

In these studies, the length of stain produced by AsH_3 on HgBr_2 paper was measured and the corresponding figure for As read from a graph obtained with standard solutions. It should be noted that variation in length of stain for a given amount of As enters into the preparation of the graph as well as into analysis of an unknown sample, thus introducing the possibility of further error in the final result. The standard deviation of a single determination of $20 \mu\text{g}$. As by the micro-titrimetric procedure was 3.7%. While it is as sensitive as the usual Gutzeit procedure, the titrimetric technique is much more objective and does not call for the same careful control of physical conditions in the evolution of AsH_3 .

Cassil & Wichmann [1939] have reported a procedure for small quantities of As very similar in principle to that described above. As was separated as AsH_3 and passed into 1 ml. 1.6% HgCl_2 . I_2 was added and the excess back-titrated with arsenite, in contrast to our direct titration. The final volume of solution titrated was about 8 ml. After the absorption of AsH_3 in HgCl_2 , one atom of arsenic was found to correspond to eight atoms of iodine. The sensitivity of the estimation must, therefore, have been identical with that observed in titrating, in a volume of 2 ml., the product of the reaction between AsH_3 and AgNO_3 , which requires two atoms of iodine for each atom of arsenic. It seems that the

sensitivity in using HgCl_2 could be further increased by decreasing the volumes of the reagents added before the back-titration. Cassil & Wichmann's accuracy was somewhat greater than that claimed for the present procedure.

SUMMARY

1. With a special tube for the absorption of AsH_3 in AgNO_3 solution and the Conway micro-burette for the titration of the arsenite formed, the iodometric estimation of As in digests of biological material was modified to cover the range 5–50 μg .

2. The recovery of 20 μg . As was 98%, the standard deviation of a single observation from the mean being 3.7%.

3. Methods for the digestion of As-containing material were examined, and the use of a mixture of HNO_3 , HClO_4 and H_2SO_4 adopted.

4. Preliminary separation of As as AsCl_3 from the digest was found to be necessary only when large samples of material were taken for analysis.

The author is indebted to Prof. G. F. Marrian for helpful criticism, to Dr G. T. Meiklejohn for making the first absorption tubes, and to all workers in these Departments, besides those already mentioned, who in carrying out arsenic estimations introduced refinements into the procedure. Permission from the Director General, Scientific Research and Development, Ministry of Supply, to publish this paper is gratefully acknowledged.

REFERENCES

- Allcroft, R. & Green, H. H. [1935]. *Biochem. J.* **29**, 824.
- Bang, I. [1925]. *Biochem. Z.* **161**, 195.
- Barnes, J. W. & Murray, C. W. [1930]. *Industr. Engng Chem.* (Anal. ed.), **2**, 29.
- Cassil, C. C. [1937]. *J. Ass. off. agric. Chem., Wash.*, **20**, 172.
- [1939]. *J. Ass. off. agric. Chem., Wash.*, **22**, 319.
- & Wichmann, H. J. [1939]. *J. Ass. off. agric. Chem., Wash.*, **22**, 436.
- Conway, E. J. [1939]. *Micro-Diffusion Analysis and Volumetric Error*. London: Crosby Lockwood.
- Cox, H. E. [1925]. *Analyst*, **50**, 3.
- First Report of the Sub-Committee of the Society of Public Analysts on the Determination of Arsenic... in Food Colouring Materials... [1930]. *Analyst*, **55**, 102.
- Hinsberg, K. & Kiese, M. [1937]. *Biochem. Z.* **290**, 39.
- How, A. E. [1938]. *Industr. Engng Chem.* (Anal. ed.), **10**, 226.
- Kahane, E. [1932]. *C.R. Acad. Sci., Paris*, **195**, 48.
- Kiese, M. [1937]. *Arch. exp. Path. Pharmac.* **186**, 337.
- Klein, D. [1920]. *J. Ass. off. agric. Chem., Wash.*, **3**, 512.
- Neller, J. R. [1929]. *J. Ass. off. agric. Chem., Wash.*, **12**, 332.
- Ramberg, L. [1921]. *Hoppe-Seyl. Z.* **114**, 262.
- Winterfeld, K., Doerle, E. & Rauch, C. [1935]. *Arch. Pharm., Berl.*, **273**, 457.

The Components of the Antigenic Complex of *Salmonella Typhimurium*

By G. G. FREEMAN, Dyson Perrins Laboratory, Oxford University

(Received 21 July 1943)

Recent work on the antigenic complex of *Eberthella typhosa* (*Bact. typhosum*) has shown that the substance may be obtained in a state approaching chemical homogeneity by trichloroacetic acid or

diethylene glycol extraction of the bacterial cells, and by tryptic digestion of the dried organisms. The complete antigenic complex may be broken down by mild acetic acid hydrolysis with the libera-

tion of the component polysaccharide, conjugated protein and lipid constituents. The polysaccharide, which is non-antigenic and relatively non-toxic, is responsible for the specific properties of the intact antigenic complex. It has been shown to be free from lactose, pentose and uronic acid residues, but it yields 40% *d*-glucose, 21% *d*-mannose and 17% *d*-galactose on hydrolysis. The polysaccharide has a minimum molecular weight of the order of 10,000 [Freeman & Anderson, 1941; Freeman, 1942]. Henderson & Morgan [1938] have shown that the antigenic complex of the O901 strain of *Eberth. typhosa* is obtained by diethyleneglycol extraction of the organisms, and exists as a polymolecular complex containing three main components, a specific polysaccharide, a protein and a phospholipin.

In the present paper the methods which have proved successful in the case of *Eberth. typhosa* have been applied to the investigation of the nature of the components of the antigenic complex of *Salmonella typhimurium* (*Bact. typhi-murium*).

The results have in general confirmed those already obtained with the closely related *Eberth. typhosa*, but quantitative differences, especially in the nature of the specific polysaccharides, which account for the immunological dissimilarity of the two species, have been found.

EXPERIMENTAL

The organism was an O-strain (L.S.H.T.M. Catalogue C3/23), which was free from Vi antigen. It was grown on a synthetic medium as described by Freeman, Challinor & Wilson [1940]. The dried bacterial bodies and several batches of antigen used in this work were prepared in the Departments of Biochemistry and of Bacteriology, London School of Hygiene and Tropical Medicine; the author is greatly indebted to Professors H. Raistrick and W. W. C. Topley for the use of these starting materials. The antigenic complex was obtained by tryptic digestion [Freeman *et al.* 1940]; the dried organisms were digested in batches of 45 g. with an average yield of 6.9% of antigen.

Purification of the antigenic complex

Before degradation, the antigenic material was fractionally precipitated from aqueous solution, (a) with ethanol and (b) with ammonium sulphate, in order to establish its homogeneity.

(a) *Ethanol fractionation of Salmonella typhimurium antigen.* 23.2 g. of antigen were dispersed in 600 ml. of water by grinding in a mortar and by mechanical shaking. The opalescent viscous solution was made 0.1N to acetic acid and centrifuged at 3000 r.p.m. for 3 hr., giving 0.24% of dark insoluble residue. The opalescent supernatant solution gave no appreciable precipitate when ethanol was added to a concentration of 50% (w/w), but when the

ethanol content was raised to 60% the bulk of the antigen separated out after standing overnight, leaving a water-clear supernatant solution, which contained little or no antigenic material. The precipitate was washed with 60% ethanol and redispersed in 1 l. of 0.1N acetic acid. Addition of ethanol to 57% concentration gave a small amount of precipitate (F0-60%/0-57%), which was removed by passage of the ethanol suspension through a de Laval Industrial Separator (F0-60%/0-57%; 1.25 g.=5.4%; N, 5.2; ash, 13.4; reducing sugar value, 58%; limit of precipitation with antiserum, 1 in 338,000; optimum, 7500-37,500), and a colourless, opalescent supernatant solution. The ethanol content of the latter was raised to 60% and the bulk of the antigen separated out leaving a clear supernatant solution. The main antigenic fraction, F0-60%/57-60%, was washed twice with absolute ethanol and twice with ether and dried *in vacuo* over P₂O₅ (19.1 g.=82.4%; N, 2.7; ash, 14.2; r.v. (reducing sugar value), 65%; $[\alpha]_D^{18}$ (in formamide, *c*=1), +106°±2°, limit of precipitation with antiserum 1 in 338,000; optimum, 7500). 50 hr. electro dialysis in cellophane membranes reduced the ash content of F0-60%/57-60% to 7.9%; N, 2.8%. The contents of the first and second 60% ethanol supernatant solutions were practically devoid of antigenic material. Total recovery in fractionation was 97.6%.

Reducing sugar value (r.v.) and specific rotation determinations on the isolated fractions were carried out as described by Freeman & Anderson [1941], and other analyses were by Dr G. Weiler.

(b) *Ammonium sulphate fractionation of antigen.*

A further attempt to fractionate the antigenic material was made by means of precipitation from aqueous solution by increasing concentrations of ammonium sulphate. A preliminary experiment showed that the bulk of the antigen was precipitated between the levels of 23-35% concentration of ammonium sulphate.

