known to contain glucose in addition.[4] The precipitation of Type XIV antiserum by tamarind seed polysaccharide (jellose)[5] was of assistance in the interpretation of the results with lung galactan, since all of the galactose in jellose, which also contains xylose and glucose, is in the form of non-reducing end groups. It appeared evident, therefore, that multiple galactose end groups sufficed to permit cross reactivity with the Type XIV system. In view of the implications of these instances of cross reactivity for a parallel study of the fine structure of the specific polysaccharide of Type XIV pneumococcus it was decided to examine the reactivity toward Type XIV antipneumococcal serum of other natural galactose-containing polysaccharides of more or less known structure. The results are given below, since they supply an alternative structural feature requisite for this type of cross reactivity and add to the presently meager knowledge of the relation between the chemical constitution of the natural antigens and their immunological specificity.

## Experimental

**Materials and Methods.**—The arabogalactan of the Jeffrey pine and its product of mild acidic hydrolysis[6] were kindly supplied by Prof. W. Z. Hassid, the gum of *Acacia pycnantha* (golden wattle),[7] karaya gum from *Cochlospermum gossypium*,[8] larch arabogalactan and its partial hydrolytic product, and carob mucilage (gum gatto)[9] by Prof. E. L. Hirst, and the galactan of the seeds of *Strychnos nux vomica*[10] by Prof. J. K. N. Jones. A commercial sample of gum arabic was in part converted to a degraded arabic acid.[11] The specific polysaccharide of Type XIV pneumococcus (S XIV) was donated by E. R. Squibb and Sons, kindness of Mr. T. D. Gerlough, and the Type XIV antipneumococcal (anti-Pn) horse serum by the Division of Laboratories of the New York State Department of Health, Dr. G. Dalldorf, Director.

Quantitative estimations of antibody nitrogen precipitated in the homologous and cross reactions were carried out[12-14] and are given in the Tables. All mixtures for analysis were allowed to stand in a bath at 0° for six days to two weeks, depending upon the extent and rapidity of interaction of the system under study. All washings were carried out with chilled saline with the tubes immersed in ice-water and centrifugations were run at 0° also.

## Results and Discussion

1. **Gum Arabic and Related Products. a. Gum Arabic.**—Because of the reactivity in Type XIV anti-Pn serum of lung galactan, with its non-reducing end groups and 1,3- and 1,6-, presumably β-, galactose linkages, it could be predicted that gum arabic, in which the galactose is similarly bound,[15] would also react. This was readily verified, as noted in Table I, but the amount of antibody nitrogen was only one-fifth as large, 45 μg., at the maximum, as that precipitated by lung galactan, about 250 μg. Possibly this is due to the presence in gum arabic of very much more glucuronic acid, which is known to be a potent determinant of immunological specificity. This will be discussed in greater detail in a forthcoming communication on the specificity of Type II pneumococcus. Few of the arabofuranose residues in gum arabic are attached to galactose end groups and presumably none to the internal 1,3- and 1,6-bound galactose units. One would therefore expect that mild hydrolytic removal of part of the arabo-

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(2) M. Heidelberger, M. L. Wolfrom, W. Brock Neely and Z. Dische, THIS JOURNAL, **77**, 3511 (1955). Now designated Paper I of this series.

(3) W. F. Goebel, P. B. Beeson and C. L. Hoagland, *J. Biol. Chem.*, **129**, 455 (1939).

(4) M. Heidelberger, S. A. Barker and M. Stacey, *Science*, **120**, 781 (1954). The glutamic acid reported in this abstract appears due to a dialyzable impurity, but glucose also occurs in a sample of one of the original preparations kindly supplied by Dr. Goebel.

(5) E. V. White and P. S. Rao, THIS JOURNAL, **75**, 2617 (1953).

(6) W. H. Wadman, A. B. Anderson and W. Z. Hassid, *ibid.*, **76**, 4097 (1954).

(7) E. L. Hirst and A. S. Perlin, *J. Chem. Soc.*, 2622 (1954).

(8) E. L. Hirst and S. Dunstan, *ibid.*, 2332 (1953).

