physical methods. A study of the effect of EDTA on the molecular weight of the enzyme to determine whether the removal of the metal present in the enzyme results in a lowering of the molecular weight should be of value in testing the validity of this hypothesis.

SUMMARY

1. The aldolase of *Aspergillus niger* has been purified by ammonium sulphate fractionation and adsorption on calcium phosphate gel.

2. The purified aldolase was electrophoretically homogeneous. The activity per milligram of the enzyme was equal to that of crystalline muscle or yeast aldolase.

3. The properties of the enzyme and the kinetics of the reaction are described.

4. The enzyme resembled yeast aldolase and differed from muscle aldolase in requiring heavy metals for its activity. It was inhibited by cysteine, pyrophosphate, ethylenediaminetetraacetic acid, *o*-phenanthroline and $\alpha\alpha'$-dipyridyl. The inhibition was reversed by bivalent zinc, manganese, iron or cobalt but not by magnesium, calcium or copper.

5. A new spectrophotometric method for the estimation of aldolase activity is described. The method is based on the increase in light absorption at 240 mμ. in the presence of hydrazine.

6. The differences between the aldolases from different tissues are briefly discussed.

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A Specific Polysaccharide of *Pasteurella pestis*

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When many strains of *Pasteurella pestis* are grown at 37° a gelatinous envelope surrounds the cells (Rowland, 1914); this envelope material will immunize mice against plague infection (Amies, 1951; Baker, Sommer, Foster, Meyer & Meyer, 1952). Schütze (1932) showed that in addition to the envelope antigen, which is heat-labile, a heat-stable somatic antigen is present in rough and smooth forms of *Past. pestis* and is common to the closely related rodent-pathogen *Pasteurella pseudotuberculosis*. It was considered that although a smooth form of colony is characteristic of most *Past. pestis* strains, including all the virulent ones, no smooth 'O' somatic antigen was present, but that the heat-stable antigen was a rough somatic antigen underlying the envelope.

The smooth 'O' somatic antigens of Gram-negative bacteria have been extensively studied (Boivin, Mesrobeanu & Mesrobeanu, 1933; Morgan, 1937; Morgan & Partridge, 1940, 1941; Freeman, 1943; Goebel, Binkley & Perlman, 1945; Davies, Morgan & Mosimann, 1954) and have proved to be protein–polysaccharide–phospholipid complexes having an immunological specificity determined by their polysaccharide moieties. These 'O' somatic antigens are generally regarded as being synonymous with endotoxins (van Heyningen, 1950). The application to *Past. pestis* of extraction methods used in these studies has failed to reveal the presence of a material of this type. By the trichloroacetic acid method of Boivin *et al.* (1933) no endotoxin was obtained from either *Past. pestis* (Girard, 1941) or from *Past. pseudotuberculosis* (Girard, 1941; Schar & Thal, 1955). Lazarus & Nozawa (1948) obtained a toxic lysate of *Past. pseudotuberculosis* but the nature of the toxic material in the preparation was not determined. *Pasteurella aviseptica*, when extracted by the trichloroacetic acid method, yielded a true 'O' somatic antigen (Pirosky, 1938), *Pasteurella tularensis* also contained what appeared to be an analogous material (Girard & Gallut, 1951) but unlike the *Past. aviseptica* product, it was not toxic.

It is well known that *Past. pestis* produces a toxin, which has been called an endotoxin by some and an exotoxin by others; this is discussed by Pollitzer (1954). The toxic substance has not yet been isolated but is likely to be a protein and not related to the Gram-negative endotoxins (Girard & Sandor, 1947).

