mixture: $\epsilon = \sum_i \frac{g_i \epsilon_i}{G}$.[10] This relationship holds if the recovery is quantitative and if the molecules are unchanged during the chromatographic separation.

In the case of the autolysate the agreement was remarkably good. The calculated summation of $\sum_i \frac{g_i \epsilon_i}{G}$ from the experimental data at 260 mμ is 0.803 while the absorption coefficient of the unseparated polysaccharides is 0.810. At 280 mμ, the values are 0.585 and 0.592, respectively. The electrodialysis procedure separated quantitatively the autolysate polysaccharides from the other substances, whereas some contaminants were present in the case of the bacterial cells. These contaminants were irreversibly adsorbed upon the column and were not recovered.

The unseparated autolysate and most polysaccharides from the autolysate were dextrorotatory. The unseparated cell material and most of the cell polysaccharides were levorotatory.

The *in vitro* serologic reactions show that 19 of the 21 polysaccharide fractions have the ability to bind antibodies. The antibody-binding abilities of the cell polysaccharides, C_1 through C_9, are uniformly more active than those from the autolysate, as indicated by the hemolytic and hemagglutination reactions.

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(10) A. Weissberger, "Physical Methods of Organic Chemistry," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1949, p. 1298.

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

Chemical Modifications of the Specific Polysaccharide of Type III Pneumococcus[1a,b] and Their Immunological Effects

BY HAROLD MARKOWITZ AND MICHAEL HEIDELBERGER

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Complete acetylation of a degraded preparation of the polysaccharide (S III) eliminated the capacity of the product to precipitate specific antiserum. Progressive deacetylation of the acetyl derivative gave progressively increasing reactivity toward antiserum. The fully deacetylated material reacted as well toward antiserum as the starting product. Undegraded S III could not be completely acetylated. After deacetylation it precipitated only one-half as much antibody as the starting material and hence was degraded by the chemical manipulations. Treatment of S III with diazomethane led to methylation of hydroxyl groups in addition to the usual esterification. After saponification to the acid, the derivative, in which two hydroxyl groups were methylated per cellobiuronic acid repeating unit, precipitated 50% as much antibody with rabbit antiserum as did the native polysaccharide. Conversion of the dimethoxy ester to the amide gave a product which did not react with antiserum. Esterification of S III to the extent of 35% and conversion of the partial ester to the amide gave products which precipitated 86% and 90% as much antibody, respectively, as the native polysaccharide. The results are discussed with reference to modern immunochemical theory.

I. Introduction

Studies on the relation of the structure of natural antigens to the specificity of antigen–antibody reactions[2] have clarified only a few of many problems demanding solution. Investigations with protein antigens, especially, have been difficult to interpret owing to the lability and the as yet unknown fine structure of proteins.

These difficulties are not as great in the case of the carbohydrates. The structure of the specific polysaccharide of the Type III pneumococcus (S III)[3] for example, is known. S III is a polycellobiuronic acid with 1,3-linkages, probably β-, between cellobiuronic acid units (Fig. 1). It has three varieties of functional groups: primary alcohol, secondary alcohol and carboxylic acid groups. Modifications were achieved by acetylation and methylation of the hydroxyl groups and by preparation of methyl esters and amides from the carboxyl groups. The immunological reactivities of the products were quantitatively studied.

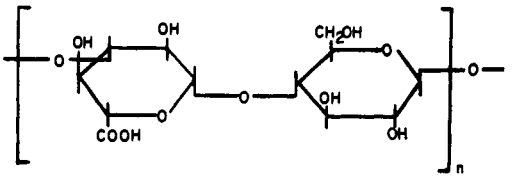

Fig. 1.—The specific polysaccharide of type III pneumococcus.

II. Experimental

Materials and Methods.—S III, lot 186[4] was purified by previously described methods.[5] Fraction 186B, obtained by precipitation with sodium sulfate, contained 5.5% ash as Na and 0.01% N. Preparation 300[4] was similarly purified and contained 5.1% ash as Na and 0.2% N. Sample 104 had undergone degradation, presumably as a result of storage as the free acid for 18 years.