The ethanol fractionated antigen (F0-60%/57-60%), 14.9 g. in 500 ml. of water, was three times fractionated between the limits of 23 and 35% concentration of ammonium sulphate. The isolated fractions were submitted to dialysis to remove the ammonium sulphate, first in collodion sacs, and then to electro dialysis for 60 hr. between cellophane membranes. The bulk of the antigenic material (10.03 g.=67.5%) was precipitated at 35% concentration of ammonium sulphate (F23-35%; N, 1.72; r.v. 65; ash, 4.2%; $[\alpha]_D^{17}$ (in formamide, *c*=1), +95°±2°; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). When the ammonium sulphate concentration was raised to 41%, a further active fraction (F35-41%) was obtained (0.779 g.=5.3%, N, 2.5; r.v. 65; ash, 5.8%; $[\alpha]_D^{17}$ (in formamide, *c*=1), +91°±9°; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). Total recovery in fractionation was 77%.

Extraction of the antigenic material with organic solvents. Extraction of the antigenic complex with organic solvents [cf. Morgan & Partridge, 1940a] yielded only 17 mg. or 0.34% of fatty fraction. It is therefore apparent that the lipid fraction which is therefore apparent that the lipid fraction which is therefore obtained from the antigenic complex on hydro-

lysis in a yield of about 4%, as described later, was an essential antigenic component and not merely an adventitious impurity.

Properties of the antigenic complex

The antigenic complex of *S. typhimurium* gave opalescent aqueous solutions even when the concentration of antigen was as low as 0.4%. The bulk of the substance remained in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolved readily in formamide and in concentrated formic acid, giving solutions which were practically free from opalescence, but it was insoluble in glacial acetic acid and in dioxan. The bulk of the substance was insoluble in concentrated aqueous phenol solutions but, on exposure to 95% aqueous phenol, the antigenic material swelled up to form a thin translucent jelly which separated out on centrifuging. 0.4% aqueous solutions of the antigen gave no precipitates on addition of an equal volume of uranyl acetate (cf. antigen of *Eberth. typhosa* Ty 2), 10% trichloroacetic acid, salicylsulphonic acid, tannic acid solution, or Esbach's reagent. Phosphotungstic acid (2% in 5% H_2SO_4) gave an immediate flocculent precipitate. The presence of tyrosine residues was indicated by positive Millon's α -nitroso- β -naphthol and Ehrlich's diazo reactions. A weakly positive Sakaguchi reaction for arginine was obtained, but Ehrlich's diazo reaction for histidine was negative. 20 mg. of the substance in 1 ml. of water gave a weakly positive biuret reaction, but the ninhydrin reaction was negative. The substance gave a strong Molisch's reaction but did not reduce Fehling's solution.

On analysis the pure antigen gave C, 48.4; H, 7.3; N, 1.8–1.9; non-volatile ash, 4–5; volatile fatty acid on hydrolysis (calculated as acetyl groups), 6.1%; $[\alpha]_D^{27}$, $+95 \pm 1^\circ$ (in formamide, $c=1$); $[\alpha]_D^{17}$, $+96^\circ$ (in water, $c=1$); reducing value (method of Hagedorn & Jensen [1923]) on hydrolysis with 2N HCl for 2 hr. in a sealed tube, 65%; organic P, 0.5; inorganic P, 0.3% (N/organic P ratio, 7). Determination of amino-N (Van Slyke) showed that 0.2% of N was present as amino-groups.

Fractionation of antigen from aqueous solution with acetone. To provide further evidence of the homogeneity of the antigenic preparation after purification by fractionation from aqueous solution, first with ethanol and then with ammonium sulphate, the substance was submitted to a further fractionation from aqueous solution with acetone.

Antigen (1.98 g.) was dissolved in 100 ml. water; the bulk of the substance (1.787 g. = 90.2%; N, 1.9; ash, 4.7; r.v. 63%; $[\alpha]_D$ (in formamide, $c=1$), $+95 \pm 1^\circ$; limit of precipitation with antiserum 1 in 1,000,000; optimum, 37,500) was precipitated between acetone concentrations of 43 and 45% (by weight). On raising the acetone concentration to 52% a further fraction (F45–52%) was ob-

tained (0.169 g. = 8.5%; N, 1.8; ash, 4.9; r.v. 65%; $[\alpha]_D$, $+97 \pm 1^\circ$; limit of precipitation with antiserum, 1 in 1,000,000; optimum, 37,500). 0.022 g. (1.1%) of substance was recovered from the supernatant solution.

Immunological properties of Salmonella typhimurium antigen. The antigen in its unfractionated state was toxic for mice, average lethal dose, 0.13–0.27 mg. [Freeman *et al.* 1940]. Mouse protection experiments have been carried out on several batches of crude antigen [cf. Raistrick & Topley, 1934; Topley, Raistrick, Wilson, Stacey, Challinor & Clark, 1937]; the material was shown to contain antigens comparable in immunological activity with that of the dried bacterial cells. It has not yet been possible to conduct mouse protection tests on the purified antigen, but this material stimulated the production of precipitins in rabbits at least as readily as the crude substance.

Formamide dissociation of the antigenic complex. Morgan & Partridge [1940a] have shown that the antigen of *Bact. dysenteriae* Shiga (a phospholipin-polysaccharide-conjugated protein complex) may be dissociated by the action of neutral formamide with the production of the free phospholipin and a polysaccharide-conjugated protein complex.

The antigen of *S. typhimurium* (1.13 g.) was submitted to dissociation by the method of Morgan & Partridge and yielded only 0.011 g. (0.9%) of a colourless, lipid fraction and 0.894 g. (79%) of 'formamide dissociated antigen'. The latter did not differ significantly in its analytical data (N, 1.8; ash, 5.2%; $[\alpha]_D^{18}$, $+101 \pm 1^\circ$), limit of precipitation with antiserum and chemical properties, from the undissociated antigenic complex. The aqueous solutions were, however, slightly less opalescent than those of the original antigen, and on addition of NaOH to pH 8.5–10, solutions of the formamide-dissociated product became almost water-clear, whereas those of the antigenic complex were still definitely opalescent.

Acetic acid hydrolysis of the antigenic complex. The antigenic complex was split up into its polysaccharide, lipid and conjugated protein components by hydrolysis with dilute acetic acid [Freeman & Anderson, 1941; Freeman, 1942].

Purified antigenic complex (1.9 g.) was dispersed in 200 ml. of 0.1N acetic acid and, after 2 hr. hydrolysis at 100°, the optical rotation of the supernatant solution had become constant ($[\alpha]_D^{18}$, $+0.69 \pm 0.01^\circ$ (1 dm.)), indicating that hydrolysis was complete.

The following fractions were obtained:

(I) Specific polysaccharide (F0–86%), 1.326 g. = 69.8%; N, nil; ash, 4.9; r.v. 93%; $[\alpha]_D^{20}$, $+105^\circ \pm 1^\circ$ (in water, $c=1$);

(II) Conjugated protein, 0.292 g. = 15.4%; N, 7.2; ash, 5.9%; $[\alpha]_D^{20}$, $-22 \pm 4^\circ$ (in 0.1N NaOH, $c=0.3$); P, 2.1%; N/P ratio, 7; a grey amorphous solid, insoluble in water, but readily soluble in dilute sodium hydroxide and reprecipitated on acidification;

(III) Lipid fraction, 0.074 g. = 3.9%, a semi-solid, colourless fatty substance;

(IV) An ethanolic residue fraction (F 86-99%), 0.16 g. = 8.4%; N, 2.7%; ash, 6.4; r.v. 49%; $[\alpha]_D^{18}$, +15° ± 1°.

Fission of the antigenic complex, as described above, is a true hydrolytic breakdown and not merely a thermal disintegration of a colloidal complex. A specimen of antigen underwent no apparent change on heating at 100° in aqueous solution for 2 hr., and gave only 2% of insoluble residue on centrifuging; on acidification with acetic acid and 2 hr. heating at 100° the supernatant solution broke up into its four components, as described above.

The specific polysaccharide component of the antigenic complex

The crude polysaccharide was purified by electro-dialysis to remove the bulk of the ash and submitted to 2-3 fractionations from aqueous solution with ethanol.

In a typical experiment 1.83 g. of crude polysaccharide in 150 ml. water were electro-dialyzed for 30 hr. in collodion membranes (Hg cathode, Pt anode, e.m.f. 100 V. d.c.), the solution concentrated *in vacuo* to about 20 ml. volume, treated with 20 mg. of norit for 30 min. at 100° and twice fractionated between the limits of 45 and 86% ethanol concentration, giving 1.42 g. = 78% of purified polysaccharide (F 45-86% (2)). This gave on analysis: C, 44.6; H, 6.9; ash, 1.5%; acetyl, nil; $[\alpha]_D^{18}$, +103° ± 1° (in water, $c=1$). $C_6H_{10}O_5$ requires C, 44.4; H, 6.2%. A specimen of the electro-dialyzed polysaccharide was re-electro-dialyzed in 5% solution for 70 hr., and the polysaccharide, reisolated by addition of ethanol to 86% concentration, then gave on analysis: C, 44.5; H, 6.9; ash, 1.5%.

It was concluded that the ash (1.5%) was combined in the polysaccharide molecule and could not be eliminated by physical means. The pure polysaccharide precipitated with *S. typhimurium* anti-serum to a dilution of 1 in 250,000. The polysaccharide gave a strongly positive Molisch's reaction but did not reduce Fehling's solution; it gave no iodine coloration. The absence of fructose residues in the molecule was demonstrated by negative Foulger's and Seliwanoff's tests. Cole's rapid furfural test, Tollens's phloroglucinol test, Bial's orcinol test and the aniline acetate test for furfural, all gave negative results, suggesting the absence of pentosé residues. The polysaccharide gave a negative reaction in Tollens's naphthoresorcinol test for uronic acids: a control test on 5 mg. of glucuronic acid under the same conditions gave a strongly positive reaction. Scherer's reaction for inositol and Sal-kowski's [1910] modification gave negative results on 10 mg. quantities of polysaccharide; 5 mg. of inositol gave strongly positive reactions, both alone and in the presence of 10 mg. of polysaccharide.