(9) E. L. Hirst and J. K. N. Jones, *ibid.*, 1278 (1948); F. Smith, THIS JOURNAL, **70**, 3249 (1948).

(10) P. Andrews, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 806 (1954).

(11) O. T. Avery, M. Heidelberger and W. F. Goebel, *J. Exper. Med.*, **42**, 709 (1925).

(12) M. Heidelberger and F. E. Kendall, *J. Exper. Med.*, **55**, 555 (1932); **61**, 559 (1935).

(13) M. Heidelberger, C. M. MacLeod, S. C. Kaiser and B. Robinson, *ibid.*, **83**, 303 (1946); R. Markham, *Biochem. J.*, **36**, 790 (1942).

(14) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., 1948.

(15) For probable structure, *cf.* F. Smith, *J. Chem. Soc.*, 1035 (1940).

furanose would have little effect on the reactivity of the gum in Type XIV anti-Pn serum and this is borne out by the values shown in Table I. The greater inhibitory effect of the hydrolyzed gum at higher concentrations is perhaps due to the splitting of other linkages and the consequent smaller size of the molecules of hydrolyzed gum.

TABLE I

PRECIPITATION OF 1.0 ML. TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM 635 BY ARABOGALACTANS AND RELATED GUMS AT 0°

The antiserum contained 47 μg. anti-C nitrogen and 920 μg. anti-S XIV nitrogen per ml. Volumes actually used ranged from 1.0 ml. 1 → 2 dilution to 2.0 ml. undiluted serum, but all results are calculated to 1.0 ml. "C-absorbed" serum refers to serum deprived of its antibody to C-substance, the somatic polysaccharide of pneumococcus.

| Amount of polysaccharide used, mg. | Antibody nitrogen (μg.) precipitated by: Gum arabic, Intact | Gum arabic, Partially hydrolyzed | Gum, *Acacia pycnantha*, Whole ser. | Gum, *Acacia pycnantha*, C-absd. | Arabogalactan, Jeffrey pine, Intact | Arabogalactan, Jeffrey pine, Partially hydrolyzed | Arabogalactan, larch, Intact | Arabogalactan, larch, Partially hydrolyzed[e] |
|---|---|---|---|---|---|---|---|---|
| 0.05 | 30 | | | | | | | |
| .1 | | 32[b] | 21 | | | 80 | | 46 |
| .2 | | | | | 108 | | 64 | |
| .3 | | 35 | 30 | | | 126 | | |
| .5 | 45 | | | 28 | | | | |
| .6 | | | | | 173 | | 85 | 72 |
| 1 | 43 | | 35 | | | 171 | | 83 |
| 2 | | | | 30 | 218 | 181 | 91 | |
| 2.5 | 37[a] | | | | | 191 | | |
| 3 | | 9 | | | 245 | | 92 | |
| 5 | | | | | | 173 | | |
| 6 | | 2 | | | 206[c] | | 90[d] | |
| 10 | | 2 | | | 187 | 138 | | |

[a] Supernatants of all tubes combined: with pine arabogalactan, 202 μg. N; the supernatant from this, with S XIV, gave 805 μg. N, total 1046. Gum arabic precipitated 41 μg. N from C-absorbed serum. [b] C-absorbed serum gave 29 μg. N, the maximum, at this level. The supernatants with 2 mg. of larch galactan, gave 73 μg. N; with 0.6 mg. of partly hydrolyzed galactan, 67 μg. N, with 1 mg., 74 μg. N. [c] Combined supernatants from these and preceding tubes, mean N already pptd., 176 μg.; with S XIV, 774 μg. N; with lung galactan, 26 μg. N, followed by S XIV, 713 μg., total 915. [d] Combined supernatants with pine arabogalactan, 113 μg. N; supernatant from this with S XIV, 779 μg. N, total 975. [e] Analyses with C-absorbed serum.