The insoluble S III acid was converted to the soluble sodium salt for serological testing by stirring a suspension with Dowex 50 in the Na form for 4 hours. The mixture was centrifuged and the supernatant, after adjusting to 3% sodium acetate concentration, treated with one volume of cold

(1) (a) A preliminary report was presented at the 122nd Meeting of the American Chemical Society in Atlantic City, N. J., September 14–19, 1952. This work was sponsored by the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army, and in part by the Harkness Research Fund of the Presbyterian Hospital. (b) Submitted by Harold Markowitz in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) Summarized in K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1946.

(3) M. Heidelberger and W. F. Goebel, *J. Biol. Chem.*, **70**, 613 (1926); **74**, 613 (1927); R. D. Hotchkiss and W. F. Goebel, *ibid.*, **121**, 195 (1937); R. E. Reeves and W. F. Goebel, *ibid.*, **139**, 511 (1941).

(4) Obtained through the courtesy of E. R. Squibb and Sons, New Brunswick, N. J.

(5) (a) M. Heidelberger, C. M. MacLeod, H. Markowitz and A. S. Roe, *J. Exper. Med.*, **91**, 341 (1950); (b) M. Heidelberger, C. M. MacLeod, H. Markowitz and M. M. DiLapi, *ibid.*, **94**, 359 (1951).

ethanol. The precipitate was centrifuged off and isolated as usual.

Ash was determined as Na by flame photometry, nitrogen by the Markham[6] technique and methoxyl by a volumetric Zeisel method.[7] Acetyl was determined as in reference 5.

Quantitative precipitin analyses were made in duplicate by the method of Heidelberger and Kendall.[8] In all of these and analogous tests the antisera were absorbed with the group-specific "C" substance of pneumococcus and were buffered at *p*H 6.7 in order to minimize saponification of acetyl or ester groupings.

Acetylation of S III.—S III acid, lot 104, was dried *in vacuo* at 78° and 0.94 g. was dispersed in 70 ml. of formamide at 45–50° in a three-necked flask equipped with reflux condenser and a mercury-sealed stirrer.[9] Twenty-five ml. of pyridine was added dropwise and stirring continued for 30 min., after which 25 ml. of acetic anhydride was added slowly at 35° and the solution stirred for 1 hour at 30°, 3 hours at room temperature, and let stand overnight. The suspension was filtered into cracked ice and the solid on the filter, 104B1, washed with cold water and dried; yield 1.02 g.

The aqueous filtrate, 104B2, was exhaustively dialyzed against water and lyophilized, yield 0.48 g. It did not react with rabbit antiserum to Type III pneumococcus.

Anal. Calcd. for $[C_{12}H_{13}O_6(OCOCH_3)_5]_n$: $COCH_3$, 39.2. Found: $COCH_3$, 104B1, 35.6; 104B2, 42.9.

Sample 104B1 was again acetylated in the same manner. The brown solid, 104C1, was washed with water and dried; yield 0.99 g.; $COCH_3$, 24.5, 26.5. It is not known why the acetyl content was so low.

One gram of lot 300, a highly polymerized S III preparation, was acetylated in the same fashion and also gave both insoluble (300B1) and soluble (300B2) products; yields: 300B1, 0.34 g., $COCH_3$, 14.7; 300B2, 0.72 g., $COCH_3$, 28, no ash as Na.

Reacetylation of 300B2 gave 0.72 g. of initially soluble product (300C) which contained 28% acetyl and became insoluble in water after lyophilization but could be readily dispersed in formamide.

Progressive Deacetylation of 104C1.—Sample 104C1 was suspended in 10 ml. of water, chilled to 0°, and treated with 1.5 *N* NaOH in 0.1-ml. portions to maintain the solution at *p*H 8. It was then exhaustively dialyzed and lyophilized; yield: 104D, 0.58 g., $COCH_3$, 14.0; N, 0.4.

Thoroughly dried 104D, 0.12 g., was suspended in 20 ml. of methanol which had been dried over Mg, cooled to 0° and anhydrous NH_3 bubbled through for 2 hours. After another 4 hours at room temperature the precipitate was filtered off, washed with absolute methanol, acetone and ether and dried; yield: 104E, 0.11 g., $COCH_3$, 5.8.