A fraction which appeared to be largely polysaccharide in nature was obtained from *Past. pestis* cells and from culture supernatant fluids by Seal (1951). The material could only be obtained from protective strains (those with envelopes) and was not present in *Past. pseudotuberculosis*. The preparation precipitated with *Past. pestis* antisera, and from a hydrolysate a sugar osazone was isolated which was thought to be that of arabinose. Baker *et al.* (1952) isolated from saline extracts of acetone-dried *Past. pestis* cells several fractions, one of which (IA) contained protein and carbohydrate. This did not appear to differ serologically from a protein fraction (IB) which protected mice from plague infection and was related to the envelope material. Chen (1952) detected in *Past. pestis* broth cultures a component which could be adsorbed on sheep erythrocytes, rendering them agglutinable by *Past. pestis* antisera; from a 25% ammonium sulphate precipitate of a saline extract of cells a fraction was obtained which also had this property and contained carbohydrate (N, 5·8%; protein, 37%). A saline extract of the phenol-insoluble residue of acetone-dried organisms was shown by Chen & Meyer (1954) to contain carbohydrate (N, 3%); the material precipitated readily with sera prepared by immunization with *Past. pestis* strain TRU (a rough strain, Schütze, 1939) but reacted weakly with anti-virulent *Past. pestis* sera. Silverman (1954) isolated fractions containing protein and carbohydrate which were related serologically to the envelope and mouse-protective antigen.

It is thus evident that *Past. pestis* produces at least one polysaccharide, possibly two, though none has yet been purified or characterized. The information available suggests, however, that no component is present which is truly analogous to the specific polysaccharides of other Gram-negative groups.

An antigenic analysis of *Past. pestis* has been described by Crumpton & Davies (1955, 1956) in which the agar-diffusion-precipitin technique of Ouchterlony (1948, 1953) was employed in conjunction with animal experiments to demonstrate the presence of at least ten antigens. With extraction products it was possible to show by comparing precipitin-line patterns with electrophoretic pictures that each line on a diffusion plate corresponded to one antigen or hapten. The characterization of these materials is in progress and one of them, a lipopolysaccharide, is described in the present paper.

MATERIALS AND METHODS

Organisms. *Past. pestis* strain Tjiwidej smooth (TS) (Otten, 1936) was used for the main polysaccharide extraction. A rough toxic variant TSR (isolated from TS), the non-toxic rough form TRU (Schütze, 1939) and a strain of *Past. pseudotuberculosis* have also been employed.

Growth and recovery of organisms. Organisms were grown in tryptic meat broth containing 1% (w/v) of galactose; 250 ml. amounts of medium were dispensed in 'Thompson' bottles (Gallenkamp and Co., London, E.C. 4), which were inoculated with 1 ml. (1 mg., dry wt., of cells) of a saline suspension which had been grown for 18 hr. on tryptic-meat agar. Incubation for 40 hr. on a reciprocating shaker at 37° gave yields of 4–5 g./l. (dry wt.). Glucose could not be substituted for galactose because excessive acid production brought growth rapidly to a standstill.

Tryptic-meat agar with or without galactose failed to support the growth of isolated organisms but allowed growth only from heavy inocula. To obtain isolated colonies for checking cultures on plates tryptic-meat agar with the addition of peptic sheep's blood was used (Herbert, 1949).

Organisms were recovered by centrifuging at 20 000 *g*, and resuspended in a small volume of cold water and poured into 10 vol. of acetone at −20°. After several changes of cold acetone the bacteria were dried *in vacuo* over H_2SO_4 and stored at 0°.

Materials for analysis. Samples of extraction products for analysis were thoroughly dialysed against distilled water at 0°–2° and dried from the frozen state. They were

further dried to constant weight *in vacuo* at 78° before making into aqueous solutions of known concentration.

Total nitrogen. This was determined by the Kjeldahl method with the distillation apparatus of Markham (1942) and the mixed bromocresol green–methyl red indicator of Ma & Zuazaga (1942).

Phosphorus. This was determined on samples containing 5–30 μg. of P by a modification of the method of Fiske & Subbarow (1925).

Sugars. Glucosamine was estimated by a modification of the colorimetric method of Elson & Morgan (1933), and aldoheptose by the H_2SO_4–cysteine reaction of Dische (1953). Total reduction was measured by the copper method of Somogyi (1937).