Evidence as to the homogeneity of the polysaccharide has been obtained from: (1) attempts to separate it into fractions with different chemical properties by fractional precipitation from aqueous solution with acetone, (2) regeneration of the substance from its triacetyl derivative in an unchanged form, and (3) ultracentrifuge experiments (see Addendum).

Fractionation of polysaccharide from aqueous solution with acetone. 1.49 g. of specific polysaccharide dissolved in 40 ml. of water, giving an almost colourless, water-clear solution, free from undissolved residue, and addition of acetone to a concentration of 40% by weight gave no opalescence, but when the acetone concentration was raised to 45% a small amount of polysaccharide was precipitated. This was separated off, triturated with ethanol, washed twice with ether, and dried *in vacuo* over phosphorus pentoxide. Further increments of acetone were added until the material had been divided into seven fractions: F 40-45%, F 45-50%, F 50-55%, F 55-60%, F 60-65%, F 65-75%, and F 75-99%. The bulk of the substance (77.6%) was precipitated between acetone concentrations of 40 and 55%, and only 6.2% remained soluble in 65% acetone. The seven fractions were compared by determination of specific optical rotation and reducing value on hydrolysis (Table 1).

Table 1. *Acetone fractionation of specific polysaccharide of S. typhimurium*

Fraction	Weight (g.)	Yield (%)	$[\alpha]_D^{20}$ (in water, $c=1$)	Reducing value on hydrolysis (percentage of that of glucose)
Unfractionated polysaccharide	1.4902	—	+103°	93
(1) F 40-45%	0.1666	11.2	+101°	85
(2) F 45-50%	0.6770	45.4	+101°	90
(3) F 50-55%	0.3131	21.0	+101°	86
(4) F 55-60%	0.0774	5.2	+101°	88
(5) F 60-65%	0.0944	6.3	+105°	84
(6) F 65-75%	0.0602	4.0	—	66
(7) F 75-99%	0.0331	2.2	—	74

Total recovery: 95.3% (results uncorrected for ash).

Fractions of the polysaccharide differing in solubility in acetone-water mixtures have been shown by specific rotation and reducing value determinations to be practically identical, and it may be concluded that the specific polysaccharide represents a chemical entity.

Acetylation of polysaccharide. On acetylation with acetic anhydride in pyridine solution at 70° for 1 hr., the substance (0.58 g.) yielded 65% of theory (0.68 g.) of crude acetyl polysaccharide. The latter was purified by extraction with boiling absolute ethanol and by reprecipitation from chloroform solution with light petroleum. The two fractions precipitated by light petroleum concentrations of 63 and 85% respectively were found to be identical on analysis, showing that the acetyl compound was homogeneous. The pure acetyl polysaccharide (triacetyl derivative) was a

colourless amorphous solid; it was insoluble, even on boiling, in absolute ethanol, methanol, ether and petroleum ether, but was readily soluble in chloroform, acetone, pyridine and acetic anhydride. It was strongly dextrorotatory, $[\alpha]_D^{18}$, $+113^\circ \pm 1^\circ$ (in chloroform, $c=1$), and gave on analysis: C, 49.9; H, 6.2; acetyl, 48.8%. $C_8H_7O_5$ ($OC \cdot CH_3$)₃ requires C, 50.0; H, 5.6; CH_3CO , 44.8%.

Regeneration of the polysaccharide from its acetyl derivative. The acetyl derivative was deacetylated under the conditions worked out for the deacetylation of the *Eberth. typhosa* triacetyl polysaccharide [Freeman, 1942]. 0.48 g. of the acetyl polysaccharide gave 0.228 g. (84.4% of theory) of specific polysaccharide, which gave on analysis: $[\alpha]_D^{18}$, $+102^\circ \pm 1^\circ$ (in water, $c=1$); C, 46.3; H, 6.85; acetyl, 0.9; ash, 0.7%, and precipitated to a dilution of 1 in 300,000 with *S. typhimurium* antiserum, optimum 1 in 150,000.

Acid hydrolysis of the specific polysaccharide. The optimal conditions of liberation of reducing sugars by hydrolysis of the polysaccharide were investigated as a preliminary step in the identification of the sugars present. The hydrolysis in $N H_2SO_4$ at 100° proceeds rapidly to about 87% (2 hr.) [Hagedorn & Jensen] and then follows a slower linear increase, reaching 93% hydrolysis after 12 hr. The unhydrolyzed polysaccharide had a reducing value, as glucose, of 1.3%. It was found that for complete hydrolysis of the polysaccharide 7 hr. heating with $N H_2SO_4$ at 100° was adequate.

Nature of the component reducing sugars

The specific polysaccharide has been shown to consist predominantly of anhydrohexose units; ketose, pentose and uronic acid residues were absent. *d*-Galactose has been shown to be present in the antigenic complex from which the polysaccharide was derived [Freeman *et al.* 1940].

The hydrolysate from 0.971 g. of specific polysaccharide, freed from H_2SO_4 and concentrated to 25 ml., gave α_D^{19} , $+1.12^\circ$ (1 dm.); total apparent glucose = 0.8115 g. (83.6% of the polysaccharide). If the reducing constituents had a reducing value equal to that of glucose, the 'apparent specific rotation' of the mixture was $[\alpha]_D^{19}$, $+34.5^\circ$.

d-Mannose-phenylhydrazone. One-half of the total neutral hydrolysate was treated with 0.2 g. phenylhydrazine and 0.4 g. 25% acetic acid [van der Haar, 1920; Freeman, 1942]; mannose phenylhydrazone crystallized out on standing at 0° , yield 0.1486 g., corresponding to a mannose content of 20.4% of the specific polysaccharide. The crude product was recrystallized from boiling aqueous ethanol (60% w/w) and gave *d*-mannose phenylhydrazone, colourless microcrystalline plates, m.p. 195° (186° on slow heating), unchanged on admixture with an authentic specimen. Found: C, 53.2; H, 6.7; N, 10.3%. $C_{12}H_{13}N_2O_5$ requires C, 53.3; H, 6.7; N, 10.4%.

d-Galactose. The excess phenylhydrazine was removed as benzaldehyde phenylhydrazone [Morgan, 1938], the filtrate concentrated *in vacuo* to dryness and the residue of sugars taken up in 2 ml. 90% acetic acid and the solution allowed to crystallize at 0° . 0.053 g. of crystalline *d*-galactose (10.8% of the polysaccharide) separated out. The product reduced Fehling's solution (m.p. 161° , mixed m.p. with authentic *d*-galactose (m.p. 164°) was 161° , $[\alpha]_D^{19}$, $+80^\circ \pm 2^\circ$ (in water, $c=1$)). A specimen (0.038 g.) yielded 90%

(0.054 g.) of *d*-galactose methylphenylhydrazone, m.p. 190° , unchanged on admixture with an authentic specimen.

A small further yield of *d*-galactose (2.0% of the polysaccharide) as the methylphenylhydrazone was obtained from the filtrate after removal of the crystalline *d*-galactose by treatment with methylphenylhydrazine (asym.) [Neuberg, 1907; Lüttke, 1929; Freeman, 1942]. Found: C, 55.0; H, 7.1; N, 10.0%. $C_{13}H_{20}N_2O_5$ requires C, 54.9; H, 7.1; N, 9.9%.

d-Glucose-*p*-nitrophenylhydrazone. Excess methylphenylhydrazine was eliminated as described above, the sugar-containing filtrate evaporated to dryness *in vacuo* and the residue taken up in 2 ml. absolute methanol and treated with *p*-nitrophenylhydrazine (0.1 g.) [van Ekenstein & Blanksma, 1903; Reclaire, 1908; Freeman, 1942]. Yellow crystalline *d*-glucose-*p*-nitrophenylhydrazone was obtained (0.044 g., 5.2% of glucose), m.p. 184° , unchanged on admixture with authentic hydrazone. Found: C, 45.7; H, 5.6; N, 13.3%. $C_{12}H_{17}O_7N_3$ requires C, 45.7; H, 5.4; N, 13.3%.

The isolations of sugars, as substituted phenylhydrazones, under conditions in which the derivative of a single hexose is obtained at each stage, provide conclusive proof of the co-existence of *d*-glucose, *d*-mannose and *d*-galactose as constituents of the specific polysaccharide. The losses of sugars in the intermediate removal of excess of substituted hydrazines with benzaldehyde are, however, sufficiently great to lower significantly the yields of hydrazones.

In a further series of experiments the neutralized hydrolysate from 1.45 g. of polysaccharide gave 0.274 g. (18.9%) of crystalline *d*-galactose. After recrystallization from 90% acetic acid, the m.p. was 165° , unchanged on admixture with authentic *d*-galactose; the initial and equilibrium rotations (in water, $c=1.7$) were: $[\alpha]_D^{15}$, $+143^\circ \pm 1^\circ$ (after 3 min.); $+80.5^\circ \pm 1^\circ$ (after 24 hr.) (these agree closely with the recorded values for α -*d*-galactose, $+144.0^\circ \rightarrow +80.5^\circ$ [Hudson & Yanovsky, 1917] and $+80.4^\circ$ (at equilibrium) [Parcus & Tollens, 1890].