Precipitates from 1.75 mg. of gum and 3.5 ml. of C-absorbed antiserum were washed as usual[12,14] except that the final washing was carried out with chilled 1.85% $Na_2SO_4$ solution in order to remove chlorides which cause errors in the analyses for sugars. The precipitates were dissociated with 5% trichloroacetic acid and recentrifuged. The insoluble portion was analyzed for nitrogen and the solution for sugars,[2,16] with the results given in Table II. With the given proportions of gum and antiserum the precipitate contains more gum than antibody nitrogen. Comparison of the ratios of the sugars in the original gum and in the specific precipitate indicates that the gum is not extensively fractionated by this potentially selective process in spite of the incorporation into the immune aggregate of only 8% of the gum added. Except for a slight concentration of galactose with respect to rhamnose and glucuronic acid, the ratios of the sugars for which analyses were made remain much the same as in the original gum.

(16) Z. Dische and M. Osnos, *Arch. Biochem.*, **22**, 169 (1949); Z. Dische, *J. Biol. Chem.*, **167**, 189 (1946).

TABLE II

PRECIPITATION OF 3.5 ML. C-ABSORBED TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM WITH 1.75 MG. GUM ARABIC

| Gum pptd.,[a] μg. | Antb. N pptd., μg. | Antb. N / gum pptd. | In precipitate, μg.: Rhamnose | Galactose | Glucuronic acid | Ratios: Gal./Rham. | Gal./Glucur. | Glucur./Rham. |
|---|---|---|---|---|---|---|---|---|
| 138 | 104 | 0.8 | 16[b] | 77[b] | 17 | 5.0[b] | 4.6 | 1.1 |
| | | | Original gum | | | 4 | 4 | 1.1 |

[a] 10/8 × sum of three sugars for which analyses are given. [b] Mean of three analyses.

**b. Gum of *Acacia pycnantha*.**—In this gum[7] arabinose and galactose are distributed in much the same fashion as in gum arabic, but there is only one-third as much glucuronic acid. One would therefore not expect it to precipitate less antibody than gum arabic, but the figures shown in Table I are comparable to the somewhat lower values characteristic of the degraded gum arabic. Possibly factors of molecular size and shape enter in this instance, as well.

**2. Arabogalactan of Jeffrey Pine.**—Relatively little of the galactose in this substance is in the form of non-reducing end groups. Evidently a favorable distribution of the 1,6- and 1,3,6-galactose

TABLE III

PRECIPITATION OF 1.0 ML. TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM 635 BY VARIOUS GALACTOSE-CONTAINING POLYSACCHARIDES AT 0°

| Amount of polysaccharide used, mg. | Antibody nitrogen (μg.) precipitated by: Human blood group A subst., data from ref. 19b | Lung galactan | Jellose | Galactan, *Strychnos nux vomica* | Karaya gum | Carob mucilage |
|---|---|---|---|---|---|---|
| 0.015 | | | | 2 | 17 | |
| .04 | | | | | 20[d] | |
| .1 | 66 | 103 | 86 | | 18 | 126 |
| .3 | 134 | 179 | | 8 | | 154 |
| .4 | | | 94[b] | | | 153[f] |
| .5 | 178 | | | | 14 | |
| .6 | | | | 12 | | 159[f] |
| 1 | 236 | 257[a] | 77 | | 8 | |
| 2 | | | 54 | 27[c] | | |
| 5 | | | | 31 | | |
| 6 | | 181 | 2 | | | |

[a] Supernatant plus jellose gave 2 μg. N; + pine arabogalactan, 24 μg. N; + S XIV, 666 μg. N, total, 923 μg. [b] Supernatant plus lung galactan gave 148 μg. N; + karaya, 16 μg. N, after which pine arabogalactan gave 162 μg. N; a supernatant from karaya and jellose in this order gave 159 μg. N with the arabogalactan. Two supernatants from absorptions with jellose, karaya and pine gave 750, 739 μg. N with S XIV, total N, 1028, 1007. Evidently when the three gums are present together, up to 10% of non-specific N may be carried down. C-absorbed serum and 0.3 mg. jellose gave 89 μg. N. [c] From C-absorbed serum. [d] Supernatant + jellose gave 93 μg. N, after which lung galactan precipitated 152 μg. N, calcd. 149. Karaya supernatants + gum arabic gave 27 μg. N; + partially hydrolyzed gum arabic, 11 μg. N; + galactan from *Strychnos nux vomica*, 7 μg. Antiserum absorbed with C substance gave 19 μg. N with karaya gum. [e] Supernatants combined; + S XIV, 784 μg. N, total 940.

linkages known to be present[6] is at least equally important for the precipitation of a portion of the Type XIV antibody (Table I), for the arabogalactan precipitates as much antibody as does lung galactan (Table III) and more than jellose and carob mucilage (see below) in which all of the galactose consists of end groups.