Chromatography. Polysaccharide samples were hydrolysed in sealed ampoules at 100° with N-H_2SO_4 for 15 hr. and neutralized with $Ba(OH)_2$. The neutral salt-free solutions were evaporated to dryness *in vacuo* and redissolved at suitable concentrations for application to paper chromatograms. Whatman no. 1 paper was used with a variety of solvent systems; papers were sprayed with Ehrlich's reagent (Partridge, 1948) to reveal hexosamine, or with anisidine hydrochloride in water-saturated butanol (Hough, Jones & Wadman, 1950) which distinguishes by colour the different classes of sugars.

Nucleic acid. The nucleic acid content of extraction products was estimated by measuring in a Unicam ultraviolet spectrophotometer the absorption at 260 mμ. of the material dissolved in 0·05N-NaOH. A purified preparation of ribonucleic acid was used as a standard for reference.

Ultracentrifuging. Preparative ultracentrifuge runs were made in a Spinco Model L Ultracentrifuge at 2–4°. Figures quoted for the force in *g* refer to the force exerted at the centres of the tubes and represent average values. Analytical ultracentrifuge runs were carried out in a Spinco Model E machine.

Electrophoresis. Examinations were made in a Spinco Electrophoresis–Diffusion Instrument Series 10 by standard techniques.

Antisera. Antisera were produced in rabbits by the intravenous injection of organisms or extraction products suspended or dissolved in sterile saline. Injections were given twice weekly until the examination of serum samples by techniques to be described indicated that the required antibodies had been formed.

Precipitation reactions in solid media. Antigens and antisera were examined by the agar-diffusion precipitin technique of Ouchterlony (1948, 1953) modified as described by Crumpton & Davies (1956).

Tube precipitation. Precipitation reactions in fluid medium were carried out in a volume of 0·5 ml., antigen or hapten solution (0·25 ml.) being added to an equal volume of antiserum. Tubes were incubated at 37° for 1 hr. and read after standing at 0° overnight.

Toxicity and animal-protection tests. These were carried out by techniques described by Crumpton & Davies (1956).

Pyrogenicity. Rabbits were injected intravenously with test material dissolved in pyrogen-free water, and rectal temperatures were recorded at intervals with a clinical thermometer inserted always to the same distance. Control animals were injected with pyrogen-free water and their temperatures were similarly recorded. Animals weighed 2·0–2·2 kg., and results are given in terms of temp. for weight of material injected/kg. of rabbit weight.

EXPERIMENTAL AND RESULTS

Extraction of polysaccharide

Preliminary extracts. An analysis of saline extracts of *Past. pestis* cells revealed that they were composed largely of protein, and that the isolation of the polysaccharide they contained might present some difficulties. Four batches of acetone-dried cells (strain TS), each weighing 10 g., were therefore extracted by different methods. Very little carbohydrate was found in the acetone-precipitated product of diethyleneglycol extraction (Morgan, 1937; Morgan & Partridge, 1940, 1941; Davies *et al.* 1954). The results of extraction with 50% (v/v) aqueous pyridine (Goebel *et al.* 1945) or 50% (v/v) aqueous glycerol (Binkley, Goebel & Perlman, 1945) were not encouraging. The product of phenol extraction by the method of Westphal, Lüderitz & Bister (1952*a*) represented about 1% of the dry wt. of the cells used and contained N, 4%, and P, 1%; this was largely composed of polysaccharide.

Phenol extraction. To remove the readily soluble protein before proceeding to the extraction of polysaccharide, acetone-dried cells of strain TS (120 g.) were suspended in 2·5% (w/v) saline (1800 ml.) at 20° and stirred for 24 hr., toluene being added to prevent mould and bacterial growth. The suspension was centrifuged at 20 000 *g* and the supernatant solution dialysed and freeze-dried (extract PIA). The sedimented cells, resuspended in saline, were re-extracted twice for periods of 24 and 12 hr., the extracted materials (PIB, PIC) being recovered by dialysis and freeze-drying. These products, some data for which are shown in Table 1, were set aside for the isolation of the proteins they contained, which will be described in subsequent communications.