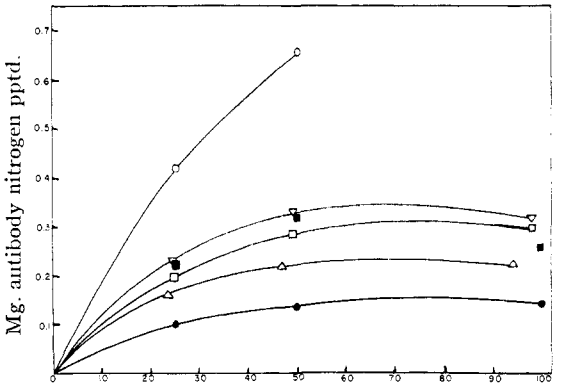

Fig. 2.—Quantitative data on precipitation of antibody by S III and acetylated derivatives: O, S III-B2[5b]; ●, 104D; △, 140E; □, 104F; ▽, 104G; ■, 104Na.

(6) R. Markham, *Biochem. J.*, **36**, 790 (1942).

(7) D. O. Hoffman and M. L. Wolfrom, *Anal. Chem.*, **19**, 225 (1947).

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

(9) J. F. Carson, Jr., and W. D. Maclay, THIS JOURNAL, **67**, 787 (1945); **68**, 1015 (1946).

Sample 104E, 0.08 g., was suspended in 10 ml. of absolute methanol and 0.5 ml. of 4 *N* $NaOCH_3$ added.[10] After thorough shaking the suspension was kept at 0° for 24 hours. The solid, 104F, was filtered off, washed with absolute methanol and dried; yield: 0.08 g., $COCH_3$, 2.2. Repetition of the procedure gave a product, 104G, with 1.5% acetyl.

Deacetylation of 300C.—Three-tenths gram of 300C was suspended in 15 ml. of *N* NaOH and kept at 0° for 48 hours. The solution was adjusted to *p*H 6 with acetic acid and 1 vol. of cold ethanol added. The precipitate, 300D, was centrifuged off, dissolved in 3% sodium acetate solution and again precipitated with 1 vol. of ethanol, washed with ethanol–water (2:1), ethanol and acetone and then filtered and dried; yield: 0.2 g.; $COCH_3$ 0.7; ash as Na, 5.7; calcd. for S III with 1 Na per –COOH, 6.4.

Precipitin tests were run on a number of the water-insoluble products after it was found that stable solutions could be obtained by dispersion of the substances in formamide and dilution with 0.85% NaCl solution; this mixture did not change the antibody N precipitated by S III. The following gave no precipitate in antipneumococcus Type III horse or rabbit serum: acetylated products 300C and 104B2 and the acetylated methyl ester 104AcMe. The deacetylated amide gave no precipitate with the rabbit serum, but a heavy one with the horse serum. Quantitative data are given in Fig. 2 and Table I.

TABLE I

PRECIPITATION OF RABBIT ANTI-PN III[a] BY S III AND METHYLATED S III PREPARATIONS

S III prepn.	Amount S III deriv. used,[b] μg.	Antibody N pptd., mg.	Supernatant tests + antiserum	Supernatant tests + S III
300	49	0.618	−	++
300	99	0.958	−	+
300	148	1.083	−	−
300	197	1.116	+	−
300D[c]	80	0.548	±	−
300D	120	.528	++	−
300D	160	.482	+++	−
300G[d]	44	.303	−	++
300G	89	.426	−	++
300G	133	.482	+	++
300G	177	.509	+	++
300H[e]	71	.382	−	+++
300H	107	.470	−	+++
300H	142	.520	−	+++
300I[f]	47 to 189	No visible ppts.		

[a] 1.0 ml. rabbit antiserum, to pneumococcus Type III, pool 58–68, diluted 1→2 with *p*H 6.57 phosphate buffer. [b] Corrected for methoxyl or acetyl content. [c] By saponification of the acetylation product, 300C. [d] Saponification product of 300Me. [e] Resaponification of 300G. [f] Amide prepared from 300Me.

Esterification of Acetylated S III and Formation of an Amide from the Ester.—Sample 104B2, the water-soluble acetyl derivative, was esterified with a tenfold excess of ethereal diazomethane.[11] After 24 hours at room temperature the suspension was filtered and the solid, 104AcMe, dried *in vacuo* over P_2O_5.