The residual sugars in solution were heated under reflux with *p*-nitrophenylhydrazine (1 g.) in methanol. *d*-Mannose-*p*-nitrophenylhydrazone (0.473 g., 18.6% mannose in terms of the polysaccharide) crystallized out as pale yellowish brown crystals from the boiling solution, m.p. 190° , unchanged on admixture with authentic *d*-mannose-*p*-nitrophenylhydrazone. On admixture with *d*-glucose-*p*-nitrophenylhydrazone (m.p. 184°) the mixed m.p. was 175° . $[\alpha]_D^{14}$, $+86^\circ \pm 1^\circ$ (in pyridine, $c=1$), which agreed with that of the authentic hydrazone ($[\alpha]_D^{15}$, $+87^\circ \pm 1^\circ$).

0.6234 g. (24.6% as *d*-glucose) of *d*-glucose-*p*-nitrophenylhydrazone separated out from the filtrate at 0° after removal of *d*-mannose-*p*-nitrophenylhydrazone.

Thus 18.9% of *d*-galactose has been isolated from the specific polysaccharide as the free sugar, in addition to yields of substituted hydrazones equivalent to 24.6% *d*-glucose, and to 20.4% *d*-mannose (20.4% as the phenylhydrazone, and in another experiment 18.6% as the *p*-nitrophenylhydrazone). Control experiments under the same conditions with similar concentrations of single pure hexoses have

shown that pure *d*-glucose gives 79.6% of the theoretical yield of the *p*-nitrophenylhydrazone and pure mannose 94.9% of the theoretical yield of *d*-mannose phenylhydrazone [Freeman, 1942]. On this basis the actual hexose contents of the specific polysaccharide are estimated to be: *d*-glucose, 31; *d*-mannose, 21.5; *d*-galactose, 18.9; total hexose, 71.4%. The latter approaches reasonably close to the reducing value of 90% (as apparent glucose) on hydrolysis of the polysaccharide.

A useful confirmation of the relative proportions of the three hexoses present in the polysaccharide hydrolysates is afforded by a comparison of the observed optical rotation of a neutralized hydrolysate with that calculated from the reducing value of the solution (Hagedorn & Jensen) in terms of apparent glucose. The hydrolysate described above had $\alpha_D^{18^\circ}$, +1.12° (1 dm.), total volume 25 ml. and total apparent glucose, 0.8115 g. In such a solution, containing 31% glucose, 21.5% mannose and 18.9% galactose, the rotation of the *d*-glucose component is +0.53°, that of the mannose component +0.10° and that of the galactose +0.50°, giving a total calculated rotation (1 dm.) of +1.13°, which agrees closely with the observed value.

The conjugated protein component of the antigenic complex

The conjugated protein fraction gave positive biuret, Millon's and Sakaguchi's reactions, showing the presence of tyrosine and arginine residues. Ehrlich's diazo reaction for histidine was also positive.

The crude substance (0.63 g.) was purified by three precipitations from 0.1N NaOH with 4N HCl [Freeman & Anderson, 1941] and gave 76% (0.48 g.) of purified conjugated protein, N, 6.2; ash, 5.1%.

Tryptic digestion of the conjugated protein. 0.0725 g. of the conjugated protein was freshly precipitated by acidifying an alkaline solution of the substance, and suspended in 15 ml. of borate buffer, pH 8.4; trypsin (0.003 g.) was added and the digest incubated at 37°. After 5 days' digestion only 5.1% of the total N had been liberated as amino-N, and 17.7% after 14 days (Van Slyke). The substance was, therefore, only slowly and very incompletely attacked by trypsin.

Acid hydrolysis of the conjugated protein. 0.01 g. quantities of the conjugated protein were hydrolyzed with 1 ml. 10N formic acid containing 3.6% HCl in sealed tubes at 100° for various periods of time and the amino-N liberated determined by the Van Slyke method [Morgan & Partridge, 1940a; Freeman & Anderson, 1941]. After 2-4 hr. hydrolysis the bulk of the conjugated protein had dissolved, giving a pale brown solution, although traces of insoluble residue were still present after 8 hr. heating. The amino-N, liberated from the conjugated protein, progressively increased to 50.5% of the total N after 4-8 hr. hydrolysis and at this stage liberation of amino-acids had reached a maximum.

The 86% ethanol-soluble fraction of the antigenic complex. In its crude state this fraction was a colourless, very hygroscopic solid, which dissolved readily in water but was insoluble in chloroform and acetone; it gave a strongly positive Molisch's reaction, showing the presence of carbohydrate residues, but negative ninhydrin and biuret reactions, from which it was concluded that the nitrogen was not present as amino-acids or peptides. The fraction reduced Fehling's solution on being boiled with it, for about 20 sec. It gave an immediate precipitate with 2% phosphotungstic acid in 5% H₂SO₄. On hydrolysis the substance yielded 21.5% of volatile fatty acid (calculated as acetyl) and a trace of glucosamine (1%). The nature of this fraction and its role in the antigenic complex are not yet understood. The compound may be a partially acetylated polysaccharide. The bulk of the volatile fatty acid component (acetyl) of the antigenic complex is present in the 86% ethanol-soluble fraction. The substance has no power of specific precipitation with *S. typhimurium* antiserum.

The lipid components of the antigenic complex

The lipid fraction liberated on acetic acid hydrolysis constitutes only 3-4% of the antigen. By dissociation of the antigenic complex with formamide a part of this lipid fraction (0.9% of the antigen) is liberated. Although other workers have isolated much larger amounts of lipid components from bacterial antigens [Morgan & Partridge, 1940a; Soru & Combiesco, 1940; Boivin & Mesrobian, 1938], our *S. typhimurium* antigen does not appear to contain more than about 5% of this component.

0.145 g. of the lipid fraction was separated by the usual methods, into a mixed fatty acid fraction (colourless solid, 0.063 g., 43%), a fraction insoluble in cold acetone, which was probably a phospholipin (0.09 g. = 6.4%), and 0.04 g. (28%) of neutral fatty substance. Insufficient of the lipid component was available for further investigation.

Preparation of the specific polysaccharide directly from the dried bacterial bodies

As in the case of *Eberth. typhosa* Ty 2, the specific polysaccharide of *S. typhimurium* may be extracted directly from the dried bacterial cells by means of dilute acetic acid at 100° as described by Freeman [1942] [cf. White, 1929, and Morgan, 1936].

28 g. of the dried cells were extracted three times for 1½ hr. at 100° with 400 ml. lots of 0.1N acetic acid. The successive extracts had optical rotations of +0.57°, +0.12° and -0.06° (1 dm., 18°), and yielded 4.3 g. (15.4%) of colourless crude polysaccharide (N, 5.9; ash, 25.9; r.v. 84%; $[\alpha]_D^{18^\circ}$, +67° ± 4° (in water, c=1)). The substance precipitated with *S. typhimurium* antiserum to a dilution of 1 in 300,000.

The polysaccharide was purified as described by Freeman [1942]; 1.88 g. of the crude material gave 0.564 g. (30%) of pure polysaccharide, which gave on analysis: C, 45.4; H, 6.8; inorganic P, 0.1%; organic P, 0.3%; N, nil. The yield of pure polysaccharide was 4.6% of the dried bacterial bodies, as compared with a yield of about 4.8% in terms of the dried bacterial bodies, by hydrolysis of the isolated antigenic complex.

A rough strain of *S. typhimurium* (100 g. dried bodies) was twice extracted with 1000 ml. lots of 0.1 N acetic acid for 1½ hr. at 100°. The extracts, which had optical rotations of +0.15° and +0.06° (1 dm., 17°), were found to contain no significant amount of the specific polysaccharide. A fraction F50-86% (1) was obtained (4.2%), which gave only a trace of precipitate with *S. typhimurium* antiserum at a dilution of 1 in 15,000. The specific polysaccharide tested at the same time precipitated to a dilution of 1 in 300,000.

Preparation of an undegraded specific polysaccharide

Morgan & Partridge [1941] have shown that by dissociation of the antigenic complex of *Bact. dysenteriae* (Shiga) with dilute NaOH, and by means of extraction with concentrated aqueous solutions of phenol, a specific non-antigenic polysaccharide was obtained, which differed markedly, in chemical and physical properties, from the ('degraded') polysaccharide obtained by means of hydrolysis with hot dilute acetic acid, and possessed the power, not shared by the 'degraded' polysaccharide, of recombining with the conjugated protein component of the antigen to produce antigenically active complexes. The antigenic complex of *S. typhimurium* has been submitted to fractional degradation by methods analogous to those described by Morgan & Partridge.

(1) *By sodium hydroxide dissociation.* The *S. typhimurium* antigen (1.34 g.), in 100 ml. of water, was treated with NaOH to a concentration of 0.05 N at 0°, as described by Morgan & Partridge; 1.13 g. (85%) of 'recovered antigen' was obtained, and this did not differ significantly from the untreated antigen in its solubility, appearance in solution and qualitative reactions. Only 0.007 g. (0.5%) of material was recovered from the alkaline alcoholic supernatant solution. This gave a negative biuret reaction. It was clear that 0.05 N sodium hydroxide had no significant dissociating effect on the antigenic complex.