In this instance, also, removal of arabofuranose by mild hydrolysis has little effect on reactivity in antiserum except that, as with gum arabic, relatively large amounts of the degraded polysaccharide show greater inhibition than do similar quantities of the intact material.

3. **Arabogalactan of the Larch.**[17]—This material contains a very much higher proportion of end groups than does the arabogalactan of the Jeffrey pine, but the remainder of the galactose also seems to be bound mainly in 1,6- and 1,3,6-linkages, to judge from the methylation studies.[17] It precipitates only about one-half as much antibody as does the pine galactan (Table I). Partial hydrolysis of larch arabogalactan has even less influence on reactivity in Type XIV-anti-Pn serum than in the case of pine arabogalactan, although the quantitative relationships of the interacting substances are somewhat altered.

4. **Galactan of** *Strychnos nux vomica.*—This polysaccharide is made up mainly of 1,4-linked galactose, but has about one non-reducing galactose end group in 28 and a few 1,3,6-galactose branch points.[10] It was reasonable, therefore, to expect weak reactivity in Type XIV antiserum and this was verified (Table III). The pattern of reactivity is different from that of any of the other galactose-containing carbohydrates, with maximum precipitation of antibody only at very high levels of galactan. This suggests either the necessity of much poorly fitting antigen to force precipitation with the cross-reactive antibody or the presence of more highly reactive material as an impurity in the galactan. If the latter alternative applies, enough of such an impurity is present to precipitate supernatants from precipitations at the 3 and 5 mg. level. When these were treated again with antiserum, 17 μg. of nitrogen was precipitated, or two-thirds as much as in the original reaction.

5. **Karaya Gum from** *Cochlospermum gossypium.*—This complex, partly acetylated product yields on hydrolysis equimolecular proportions of L-rhamnose, D-galactose and D-galacturonic acid, with traces of a ketohexose. Roughly two-thirds of the galactose is accounted for on methylation as non-reducing end groups, while the remainder appears to be involved mainly in 1,4-linkages.[8] Because of the galactose end groups it was predicted that the gum would react with Type XIV antiserum. The positive result is shown in Table III. As in the case of gum arabic, the extent of reaction is surprisingly small considering the high proportion of galactose end groups and again the reason is possibly to be found in the high uronic acid content, which might deviate the principal immunological specificity, if any, into one oriented toward galacturonic acid. Any such specificity, however, is different from that of the capsular polysaccharide of Type I pneumococcus, the only pneumococcal substance at present known to contain galacturonic acid. Karaya gum gives practically no precipitate with Type I anti-Pn horse serum. It is evident, therefore, that the linkages of galacturonic acid are different in karaya gum and in the Type I polysaccharide.

6. **Carob Mucilage, Gum Gatto.**—This gum is a galactomannan, in which all of the galactose, roughly 20%, consists of non-reducing end groups.[9] For this reason it was tested against the Type XIV antiserum and reacted heavily, as shown in Table III, precipitating about 17% of the antibody. Qualitative tests with two other galactomannans of similar structure, from guar and the Kentucky coffee bean, kindly furnished by Prof. F. Smith, also showed heavy precipitation. All three of these mucilages reacted strongly in Type VI and Type XII anti-Pn horse sera, as well, and will therefore be discussed as a separate group in another communication.

7. **Lung Galactan and Tamarind Seed Polysaccharide.**—Immunochemical properties of these substances were given in an earlier paper.[2] Data on their reactions in Type XIV anti-Pn sera are recorded in Table III for comparison with those given by the polysaccharides previously discussed.