Anal. Calcd. for $[C_{11}H_{12}O_4(OCOCH_3)_5COOCH_3]_n$: OMe, 5.5. Found: OMe, 3.6.

A portion of the ester, 0.32 g., suspended in absolute methanol, was chilled to 0° and anhydrous NH_3 passed in for 15 min. Because of incomplete deacetylation in the shorter period previously used the mixture was allowed to stand 24 hr. at 0° and again at room temperature and was centrifuged in the cold and the precipitate, 104N, washed with methanol and ether and dried; yield 0.22 g.

Anal. Calcd. for $(C_{11}H_{17}O_9CONH_2)_n$: N, 4.15. Found: N, 2.75; $COCH_3$, 0.

Methylation of S III with Diazomethane.—0.2 g. of S 300 was dissolved in 20 ml. of water, chilled to 0° and 14 ml. of

(10) G. Zemplèn, A. Gerecs and I. Hadácsy, *Ber.*, **69**, 1827 (1936).

(11) F. Arndt, "Organic Syntheses," Coll. Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 165.

cold 1:1 HCl added.[12] The precipitate of the free acid (300A) was centrifuged off in the cold, washed with ethanol–water (1:1) until free of Cl^-, then with ethanol, redistilled acetone and ether, and dried over P_2O_5 *in vacuo;* yield 0.16 g. The dry 300A was treated with diazomethane as described previously. Copious evolution of nitrogen was noted. After 4 hours at 0° and 32 hours at room temperature, N_2 was bubbled through the mixture to remove diazomethane and the suspension filtered. The solid, 300Me, was dried over P_2O_5 *in vacuo;* yield 0.19 g.; no precipitate with rabbit antiserum.

Anal. Calcd. for $(C_{11}H_{17}O_9COOCH_3)_n$: OMe, 8.8; for $[C_{11}H_{15}O_7(OCH_3)_2COOCH_3]_n$; OMe, 24.5. Found: OMe, 25.7.

Saponification of 300Me.—A suspension of 0.07 g. of 300Me in 2 ml. of water was dissolved by dropwise addition of 0.1 *N* NaOH at intervals during 1.5 hr. After neutralization with *N* HCl the solution was dialyzed against water until chloride-free and lyophilized; yield of 300G, 0.07 g.; OMe, 19.5; ash as Na, 3.9.

Product 300G was again treated with excess 0.1 *N* NaOH at 0° for 24 hours and the product (300H) isolated in the same fashion.

Anal. Calcd. for $[C_{11}H_{15}O_7(OCH_3)_2COOH]_n$: OMe, 16.9. Found: OMe, 17.4.

Preparation of an Amide from 300 Me.—A suspension of 0.06 g. of 300 Me in absolute methanol was treated with anhydrous NH_3 as before. The product, 300I, was isolated as usual, yield 0.06 g.

Anal. Calcd. for $[C_{11}H_{15}O_7(OCH_3)_2CONH_2]_n$: OMe, 17.0; N, 3.8. Found: OMe, 20.0; N, 3.9.

Quantitative precipitin curves for the methylated S III derivatives are given in Table I.

Esterification of S III with HCl and Methanol.—The free acid was prepared from fraction 186B as described for 300A. A suspension of 0.3 g. of the finely divided acid in 10 ml. of 0.1 *N* methanolic hydrogen chloride was shaken for 4 days at 0° and filtered.[13] The precipitate, 186E, was washed with absolute methanol and dried over P_2O_5; yield 0.35 g.

Anal. Calcd. for $(C_{11}H_{17}O_9COOCH_3)_n$: OMe, 8.8. Found: OMe, 3.1; % esterification, 35.

The ester swells but does not dissolve in water unless first dispersed in a small volume of formamide.

Preparation of an Amide from the Ester.—Dry 186E, 0.1 g., was suspended in 20 ml. of absolute methanol and treated with NH_3 as before; yield of 186F 0.12 g.; N, 3.0; soluble in water and physiological saline. Excess N over the theoretical is probably present as the ammonium salt. Conversion of 3.1% OMe to NH_2 would give 1.4% N.