The residue remaining after saline extraction proved to be composed of intact cells, although 28% of their original dry wt. had been removed. This cell residue was suspended in water (250 ml.) and warmed to 65°, and an equal vol. of 90% (w/v) aqueous phenol at 65° was added; the mixture was stirred for 45 min. and cooled to 0°, whereupon a separation of water and phenol phases occurred which was hastened by centrifuging. The clear aqueous phase was removed and the addition of 3 vol. of ethanol at −10° to the solution precipitated most of the material it contained; only a small fraction with a high N content was recovered from the 3 vol. of ethanol supernatant fluid. On redissolving the fraction which had been precipitated by 3 vol. of ethanol, a very opalescent aqueous solution was obtained from which nearly all of the material was precipitated by the addition of 1 vol. of cold ethanol. Examination of the ultraviolet-absorption spectrum of a sample of the material revealed the presence of nucleic acid. To the redissolved 50% (v/v) ethanol precipitate at 0° cold HCl was added, to pH 2; this precipitated some material which on recovery proved to be very largely composed of nucleic acid. The fraction soluble at pH 2 after neutralization, dialysis and freeze-drying contained N, 2·2% and P, 2·1% Material recovered from the phenol phase (PIR) was poorly soluble and consisted mainly of denatured protein (see Table 1).

Table 1. *Materials extracted from* Pasteurella pestis *cells (strain TS, 120 g.) with saline (2·5 %) and with phenol (45 %). Fractions* PIR *and* PIR 1 *are derived from the phenol and aqueous phases respectively*

Extract	Period of extraction (hr.)	Wt. (g.)	Yield (%)	N (%)	P (%)	Mouse LD_{50} (μg.) and 95 % confidence limits
Saline						
PIA	24	27·5	28	14·7	1·7	22...29...36
PIB	24	4·6		13·4	1·7	10...13...16
PIC	12	1·3		12·0	1·1	7...9...12
Phenol						
PIR	0·75	—	—	13·7	0·5	150
PIR 1		2·8	2·3	2·2	2·1	Over 500

Table 2. *Materials obtained from phenol extracts of* Pasteurella pestis *cells by fractionation with ethanol. Fractions* PER 11 *to* PER 13 *derived from the first,* PER 22 *and* PER 23 *from the second phenol extract*

Ethanol (%)	Fraction	Wt. (g.)	N (%)	P (%)	Nucleic acid (%)	Heptose estimated as D-glycero-D-galactoheptose (%)
0–15	PER 11	4·5	1·6	2·4	Nil	28
15–50	PER 12	1·5	2·0	3·0	20	18
Over 50	PER 13	0·07	9·1	2·4	12	5
0–66	PER 22	0·74	2·7	2·6	10	20
Over 66	PER 23	0·43	5·7	1·8	10	5

After freeze-drying, the material which had remained in solution at pH 2 proved to be poorly soluble in water and even less soluble in the presence of salt. It gave a strong Mölisch reaction but was biuret-negative; some absorption at 260 mμ. indicated the presence of about 3 % of nucleic acid. When tested against an antiserum prepared in rabbits by injecting *Past. pestis* cells (strain TS grown at 20°) the preparation precipitated at a dilution of 1 in 5 million; no precipitation occurred with normal serum below a dilution of 1 in 100, and the precipitation at this level appeared to be due to the poor solubility of the substance. Mild hydrolysis of a sample of this material (PIR 1) resulted in the separation of a chloroform-soluble lipid, leaving a simple polysaccharide in solution. The original substance was thus shown to be a lipopolysaccharide.

Larger-scale extraction and purification of lipopolysaccharide

Cells of strain TS grown at 37° (500 g.) were divided into two equal parts and both handled in the following way. Cells were extracted successively for 6, 12 and 24 hr. periods with 2·5 % (w/v) saline at 20° and the extracts were preserved for the examination of the protein antigens they contained. The sedimented cells remaining after the third saline extraction were resuspended in 1 l. of distilled water and extracted twice with phenol at 65° according to the method of Westphal *et al.* (1952*a*) already described. The first and second extracts of each batch (aqueous phases) were kept separately (PER 1 and PER 2, PFR 1 and PFR 2).