8. **Blood Group Substances.**—The cross precipitation of these substances in Type XIV anti-Pn serum, first noted for the A substance by Goebel, Beeson and Hoagland[18] and quantitatively studied by Kabat and co-workers[19] are, in general, of similar magnitude to those encountered in the present study. One of the most highly reactive human blood group A substances is included in Table III for comparison. The cross reactivity could be increased and the blood group reactivity decreased by mild acid hydrolysis, with elimination of fucose and other side chains from a more central chain of galactose and amino-sugar residues.[19d] At present it is not known whether the cross reactivity in Type XIV anti-Pn serum is due to the galactose residues, or to the N-acetylglucosamine or both. On the basis of the work presently reported, however, this instance of cross reactivity and its increase on mild hydrolysis could be accounted for if the blood group substances contained galactose residues bound in the 1,3- and/or 1,6- and/or 1,3,6-positions, as well as, or including galactose units which become non-reducing end-groups after removal of attached fucose or oligosaccharide residues on mild hydrolysis. These easily hydrolyzable portions have been shown to be determinants of blood group specificity.[20]

Another type XIV anti-Pn horse serum, 867, contained 215 μg. of anti-S XIV N per ml. A partially purified antibody solution prepared from

(17) W. G. Campbell, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 774 (1948).

(18) W. F. Goebel, P. B. Beeson and C. L. Hoagland, *J. Biol. Chem.*, **129**, 455 (1939); W. F. Goebel and P. B. Beeson, *J. Exper. Med.*, **70**, 239 (1939).

(19) (a) E. A. Kabat, A. Bendich, A. E. Bezer and V. Knaub, *ibid.*, **87**, 295 (1948); (b) E. A. Kabat, H. Baer, A. E. Bezer and V. Knaub, *ibid.*, **88**, 43 (1948); (c) H. Baer, Z. Dische and E. A. Kabat, *ibid.*, **88**, 59 (1948); (d) S. Leskowitz and E. A. Kabat, THIS JOURNAL, **76**, 4887, 5060 (1954).

(20) E. A. Kabat and S. Leskowitz, *Federation Proc.*, **14**, 467 (1955).

this[21] contained 462 μg. of anti-S XIV N per ml. With lung galactan it precipitated a maximum of 34 μg. of N per ml.; the supernatant gave 392 μg. of N with S XIV. The antibody solution precipitated 13 μg. of N with jellose; the supernatant from this gave 15 μg. more N with the arabogalactan of Jeffrey pine.

The results on the cross reactions of the various polysaccharides in Type XIV anti-Pn sera do more than merely establish the types of galactose linkages which suffice to induce this reactivity. Because of their generality, as shown by the success of predictions noted above as to which gums would react, they provide a powerful aid to the carbohydrate chemist, since a simple serological test may show the presence or absence of one or more of the linkages in question in certain galactose-containing polysaccharides of unknown structure, particularly those which do not include glucose or N-acetylglucosamine. Demonstration has already been made of some of the potentialities of other anti-Pn sera in which multiple recurrences of glucuronic acid[2] or glucose[22] or cellobiuronic acid[23] in a polysaccharide involve cross reactivity, and other instances are at present under study.

(21) L. D. Felton, *J. Infect. Dis.*, **42**, 248 (1928), and earlier papers.

(22) (a) M. Heidelberger and A. C. Aisenberg, *Proc. Nat. Acad. Sci.*, **39**, 453 (1953); (b) *J. Exper. Med.*, **99**, 343 (1954).

(23) M. Heidelberger and G. L. Hobby, *Proc. Nat. Acad. Sci.*, **28**, 516 (1942).