Saponification of 186E.—After saponification of 0.05 g. of 186E with 0.1 *N* NaOH in the cold as before 0.05 g. of a product, 186G, with 0.1% OMe was obtained. The quantitative precipitin reactions of this and the previous substances discussed may be found in Table II.

TABLE II

PRECIPITATION OF RABBIT ANTI-PN III[a] BY S III AND ITS ESTER AND AMIDE DERIVATIVES

S III prepn.	Amount S III used,[b] mg.	Antibody N pptd.,[c] mg.	Supernatant tests + antiserum	Supernatant tests + S III
186B[d]	0.082	0.883	−	+++
186B	.163	1.111	+	−
186B	.245	1.178	++++	−
186E[e]	.071	0.699	−	+++
186E	.106	.855	−	+
186E	.141	.938	+	+
186E	.212	.950		
186E	.283	.867		
186E	.424	.782		
186F[f]	.082	.828	−	+++
186F	.164	1.019	+	−
186F	.246	0.990		
186F	.820	.717		
186G[g]	.054	.699	−	++++
186G	.108	.990	+	−
186G	.542	.830		
186G	1.084	.503		
300H[h]	0.071	.382	−	+++
300H	.107	.469	−	+++
300H	.143	.523	−	+++
300H	.357	.473		
300H	.714	.341		

[a] 1.0 ml. of rabbit anti-Pn III, pool 3A, diluted 1→2 with phosphate buffer. [b] Corrected for methoxyl where necessary. [c] Corrected for antigen N in the specific precipitate where necessary. [d] Na salt. [e] Methyl ester; OMe, 3.1. [f] Amide; N, 3.0. [g] Na salt of saponified ester, 186E; OMe, 0.1. [h] Na salt of resaponified dimethoxy ester, 300Me; OMe, 17.4.

III. Discussion

The specific polysaccharide of Type I pneumococcus (S I) has been shown to yield with diazomethane an N-methylated methyl ester[14] which did not precipitate horse antiserum to Type I pneumococcus. On saponification of the ester the product again reacted with antiserum. These exploratory reactions were carried out with an immunologically active polysaccharide, the chemical structure of which has not been elucidated. Moreover, in this instance the methylation not only esterified the carboxyl groups but also substituted the amino group and one of the hydroxyl groups of the repeating unit.[14]

The polysaccharide chosen for the present work, S III, is known to be a polycellobiuronic acid and does not contain nitrogen, so that only the reactions of hydroxyl and carboxyl groups need to be considered. One method used to modify S III was the acetylation of primary and secondary alcohol groups. Acetylated S III, 104B1, from a degraded sample, showed almost the theoretical acetyl content. When deacetylated stepwise it gave products which precipitated progressively more antibody from rabbit antiserum as their acetyl content was reduced (Fig. 2). The completely deacetylated product precipitated as much antibody as did the starting material, indicating that the lowered reactivity observed with partially acetylated samples was due solely to the acetyl groups introduced and not to any accompanying degradative changes. When, however, a similarly mild procedure was used with a highly polymerized S III preparation (no. 300) the result was complicated by occurrence of depolymerization, for upon deacetylation the product, 300D, precipitated only about as much antibody from the antipneumococcus Type III rabbit serum as did the degraded sample 104Na, *i.e.*, about one-half as much as the starting material, lot 300 (Table I).

Diazomethane usually reacts with carboxyl groups, phenolic hydroxyl groups[15] and with acidic hydroxyls, as in ascorbic acid.[16] Diazomethane also has been found to methylate non-acidic hydroxyl groups of some aliphatic alcohols and poly-

(12) M. Heidelberger and O. T. Avery, *J. Exper. Med.*, **40**, 301 (1924); W. F. Goebel, *J. Biol. Chem.*, **89**, 395 (1930).

(13) E. F. Jansen and R. Jang, THIS JOURNAL, **68**, 1475 (1946).

(14) B. F. Chow and W. F. Goebel, *J. Exper. Med.*, **62**, 179 (1935).

(15) L. I. Smith, *Chem. Revs.*, **23**, 193 (1938).

(16) E. G. E. Hawkins, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 246 (1939).

saccharides[17] and to react with saccharic and trihydroxyglutaric acids[18] to produce mixtures which include unsaturated acids containing $-OCH_3$ and $-COOCH_3$ groupings.