'Recovered antigen' from the previous experiment was treated with 0.25 N NaOH. An ethanol precipitated fraction (F68% (2), 1.01 g.=94%; N, 1.2; ash, 2.2%; $[\alpha]_D^{18}$, +104° ± 1° (in formamide, *c*=1)) and a protein-like fraction soluble in alkaline ethanol, were obtained. The latter (0.041 g.=3.9%; N, 9.0%; ash, nil) was a colourless, amorphous solid, which was insoluble in water but dissolved freely in 0.1 N NaOH and was slowly precipitated from alkaline solution on acidification with 2 N HCl. The sub-

stance gave positive biuret, Sakaguchi's and Ehrlich's diazo reactions, indicating the presence of peptides and arginine and histidine residues. The compound was amphoteric; it dissolved slowly in N acetic acid at 40° as well as in dilute alkali. After reprecipitation from alkaline solution and from solution in N acetic acid, the substance remained virtually unchanged, N=9.3%.

The recovered 'polysaccharide' (F68% (2), 0.91 g.) was submitted to two further dissociations with 0.25 N alkali and gave 0.68 g. (75%) of 'undegraded polysaccharide' (F68% (4), N, 0.5%), equivalent to 60% of the undissociated antigen. The undegraded polysaccharide gave aqueous solutions very much less opalescent than those of the original antigenic complex. 0.021 g. (2.3%) of amphoteric protein fraction was also obtained from the third and fourth alkali treatments of the antigen.

In order to establish the homogeneity of the undegraded polysaccharide, F68% (4) (0.62 g.) was fractionally precipitated from aqueous solution with ethanol. Three fractions were isolated:

(a) F55-60% (0.56 g.=90.9%; C, 47.1; H, 7.3; acetyl 3.5; ash, 6.3; R.V. 73%; $[\alpha]_D^{18}$, +112° ± 1°; limit of precipitation with antiserum, 1 in 300,000, optimum 150,000),

(b) F60-70% (0.004 g.=0.7%), and

(c) F70-99% (0.048 g.=7.8%; R.V. 75%; limit of precipitation with antiserum 1 in 150,000; optimum 1500), and it was concluded from the results that the alkali-dissociated polysaccharide, F68% (4), was chemically homogeneous. Further evidence on the homogeneity of this substance was provided by ultracentrifugal sedimentation (see Addendum).

(2) *By extraction of the antigenic complex with concentrated aqueous phenol solutions.* *Salmonella typhimurium* antigenic complex (0.984 g.) was repeatedly extracted with 80% aqueous phenol and 95% aqueous phenol; the former yielded 0.027 g. (2.7%) of amphoteric protein and the latter none. The 'recovered polysaccharide' (1) (0.75 g.=76.5%; $[\alpha]_D^{18}$, +81° ± 3° (in formamide, *c*=1)) was an amorphous substance which gave very opalescent aqueous solutions. Recovered polysaccharide (1) (0.722 g.) was three times re-extracted with 80% phenol and gave 0.621 g. (86.0%) of recovered polysaccharide (2) (N, 0.5%). The latter gave very opalescent aqueous solutions similar to those of the original antigen, and was precipitated from solution by 2% phosphotungstic acid in 5% H₂SO₄.

In order to investigate the homogeneity of recovered polysaccharide (2), the material (0.559 g.) was fractionally precipitated from aqueous solution (50 ml.) with ethanol. The properties of the fractions isolated are summarized in Table 2. Fraction F50-60% gave on analysis, C, 49.1; H, 7.9; ash, 5.5%.

Table 2. Analytical data for fractions of *S. typhimurium* 'undegraded polysaccharide' prepared by phenolic extraction

	Insoluble residue	Ethanol fractions of 'polysaccharide'		
		F50-60%	F60-65%	F65-99%
Weight (g.)	0.069	0.298	0.112	0.019
Yield (%)	12.3	53.4	20.1	3.5
N (%)	—	0.9	—	—
Reducing value on hydrolysis (percentage of that of glucose)	—	66	72	—
Dilution limit of precipitation with <i>S. typhimurium</i> antiserum (1 part dissolved in:)	—	1,000,000	1,000,000	—
Dilution giving optimal precipitation (1 part dissolved in:)	—	300,000	300,000	—

Total recovery; 89.3%.

*Recombination of Salmonella typhimurium
'undegraded polysaccharides' with proteins*

Antigenically active artificial complexes of the specific polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga) have been obtained by Morgan & Partridge [1940 a, b] by dissolving the isolated components in formamide and precipitating the complex with ethanol, and later, using an undegraded specific polysaccharide, simply by mixing the components of the complex in alkaline solution [Morgan & Partridge, 1941]. The observation that bacterial polysaccharides possess the power of forming acid-soluble complexes with proteins, which are normally insoluble in aqueous solution at acid reaction, has been confirmed and extended, to the undegraded polysaccharide of *S. typhimurium*. If a solution of the undegraded polysaccharide and an alkaline solution of the protein are mixed and acidified with acetic acid, a soluble, stable complex is formed, and the protein is no longer precipitated by the addition of trichloroacetic acid or other protein precipitants, provided that an excess of the protein above that equivalent to the combining power of the undegraded polysaccharide has not been used.

By this means the 'undegraded polysaccharides' prepared by alkaline dissociation and by phenolic extraction of the *S. typhimurium* antigenic complex were found to form acid-soluble complexes with the conjugated proteins of *S. typhimurium* and *Eberth. typhosa* Ty2. When the specific polysaccharide (acetic acid hydrolytic product) replaced the undegraded polysaccharide, the admixed proteins were immediately precipitated by acid. The alkali-dissociated undegraded polysaccharide had the power of rendering soluble at least an equal weight of conjugated protein (*S. typhimurium*), but the phenol-extracted 'polysaccharide' was saturated by more than 40% of protein; this shows that the two 'undegraded polysaccharides' are clearly different.

The alkali-dissociated undegraded polysaccharide formed trichloroacetic acid-soluble complexes on admixture in alkaline solution with edestin, human serum pseudoglobulin, casein, gliadin and rabbit myosin. When the polysaccharide was mixed with rabbit myosin in neutral salt solution, no acid-soluble complex was formed. Control experiments with *S. typhimurium* conjugated protein and with mixtures of the above proteins with degraded polysaccharide, gum arabic or soluble starch, all gave negative results.

The undegraded polysaccharide lost its power of combining with the conjugated protein after 1 hr. heating in 0.1N acetic acid solution at 100°, but there was no appreciable loss of this activity after 30 min. at 50°, or 5 min. at 100°, in acetic acid. After 1 hr. hydrolysis at 100° with 0.1N acetic acid

the undegraded polysaccharide solution had lost its opalescence and had deposited a trace of precipitate (2.1%). At this stage the hydrolysate contained two polysaccharides, which were separated by fractional precipitation with ethanol: (a) (F0-86%), 80.2%, $[\alpha]_D^{20}$, $+100 \pm 1^\circ$; this substance was identical with the degraded polysaccharide prepared by acetic acid hydrolysis of the antigenic complex; and (b) (F86-99%), 0.032 g. = 10.6%; acetyl, 13.2%; insufficient of this component was obtained for adequate purification, but the evidence available strongly suggests that the substance, which was a very hygroscopic acetyl polysaccharide, consisted predominantly of the acetyl polysaccharide previously isolated directly from the antigenic complex.

An artificial complex of the undegraded polysaccharide and the conjugated protein behaved, on heating at 100° in 0.1N acetic acid solution, in a manner identical with that of the native antigenic complex. An artificial complex of undegraded polysaccharide and horse serum albumin was stable in neutral solution at 100° for 2 hr., giving no precipitation of the albumin.

Combination of undegraded polysaccharide and horse serum albumin. Determination of saturation value. The undegraded polysaccharide was found to form a trichloroacetic acid-soluble complex with horse serum albumin when the two components were mixed either in neutral or in alkaline solution at 30°. (The horse serum albumin was a specimen of lyophil-dried protein kindly supplied by Dr A. G. Ogston; see Addendum.) When the ratio protein/polysaccharide did not exceed 3.5, trichloroacetic acid-soluble complexes were formed, but increase of protein above this proportion resulted in heavy precipitates on addition of trichloroacetic acid. In the neighbourhood of the equivalence zone addition of the precipitant produced very opalescent solutions, from which a small amount of protein separated out on standing.

The nitrogen contents of the precipitates and supernatant solutions obtained from several undegraded polysaccharide-albumin mixtures, by treatment with 20% trichloroacetic acid, were determined after drying by the Dumas method. It was found that when the ratio protein/polysaccharide did not exceed 3.5, practically the whole of the protein remained in the supernatant solution, but that when this figure was exceeded the whole of the protein was precipitated, practically none remaining in the supernatant solution.

Titration curves of artificial polysaccharide-protein complexes

In order to investigate the changes which take place in the solubility and buffering power of proteins in the presence of *S. typhimurium* undegraded polysaccharide, (a) horse serum albumin and

(b) *S. typhimurium* conjugated protein, alone and in the presence of the undegraded polysaccharide, were titrated at 19° with 0.01N HCl and NaOH, and the pH determined at intervals with a glass electrode and valve electrometer pH meter.

The following solutions were titrated:

- (i) 5 ml. 1% aqueous albumin + 4 ml. water.
- (ii) 5 ml. 1% aqueous albumin + 4 ml. 0.5% polysaccharide.
- (iii) 5 ml. 1% conjugated protein + 4 ml. water.
- (iv) 5 ml. 1% conjugated protein + 4 ml. 0.5% polysaccharide.
- (v) 5 ml. water + 4 ml. 0.5% polysaccharide.
- (vi) 9 ml. distilled water.

The conjugated protein solution was prepared by dissolving the substance (0.14 g.) in 2.5 ml. 0.1N NaOH, adding 1 ml. 0.1N HCl to the solution and diluting to 14 ml. with water.