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# Casein: Monomers and Polymers[1]

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A method is described for preparing a mixture of α- and β-caseins at constant *p*H and reduced temperature. This casein is termed soluble casein since it is in equilibrium with the casein in the casein micelles of milk and is readily soluble at neutral *p*H. The behavior of soluble casein has been examined by physico-chemical means as a function of *p*H, temperature, ionic strength and protein concentration. At *p*H 12 soluble casein is completely and reversibly dissociated into monomers having an average $S_{20}^0 = 1.18 \times 10^{-13}$ sec. and $D_{20}^0 = 7.11 \times 10^{-7}$ cm.$^2$ per sec. An average monomer molecular weight of 15,000 is calculated. A detailed consideration of the mixture suggests that the molecular weight of the α-casein monomer probably lies in the range 13,000 to 15,000 and that the molecular weight of the β-casein monomer lies in the range 15,000 to 25,000. Analyzing values of $S$ and $D$ and of measured viscosity increments, yields an average axial ratio of 12.0 for the anhydrous particle and a hydration of 0.3 g. water per gram protein. If a casein solution at *p*H 12 and temperatures near 0° (monomers) is adjusted to progressivly lower *p*H values, a peak representing α-casein polymers appears in the ultracentrifuge at *p*H 10.8. These α-casein polymers increase in sedimentation constant from 4.0 to 5.8 $S$ (8°) as the *p*H is decreased to *p*H 7. β-Casein remains in the monomeric form. At *p*H 7 and 0°, the ultracentrifuge and electrophoretic data are in excellent agreement. If at *p*H 7 the temperature is increased from 0°, the β-casein peak disappears and polymers containing both α- and β-casein appear, the average sedimentation constant increasing progressively from $S_{20} \sim 4.4$ $S$ at 4° to $S_{20} \sim 9.3$ $S$ at 32°. Beyond 32° there is a degeneration in the pattern. In all cases the polymers are characteristic of a given set of conditions and center around a preferred size. A re-examination of the properties of casein prepared from acid-precipitates suggests that such preparative procedures lead to some aggregation, particularly of α-casein, of dubious reversibility.

Since its preparation in 1838 by Mulder,[3] casein has been subjected to extensive study. Nevertheless, its physical and chemical properties have eluded precise definition. This may be due, in part, to the fact that most of these studies have been carried out on caseins prepared using modifications of the acid-precipitation method described by Hammarsten[4] in 1883. Linderstrom-Lang and Kodama,[5] in examining the solubility behavior of casein prepared in this way, first demonstrated the presence of several components. Mellander[6] and Warner[7] have since characterized these components electrophoretically, and more recently Hipp, Groves, Custer and McMeekin[8,9] have devised methods for fractionating casein by chemical means.

With the exception of the electrophoretic studies, upon which are based the designations of the various casein fractions as α-, β- and γ-casein, the results of physical examinations have not been in satisfactory agreement. Svedberg, Carpenter, and Carpenter[10,11] and Pedersen[12] examined casein by ultracentrifugation and obtained only polydisperse sedimentation patterns, highly dependent in form on small modifications in preparative procedure. These patterns generally exhibited from six to nine peaks, representing components ranging upward in molecular weight from about 75,000. On the other hand, osmotic pressure measurements by Burk and Greenberg[13] on acid-precipitated casein

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(2) Predoctoral Fellow of the National Science Foundation, 1952–1955. The work reported here will constitute part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A portion was reported in: P. H. von Hippel, Master's Thesis, Department of Biology, Massachusetts Institute of Technology, June, 1953.

(3) G. J. Mulder, *Ann. Pharm.*, **28**, 73 (1838).

(4) O. Hammarsten, *Z. physiol. Chem.*, **7**, 227 (1883).

(5) K. Linderstrom-Lang and S. Kodama, *Compt. rend. trav. lab. Carlsberg*, **16**, No. 1 (1925).

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(7) R. Warner, THIS JOURNAL, **66**, 1725 (1944).

(8) N. Hipp, M. Groves, J. Custer and T. McMeekin, *J. Dairy Sci.*, **35**, 272 (1952).

(9) N. Hipp, M. Groves, J. Custer and T. McMeekin, THIS JOURNAL, **72**, 4928 (1950).

(10) T. Svedberg, L. Carpenter and D. Carpenter, *ibid.*, **52**, 241 (1930).

(11) T. Svedberg, L. Carpenter and D. Carpenter, *ibid.*, **52**, 701 (1930).

(12) K. Pedersen, *Biochem. J.*, **30**, 948 (1936).

(13) N. Burk and D. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).