Treatment of the S III acid with moist ethereal diazomethane yielded a product, 300Me, with 25.7% methoxyl as against the 8.8% calculated for the methyl ester. When saponified with alkali the resulting acid, 300H, still contained 17.4% methoxyl, indicating that two of the five available alcoholic hydroxyl groups per cellobiuronic acid unit were methylated. 300H precipitated almost one-half as much antibody as did the native polysaccharide (Table I). Conversion of the serologically inactive methylated S III ester to the amide gave a substance which also did not react with antiserum. These results confirm the conclusions of Chow and Goebel with SI[14] insofar as the cross-reactions of the methylated S III derivatives in Type III antiserum are concerned.

Because these authors stressed the importance of the carboxyl groups of S I for its reactivity with antiserum attempts were made to prepare fully esterified S III. This could not be effected by any of the methods tried. A fully acetylated preparation obtained from a degraded but serologically reactive sample of S III, 104, was esterified with diazomethane and the ester converted to the amide. This substance, 104N, with all hydroxyl groups and 34% of the carboxyl groups free (if it be assumed that all ester –OMe was converted to $-NH_2$) did not react with the rabbit antiserum mainly used in these studies, but did show a precipitin reaction with horse antiserum. On the other hand, when a relatively undegraded S III was converted into a methyl ester, 186E, with 65% of its carboxyl groups unreacted, and the ester was converted into an amide, this product, 186F, reacted well with rabbit antiserum, precipitating 91% as much antibody as the native S III. The ester, 186E, precipitated 86% of the total antibody. These results also indicate that 186E had not been appreciably degraded by esterification with HCl and MeOH, otherwise much less rabbit antibody would have been precipitated. While Chow and Goebel[14] showed that esterification of all of the carboxyl groups with simultaneous methylation of an $-NH_2$ and –OH converted S I into a serologically inactive substance the present data demonstrate that about one-third of the carboxyl groups of S III may be altered without seriously interfering with the ability of the polysaccharide to precipitate antibody. Evidently then, all of the carboxyl groups of an antigenic polyelectrolyte of this kind are not required for satisfactory fitting with the homologous rabbit antibody.

When, however, only one-third of the carboxyl groups of the degraded amide, 104N, were free, this did not suffice for formation of a precipitate with antibody formed in the rabbit, but did allow precipitation with antibody formed in the horse. The differences in behavior of the antibodies in the sera of the two species are probably due to the large size (*ca.* 10^6) of the antibodies in the horse, while in the rabbit these have the normal molecular weight of globulins.[19] The greater ease with which precipitation occurs with horse sera was also noted with partial hydrolytic products of S III[20] which precipitate with antisera prepared in the horse and not in rabbit antisera.

In terms of modern immunochemical theory both antigen and antibody should possess certain stereochemical configurations for precipitation to occur. It is usually considered, also, that both antigen and antibody possess multiple reactive sites.[21] It then would be apparent why alteration of a few of these sites on the antigen by substitution of $-COCH_3$ for the H of –OH, or by insertion of –COOMe or $-CONH_2$ for –COOH might cause diminished reactivity with antibody as a result of impaired fitting together, while blocking of all the sites would completely abolish the reaction.

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(17) L. Schmid, *Ber.*, **58B**, 1963 (1925); M. Nierenstein, *J. Chem. Soc.*, **60B**,1820 (1927).

(18) Th. O. Schmidt and H. Kraft, *Ber.*, **74B**, 33 (1941); Th. O. Schmidt and H. Zeiser, *ibid.*, **67**, 2120 (1934); Th. O. Schmidt, H. Zeiser and H. Dippold, *ibid.*, **70B**, 2402 (1937).

(19) K. Goodner, F. L. Horsfall, Jr., and J. H. Bauer, *Proc. Soc. Exp. Biol. Med.*, **34**, 617 (1936); M. Heidelberger, K. O. Pedersoc and A. Tiselius, *Nature*, **138**, 165 (1936); M. Heidelberger and K.On. Pedersen, *J. Exper. Med.*, **65**, 393 (1937).

(20) M. Heidelberger and F. E. Kendall, *ibid.*, **57**, 373 (1933).

(21) M. Heidelberger and F. E. Kendall, *ibid.*, **61**, 563 (1935).