To avoid subjecting the material to acid conditions nucleic acid was removed from fraction PER 1 by ethanol fractionation. To the material dissolved in cold water (800 ml.) ethanol at −10° was added, and with 15 % (v/v) of ethanol a flocculent precipitate was obtained. The further addition of ethanol to the supernatant solution caused this to become progressively more opalescent until, with 50 % (v/v) of ethanol, a flocculent precipitate again appeared (PER 12). A small fraction (PER 13) having a relatively high N content was recovered from the 50 % ethanol supernatant fluid by evaporation, concentration *in vacuo* at 16°, dialysis and freeze-drying. The 15 % (v/v) ethanol precipitate was redissolved and reprecipitated twice at the 15 % (v/v) ethanol concn., dialysed and freeze-dried (PER 11).

At this stage the lipopolysaccharide was found to contain an aldoheptose sugar (see below). Relative estimates of the amounts of heptose in different fractions could readily be obtained, and the increase in heptose content was used to follow the purification of the polysaccharide. Analytical data for some of the fractions obtained are shown in Table 2.

The second extract of the first batch (PER 2) was dissolved in water (400 ml.), cooled to 0°, and ethanol at −10° was added to give a concn. of 15 % (v/v). Unlike the behaviour of the first extract no precipitation occurred at this concn., but a flocculent precipitate was recovered after 2 vol. of ethanol had been added (PER 22). The material remaining in the 2 vol. of ethanol supernatant fluid was also recovered (PER 23). Fraction PER 22 was reprecipitated between 15 and 66 % (v/v) ethanol concentration before recovery.

Whereas a separation of lipopolysaccharide (PER 11) from nucleic acid had been achieved with the product of the first extract, this was not the case in the second (Table 2). Accordingly, material PER 22 dissolved in water (250 ml.) was centrifuged at 100 000 *g* for 4 hr. and the supernatant fluid was removed. The sediment was redissolved in the original volume of water and centrifuged in the same way a second time. The combined supernatant solutions yielded nucleic acid with a small amount of polysaccharide, whereas the sedimented fraction did not contain detectable amounts of nucleic acid.

Extracts PFR 1 and PFR 2 were precipitated twice with 2 vol. of ethanol and freed from nucleic acid by the method of high-speed centrifuging as employed for PER 22. The lipopolysaccharides (PER 22 and PFR 22) obtained from the two second phenol extracts did not differ in any analytical or serological property from those of the two first extracts, and the reason for their different behaviour during ethanol fractionation was judged to be due to differences in particle size. Accordingly, these four fractions, which had been freed from nucleic acid, were pooled and designated PR 1; this weighed 10 g. and represented a 2% yield from the bacteria.

The material PR 1 was almost insoluble in the presence of salt and only poorly soluble in distilled water. At 1% (w/v) concentration a cloudy viscous solution was obtained and when a sample of this was examined in the analytical ultracentrifuge a sharp peak which sedimented rapidly was preceded by an accumulation of material on the bottom of the cell. A sample of PR 1 (3 g.) was suspended in distilled water (600 ml.) and allowed to soak for 1 week with occasional shaking, and centrifuged at 12 000 *g* for 1 hr. The supernatant solution (PR 1*a*), after samples had been dried to constant weight to estimate its concentration, was adjusted to 1% (w/v) of total solids after evaporation under reduced pressure at 16°. The sedimented fraction (PR 1*b*) after recovery by freeze-drying did not differ analytically from that which remained in solution but was evidently of larger particle size. Freeze-dried samples of the more soluble fraction PR 1*a* were again poorly soluble when shaken in water. Accordingly, the material PR 1*a* which had been preserved in solution was regarded as a representative sample of PR 1 for use in all experiments which involved optical methods of analysis.

Examination for immunological homogeneity

When cells of *Past. pestis* (strain TS) grown at 37° were tested against their homologous antiserum by the agar-diffusion precipitin technique of Ouchterlony (1948, 1953) at least ten lines of precipitation could be demonstrated, nearly all of which were shown to be due to different antigens of this organism (Crumpton & Davies, 1956). The majority of these lines were due to protein antigens, many of which because of their relatively sharp flocculation zones gave sharply defined lines. The lipopolysaccharide PR 1*a* precipitated over a wide range of serum dilutions (see below) and gave a rather poorly defined zone. This could not readily be detected underlying the pattern of lines produced when whole organisms were examined by this method.