Between pH 5.8 and 4.5 in titration (iii) each successive addition of acid produced a slight precipitate, which rapidly redissolved on stirring; at pH 4.47 a permanent precipitate of conjugated protein appeared which separated out on standing, leaving a clear supernatant solution. In the presence of the undegraded polysaccharide (titration (iv)) there was no change in the appearance of the solution and no precipitation of protein.

The undegraded polysaccharide had no appreciable buffering power; titrations (v) and (vi) gave practically identical results. The presence of the undegraded polysaccharide brought about no significant change in the buffering power of serum albumin (Fig. 1) on the acid side of pH 6, but in the range of pH 6–10 there was an appreciable increase of buffering power. The displacement of the serum albumin curve in the presence of the undegraded polysaccharide occurred in the same pH range and in the same sense as that produced by formaldehyde [Prideaux & Woods, 1933], but was not nearly as great. In the experiments of Prideaux & Woods, 5–6 ml. more 0.1N alkali were required to bring the albumin to pH 9 in the presence of formaldehyde, whereas only 12 ml. more 0.01N alkali would be required to bring a corresponding quantity of albumin to pH 9 in the presence of undegraded polysaccharide. In the titration curves of *S. typhimurium* conjugated protein (Fig. 2) there was a marked decrease of buffering power, in the presence of the polysaccharide, in the pH range 4–7, which coincided with the range of solubility of the conjugated protein on the acid side of neutrality.

Katchalsky [1941] observed depression of the pH when glucose was added to solutions of various amino-acids and peptides, from which he has determined the equilibrium constants of the interaction of the two components.

It is clear that interaction or combination takes place between the protein and undegraded poly-

saccharide molecules, with the production of complexes which behave towards acids and alkalis differently from the untreated protein. Nothing is

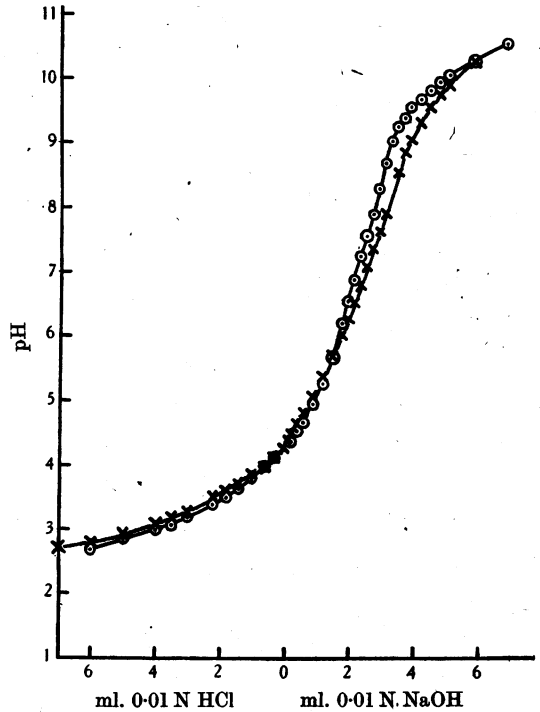


Fig. 1. Titration curve of serum albumin in presence and absence of *Salmonella typhimurium* undegraded polysaccharide. ⊙—⊙ Serum albumin, ×—× serum albumin + polysaccharide.

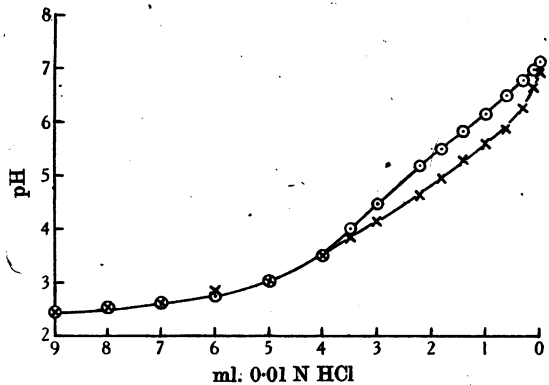


Fig. 2. Titration curve of *Salmonella typhimurium* conjugated protein in presence and absence of undegraded polysaccharide. ⊙—⊙ Conjugated protein, ×—× conjugated protein + polysaccharide.

known of the nature of the protein-polysaccharide bond, but this may be salt-like, or possibly of the Schiff's base type through a polysaccharide alde-

lydic group and an amino-group in the protein. The evidence available suggests that the combination is not analogous to the interaction of formaldehyde with amino-groups, which involves a considerable pH shift and alteration of the pK's of the amino-groups, whereas in the formation of the undegraded polysaccharide-protein complex there is a very small pH shift, and the pK's of the amino-groups are not seriously changed despite the formation of an apparently stable complex, in which the polysaccharide renders the protein more soluble in the presence of acid precipitants. These conclusions regarding the existence of an artificial protein-polysaccharide complex are confirmed by ultracentrifugal determination of sedimentation constants (see Addendum).

Antigenicity of undegraded polysaccharides and an artificial polysaccharide-conjugated protein complex

The following fractions were tested for antigenicity in rabbits:

- (i) Undegraded polysaccharide (phenol-dissociated product).
- (ii) Undegraded polysaccharide (alkali-dissociated product).
- (iii) 'Degraded' specific polysaccharide.
- (iv) *Salmonella typhimurium* conjugated protein (dissolved in phosphate buffer (pH 8.4) plus saline).
- (v) Artificial polysaccharide-conjugated protein complex (70 mg. undegraded polysaccharide, alkali dissociated, +20 mg. conjugated protein).
- (vi) *Salmonella typhimurium* antigenic complex. (The author is indebted to Dr A. R. Martin for the rabbit immunizations.) The rabbit antisera were tested for the presence of specific precipitins against solutions of the homologous antigens, and for their power of agglutinating killed O-suspensions of *Salmonella typhimurium* (Table 3).

The phenolic-dissociation product and the artificial polysaccharide-conjugated protein complex were powerfully antigenic, and gave rise to antisera which precipitated to high dilutions with the homologous antigens, and agglutinated *S. typhimurium* suspensions to about the same dilution as an antiserum prepared against the purified antigenic complex. The alkali-dissociation product (undegraded polysaccharide) and the degraded specific polysaccharide, on the other hand, had no detectable antigenic activity. The phenolic- and alkali-dissociated products, and the degraded polysaccharide, precipitated with an antiserum prepared against the original antigenic complex to dilutions of 1 in 1,000,000 (optimum 1 in 150,000), 1 in 300,000 (optimum 1 in 15,000) and 1 in 300,000 respectively. *S. typhimurium* conjugated protein was feebly antigenic, giving rise to homologous precipitins, but sera 296 A and B were free from *S. typhimurium* agglutinins and gave no precipitates with the original antigenic complex. By combination of the inactive undegraded polysaccharide and the feebly antigenic conjugated protein, which had no *S. typhimurium* specificity, a powerfully antigenic artificial complex was obtained, which gave rise to antisera which precipitated the homologous antigen and the original antigenic complex (to dilutions of 1 in 600,000, optimum 1 in 15,000) and agglutinated *S. typhimurium* suspensions.

The phenolic-dissociated undegraded polysaccharide precipitated with antisera prepared against the parent antigenic complex in a manner almost identical with that of the alkali-dissociated undegraded polysaccharide, had similar chemical and physical properties and also possessed the power of recombining with the conjugated protein com-

Table 3. *Antigenicity of fractions from S. typhimurium*

Material used as antigen	Rabbit no.	Precipitation test. Dilution of homologous antigen				Agglutination test. Dilution of immune serum					
		1 in				1 in					
		1500	15,000	150,000	300,000	40	80	160	320	640	1280
(i) Undegraded polysaccharide (phenol-dissociated product)	263A	++	++++	+++	+++	4	4	4	3	1	0
	263B	++	+++ (optimum)	+++	+	4	4	4	3	1	0
(ii) Undegraded polysaccharide (alkali-dissociated product)	266A	—	—	—	—	0	0	0	0	0	0
	266B	—	—	—	—	0	0	0	0	0	0
(iii) 'Degraded' specific polysaccharide	265A	—	—	—	—	0	0	0	0	0	0
	265B	—	—	—	—	0	0	0	0	0	0
(iv) <i>S. typhimurium</i> conjugated protein	296A	++	++	++	—	0	0	0	0	0	0
	296B	++	++	++	—	0	0	0	0	0	0
		(optimum)									
(v) Artificial polysaccharide-conjugated protein complex	298A	++	+++	+	—	4	4	4	3	1	0
	298B	+++	++++ (optimum)	++	+	4	4	4	2	1	0
(vi) <i>S. typhimurium</i> antigenic complex	230	+	+++ (optimum)	+++	++	4	4	4	4	2	0

4, complete agglutination; 3, almost complete agglutination; 2, incomplete agglutination; 1, trace of agglutination; 0, no agglutination.

ponent, though not so powerfully as the alkali dissociation product. It was clearly different from the latter, as shown by its antigenic properties in rabbits, though this property may be traceable to residual small amounts of the antigenic complex still undecomposed by repeated phenolic extractions.