In Fig. 1 a photograph shows an agar-diffusion plate in use as a test for immunological homogeneity according to the technique described by Crumpton & Davies (1956). The reservoir *A* contains acetone-dried cells (10 mg.) of *Past. pestis* (strain TS) grown at 37°; reservoir *B* contains a serum prepared by immunizing rabbits with living cells of this strain. Between these 2 reservoirs ten lines of precipitation appeared; some of these can be seen but others were not clear enough to appear on the photograph. The lipopolysaccharide PR 1*a* (3 mg.) contained in reservoir *C* gives only one line when reacting with the complex antiserum in reservoir *B*, which shows that the material contains only one of the specific substances (antigen or hapten) which this organism produces. It has been found that as little as 1 μg. of some *Past. pestis* antigens can be detected on diffusion plates under these conditions with the serum used in this instance, which demonstrates that less than 1 part in 3000 of such a contaminating material could be present in PR 1*a*.

A more exacting test is illustrated on the same photograph (Fig. 1). Two rabbits were injected intravenously with fraction PR 1: two injections were given each week for 5 weeks, 0·25 mg. being injected on each occasion. No antibodies of any kind could be demonstrated in these sera, showing that the lipopolysaccharide is a hapten and that the sample was free from detectable amounts of contaminating antigens. An antigen having the specificity of the polysaccharide was made in the following way. A sample of PR 1 (3 ml.; 1%, w/v, in water) which was viscous and cloudy was added to 1 ml. of a 1% (w/v) solution of the conjugated-protein component of the 'O' somatic antigenic

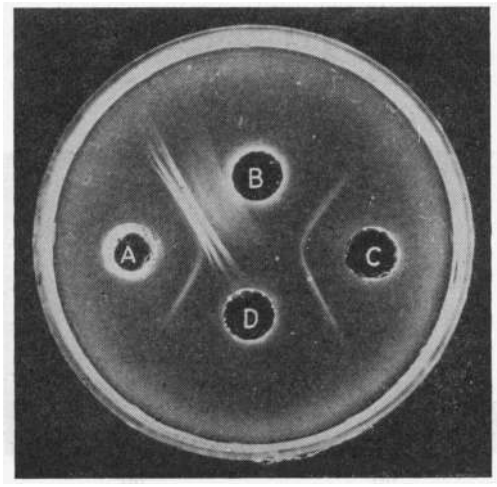

Fig. 1. Agar-diffusion precipitin pattern of *Past. pestis* cells and lipopolysaccharide. Reservoir *A*, cells of *Past. pestis* (TS) grown at 37° (10 mg.); reservoir *B*, a homologous antiserum (0·2 ml.); reservoir *C*, purified lipopolysaccharide (3 mg.); reservoir *D*, specific anti-polysaccharide serum (0·2 ml.).

complex of *Shigella dysenteriae* (Partridge & Morgan, 1940; Morgan, 1943; Davies & Morgan, 1953) dissolved in 0·05 N-NaOH. The mixture was allowed to stand at 20° for several hours, after which time it was no longer viscous, but had become a stable opalescent solution. Dilute HCl was added to pH 4 and the solution was centrifuged. A trace of insoluble residue which sedimented was removed and the supernatant solution was neutralized to pH 7. It will be noted that both of the reagents were acid-insoluble materials. The artificial complex was used to immunize three rabbits, 0·25 mg. being injected twice weekly. After four injections antibodies appeared in each, which precipitated with the lipopolysaccharide hapten; six further injections were given and one of the sera then obtained was placed in reservoir *D* (Fig. 1). This serum can be seen to give only one line when reacting with whole *Past. pestis* cells (reservoir *A*) and one line when reacting with lipopolysaccharide (reservoir *C*). The arc formed on the right of the plate demonstrates the specific identity of the antibody reacting from the complete and univalent sera.