DISCUSSION

In its nature and properties the antigen of *S. typhimurium* shows a close similarity to the corresponding complex from *Eberth. typhosa* Ty2. Both antigens exist as polysaccharide-conjugated protein-lipid complexes, which are readily broken down into their components by hydrolysis. There is also a fourth component which has not yet been isolated in a pure condition, but is described provisionally as a partially acetylated polysaccharide. When the antigenic complex is degraded, its antigenic properties are lost but the polysaccharide component retains the specific properties of the complex. The latter undergoes partial degradation, with the elimination of one or more of its components, on treatment with formamide, dilute alkali and concentrated aqueous phenol. Dilute alkali and phenol liberate an amphoteric protein, which differs from the conjugated protein in being soluble at acid reactions, and an 'undegraded polysaccharide', intermediate in properties between the antigenic complex and the specific polysaccharide. The undegraded polysaccharide differs very significantly from the specific polysaccharide, in possessing the power of combining with proteins to form stable complexes, and there is evidence that it consists of a complex of the specific polysaccharide and the acetyl polysaccharide.

Previous workers have isolated antigenic complexes from *S. typhimurium* but some of the earlier preparations were undoubtedly contaminated with materials derived from the culture media and evidence of their chemical homogeneity is not always available. Raistrick & Topley [1934] prepared antigenic fractions which contained 4-6.5% nitrogen and gave 23.1-37.8% reducing sugars on hydrolysis. Topley *et al.* [1937] describe an antigenic fraction which contained 4.2-4.8% N. Boivin's antigen from the same organism contained 1.83-2.20% N; r.v. 40% on hydrolysis [Boivin & Mesrobian, 1934]. At an earlier stage of this investigation, Freeman *et al.* [1940] obtained the antigen for the first time from organisms grown on a synthetic medium; it contained N, 4.2%, and gave 50% of reducing sugars on hydrolysis, $[\alpha]_{5461} + 76^\circ$. These data relate to the crude fraction which was the starting material in the ethanol and ammonium sulphate fractionations in the present work. These figures illustrate the difficulties of obtaining pure antigenic fractions from the bacterial cell, and

emphasize the need for careful fractionation of the primary extraction products.

In their behaviour on hydrolysis, and in the properties of their conjugated protein and specific polysaccharide components, the antigenic complexes of *S. typhimurium* and *Eberth. typhosa* display a marked similarity, but there are considerable quantitative differences of nitrogen content, reducing value on hydrolysis and specific optical rotation. The conjugated proteins of the two antigenic complexes are markedly similar in their physical properties, N contents, constituent amino-acids and liberation of amino-nitrogen on tryptic digestion and acidic hydrolysis.

As was to be expected from the immunological data for the two organisms, cross precipitation reactions between the specific polysaccharides of *S. typhimurium* and *Eberth. typhosa* Ty2 were negative; the chemical data confirm that the two substances are different. Both polysaccharides, however, consist entirely of the same hexose units (*d*-glucose, *d*-mannose and *d*-galactose), which are present in approximately the same relative amounts. Differences in the structural arrangement of the constituent sugars, which may account for the serological dissimilarity of the two specific polysaccharides, provide an interesting subject for investigation when more of the specific substances become available.

The work of Morgan & Partridge [1941] revealed new methods of degradation of the parent antigenic complex, which do not involve high temperatures or extremes of acidity. These have been used successfully to prepare undegraded polysaccharides from *S. typhimurium*, which were intermediate in properties and composition between the parent antigenic complex and the degraded polysaccharide. The undegraded polysaccharides possess the remarkable property of forming stable complexes with proteins, from which the latter are not precipitable by acid protein precipitants. The alkali- and phenol-dissociation products (undegraded polysaccharides) display an important difference in that the former is non-antigenic, whilst the latter still stimulates formation of precipitins in rabbits. This antigenicity is thought to be due to residual traces of undecomposed antigenic complex.

Morgan & Partridge also showed how the polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga), lacking specific antigenicity, could be recombined to form complexes which gave rise to powerful specific antibodies on injection into animals. This has been confirmed and extended to *S. typhimurium*; mixture of the non-antigenic alkali-dissociated polysaccharide and the conjugated protein (feebly antigenic but with no *S. typhimurium* specificity) in alkaline solution, followed by acidification, produced a powerfully antigenic

complex which gave rise to *S. typhimurium* precipitins and agglutinins on injection into rabbits. Ultracentrifugal studies of mixtures of the undegraded polysaccharide with albumin also clearly showed the presence of a new complex.

SUMMARY

1. By means of ethanol and ammonium sulphate fractionation from aqueous solution the antigenic complex of *Salmonella typhimurium* has been obtained in a form which is believed to represent a chemical entity.

2. On acetic acid hydrolysis the antigenic complex is broken down into four components: (a) a specific polysaccharide (69%), (b) an insoluble conjugated protein (16%), (c) a mixed lipid fraction (3-4%) and (d) an alcohol-soluble acetyl-polysaccharide (about 8%).

3. The specific polysaccharide is non-antigenic.

It is dextrorotatory, $[\alpha]_D^{20}$, $+103^\circ \pm 1^\circ$; it is free from nitrogen and yields 93% of reducing sugars on acidic hydrolysis. The polysaccharide is free from ketose, pentose and uronic acid residues, and yields 31% *d*-glucose, 21.5% *d*-mannose and 19% *d*-galactose on hydrolysis.

4. The antigenic complex has also been dissociated by means of precipitation from weakly alkaline solution, yielding a small amount of an amphoteric protein, and a non-antigenic 'undegraded polysaccharide' which reacts specifically with *Salmonella typhimurium* antiserum to high dilutions. This polysaccharide has the power of forming complexes with proteins such that the protein becomes no longer precipitable by acidic protein precipitants.

The author wishes to thank Prof. Sir Robert Robinson for his interest in this work and Imperial Chemical (Pharmaceuticals) Limited for permission to publish this account of it.

ADDENDUM

Examination in the Ultracentrifuge

By A. G. OGSTON (with technical assistance by Mrs P. M. HODSON),
Department of Biochemistry, Oxford

Various samples derived from the antigen of *Salmonella typhimurium* were examined in a Svedberg oil-turbine ultracentrifuge, by the 'diagonal schlieren' method of Philpot [1938]; where it was possible, the total refractive increments of the dissolved substances were measured, and the refractive increments appearing in different boundaries determined by integration of areas in the diagrams [Philpot, 1939].

(1) 'Degraded' polysaccharide: 1% solution unbuffered, in 0.1M NaCl; $S_{20}^w = 1.25 \times 10^{-13}$ is close to the value given by Philpot [1942] for the polysaccharide derived from *Eberth. typhosa* Ty2. The form of the sedimentation diagram (Fig. 3) indicates fair homogeneity and probably a not very asymmetric shape of particle (less so than that of *Eberth. typhosa* polysaccharide): if we assume a partial specific volume of 0.62, this gives a minimum molecular weight of 3600. Only 80% of the total refractive increment appears in the boundary, which shows that there is some non-sedimenting or highly heterogeneous material.

(2) Undegraded polysaccharide: 0.5% in 0.01M phosphate buffer, pH 6.8 and 0.2M NaCl: this presents a very different picture. There are at least three molecular species present of $S_{20}^w = 13.1$, 7.6 and 1.72×10^{-13} respectively: the diagram (Fig. 3) indicates also some rather heterogeneous material of sedimentation constant greater than 13×10^{-13} ,

and possibly a small component of sedimentation constant less than 1.72×10^{-13} . The form of boundary shown by the component of $S_{20}^w = 7.6$ suggests that its particles are asymmetric. The component of $S_{20}^w = 1.72$ may be identical with the 'degraded' polysaccharide.

(3) In view of the remarkable power of the undegraded polysaccharide to prevent the precipitation of proteins by trichloroacetic acid, a solution of it with crystalline horse serum albumin was compared with solutions of the polysaccharide and of albumin separately. The solution was 0.5% with respect to polysaccharide, 1% with respect to albumin, in 0.01M phosphate buffer pH 6.8 and 0.2M NaCl. The runs were carried out as nearly as possible identically with respect to speed, temperature, etc. and photographs were taken at corresponding times, with identical settings of the optical system (Fig. 4). The mixture contains two main components: one, of $S_{20}^w = 3.57 \times 10^{-13}$, comprises 70% of the total, has a sedimentation constant significantly lower than that of serum albumin alone (4.11×10^{-13}) and the shape of the boundary suggests a more asymmetric particle; the other, of $S_{20}^w = 5.11 \times 10^{-13}$, comprises 30% of the total and appears to be somewhat heterogeneous. There is no sign in the diagram of the mixture of any of the components of the polysaccharide, and the results provide clear evidence of complex formation be-

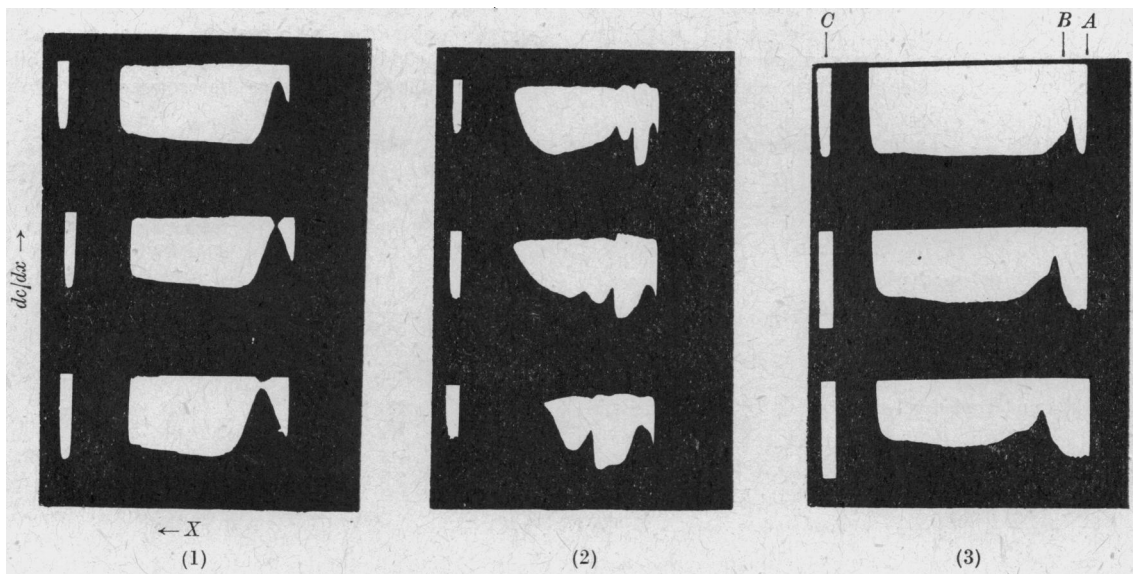


Fig. 3. (1) degraded polysaccharide: 1%, 20 min. intervals, 1080 r.p.s.; (2) undegraded polysaccharide: 0.5%, 10 min. intervals, 1000 r.p.s.; (3) antigen: 0.5%, 10 min. intervals, 370 r.p.s.

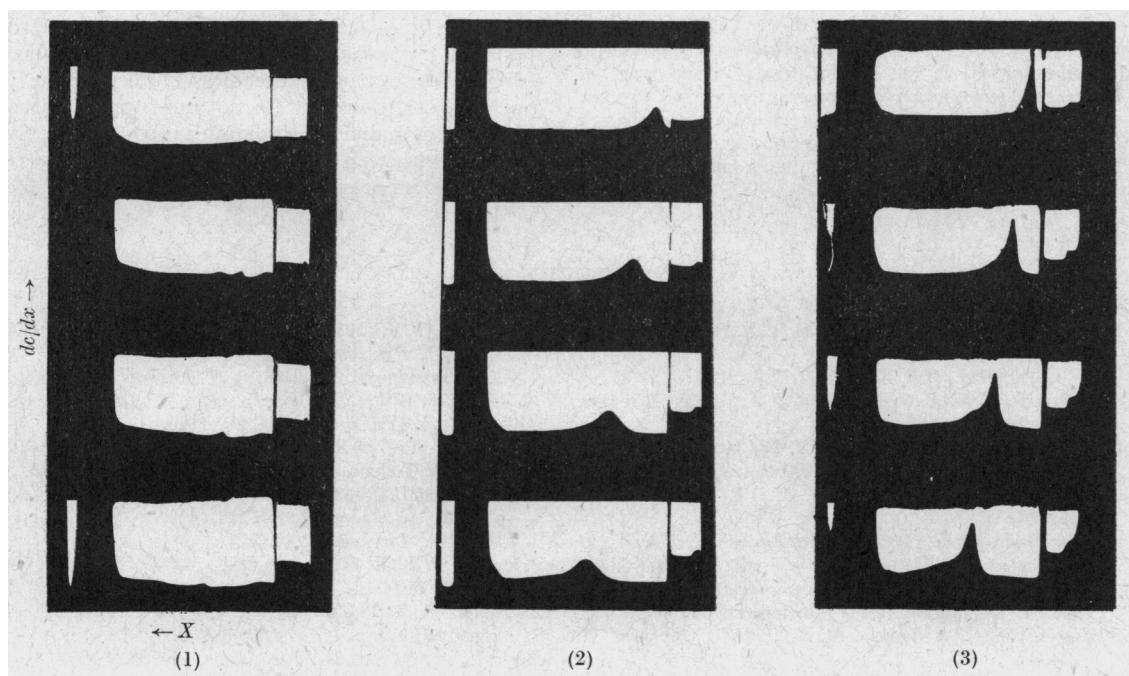


Fig. 4. (1) undegraded polysaccharide: 0.5%; (2) serum albumin: 1%; (3) undegraded polysaccharide: 0.5% + serum albumin, 1%, 20 min. intervals, 1050 r.p.s. Identical times and optical settings for all three sets of diagrams.

X = distance from centre of rotation; dc/dx = concentration gradient; A = meniscus; B = sedimentation boundary; C = index.

tween the protein and all components of the polysaccharide. This is in agreement with the titration data and the prevention of precipitation of the protein by trichloroacetic acid.*

resembles these rather closely, suggesting a similar type of combination with protein. Again, the main component (70%) seems to be homogeneous, while the other (30%) is rather heterogeneous. Only

Table 4. *Sedimentation constants of Salmonella typhimurium fractions*

Substance	Sedimentation constant ($S_{20}^w \times 10^{-13}$)	M (assumed value of V^* in brackets)	Fraction of total refracting material	Specific refractive increment
Degraded polysaccharide	1.25	3,600 (0.62)	0.82	0.0012
Undegraded polysaccharide (0.5%)	1.72	5,800 (0.62)	—	0.0012
	7.60	54,000 (0.62)		
	13.10	117,000 (0.62)		
Serum albumin	4.11	70,000	0.73	—
0.5% undegraded polysaccharide + 1% serum albumin	3.57	28,000 (0.71)	0.60	—
	5.11	48,000 (0.71)	0.23	—
Antigen (0.526%)	56.8	1.5×10^6 (0.68)	0.31	0.00115
	79.7	2.5×10^6 (0.68)	0.14	—

* Partial specific volume.

(4) The bacterial antigen (Fig. 3) shows two sedimentation boundaries of $S_{20}^w = 79.7$ and 56.8×10^{-13} . Although the sedimentation rates are much greater than those of the polysaccharide-serum albumin mixture, the general character of the components

* The horse serum albumin was prepared from horse serum in 1938 by the method of Hewitt [1938] somewhat modified. It was free from carbohydrate and appeared to be homogeneous. It was lyophil dried. In the course of five years it seems to have become heterogeneous to some extent; the specific refractive increment in the boundary is only 0.00136 compared with 0.00185 when it was made. The sedimentation constant of the homogeneous part remains unchanged.

about 50% of the total refracting material is present in these components. There is also a small amount of material which sediments very slowly.

The results are summarized in Table 4: column 2 gives the sedimentation constant in water at 20°; column 3 gives estimates of the molecular weights: it must be emphasized that these are calculated for spherical particles on the basis of the assumed values of the partial specific volumes given in brackets, and therefore do no more than indicate the orders of magnitude; column 4 gives the fraction of total refracting material appearing in the respective boundaries.

REFERENCES

- Boivin, A. & Mesrobian, L. [1934]. *C.R. Soc. Biol., Paris*, 115, 304, 309.
 ——— [1938]. *C.R. Soc. Biol., Paris*, 128, 5.
 van Ekenstein, W. A. & Blanksma, J. J. [1903]. *Rec. trav. chim. Pays-Bas*, 22, 434.
 Freeman, G. G. [1942]. *Biochem. J.* 36, 340.
 ——— & Anderson, T. H. [1941]. *Biochem. J.* 35, 564.
 ——— Challinor, S. W. & Wilson, J. [1940]. *Biochem. J.* 34, 307.
 van der Haar, A. W. [1920]. *Anleitung zum Nachweis, zur Trennung und Bestimmung der reinen und aus Glucosiden u.s.w. erhaltenen Monosaccharide und Aldehydsäuren*. Berlin: Gebrüder Bornträger.
 Hagedorn, H. C. & Jensen, B. N. [1923]. *Biochem. Z.* 135, 46.
 Henderson, D. W. & Morgan, W. T. J. [1938]. *Brit. J. exp. Path.* 19, 82.
 Hewitt, L. J. [1938]. *Biochem. J.* 32, 26.
 Hudson, C. S. & Yanovsky, E. [1917]. *J. Amer. chem. Soc.* 39, 1020, 1035.
 Katchalsky, A. [1941]. *Biochem. J.* 35, 1024.
 Lüdtke, M. [1929]. *Biochem. Z.* 212, 419.
 Morgan, W. T. J. [1936]. *Biochem. J.* 30, 909.
 ——— [1938]. *Helv. chim. Acta*, 21, 469.
 ——— & Partridge, S. M. [1940a]. *Biochem. J.* 34, 169.
 ——— [1940b]. *Brit. J. exp. Path.* 21, 180.
 ——— [1941]. *Biochem. J.* 35, 1140.
 Neuberg, C. [1907]. *Biochem. Z.* 3, 519.
 Pareus, E. & Tollens, B. [1890]. *Liebigs Ann.* 257, 169.
 Philpot, J. St L. [1938]. *Nature, Lond.*, 141, 283.
 ——— [1939]. *Biochem. J.* 33, 1725.
 ——— [1942]. *Biochem. J.* 36, 355.
 Prideaux, E. B. R. & Woods, D. E. [1933]. *Proc. roy. Soc. B*, 114, 110.
 Raistrick, H. & Topley, W. W. C. [1934]. *Brit. J. exp. Path.* 15, 113.
 Reclaire, A. [1908]. *Ber. dtsh. chem. Ges.* 41, 3665.
 Salkowski, E. [1910]. *Hoppe-Seyl. Z.* 69, 478.
 Soru, E. & Combiesco, C. [1940]. *C.R. Soc. Biol., Paris*, 133, 498.
 Topley, W. W. C., Raistrick, H., Wilson, J., Stacey, M., Challinor, S. W. & Clark, R. O. J. [1937]. *Lancet*, 1, 252.
 White, P. B. [1929]. *J. Path. Bact.* 32, 85.