It is clear from these tests that the lipopolysaccharide shows no evidence of immunological inhomogeneity, but the presence of serologically inactive contaminants cannot be detected by these tests.

Examination for physical homogeneity

Ultracentrifuge. Because of the poor solubility of the lipopolysaccharide in the presence of salt a sample of fraction PR 1*a* was examined in water, in the belief that like other lipopolysaccharides (Davies, Morgan & Record, 1955) the preparation would not be highly charged. At 0·8 % (w/v) concentration a sharp peak sedimented rapidly but was preceded by a small amount of material accumulating on the base of the cell. It seemed likely, in view of the method by which fractions PR 1*a* and PR 1*b* were separated, that the most rapidly sedimenting substance was itself composed of lipopolysaccharide which had not been truly in solution. At 0·2 % (w/v) concentration in water the material gave what appeared to be a true solution, and a photograph obtained in this run is shown in Fig. 2*a*. No evidence of inhomogeneity can be seen. The sedimentation coefficient has not been measured but the particle size is evidently very large and of a similar order to that of the 'O' somatic antigen of *Sh. dysenteriae* (Davies *et al.* 1954).

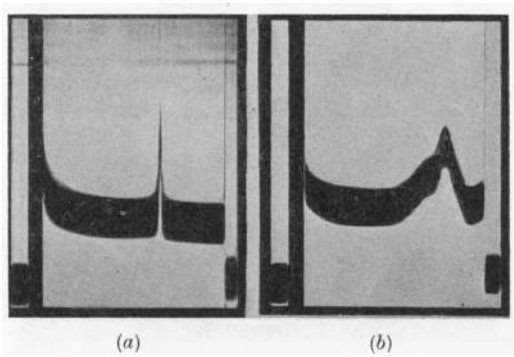

Fig. 2. Sedimentation patterns of *Past. pestis* polysaccharide preparations. Force, 260 000 *g*, migration from right to left. (*a*) Lipopolysaccharide PR 1*a*, 0·2 % (w/v) in water; exposure at 4 min. (*b*) *Past. pestis* lipopolysaccharide–'Shiga' conjugated-protein complex, 1 % (w/v) at pH 7·0 (phosphate–NaCl, $I = 0·2$); exposure at 12 min.

The soluble complex obtained by combining fraction PR 1 with the 'Shiga' conjugated protein contained 85 % of its weight of lipopolysaccharide and 15 % of the protein, these figures having been calculated from the N and P contents of the starting materials and product. This complex, dissolved in phosphate buffer at pH 7·0 ($I = 0·1$, and containing 0·1 M-NaCl) was examined in the ultracentrifuge at 1 % (w/v) concentration. Two major components were revealed and a third was present in small amount (Fig. 2*b*); none of these appeared to represent either of the reactants. A similar picture had previously been obtained with the undegraded polysaccharide and conjugated protein of *Sh. dysenteriae*, both of which had been shown to satisfy the usual criteria of physical homogeneity (Davies, unpublished observations). The meaning of these results is not yet understood.

Electrophoresis. Material PR 1 could not be examined by electrophoresis because of its insolubility in salt solution. A sample of *Past. pestis* lipopolysaccharide–'Shiga' conjugated-protein complex, which had been shown to be immunologically homogeneous but to have slight dispersion in the ultracentrifuge, was tested, however, at 0·8 % (w/v) concentration in phosphate–NaCl buffer, pH 7·0 ($I = 0·2$). The ascending and descending boundaries are shown in Fig. 3*a* and *b* respectively. Tracings from the photographic plate were necessary as the print reproduced inadequately the complete refractive-index-gradient curve because of opalescence. The absorption boundary was coincident with the main component boundary and examination of the ascending (anode) limb pattern did not reveal the presence of any faster-moving component. Similarly, the descending (cathode) limb pattern did not reveal the presence of any slower-moving component. Examination of the boundary shows an asymmetry which suggests that more than one component may have been present. A small disturbance on the leading edge of the ascending boundary is almost certainly due to a rather poor initial boundary.

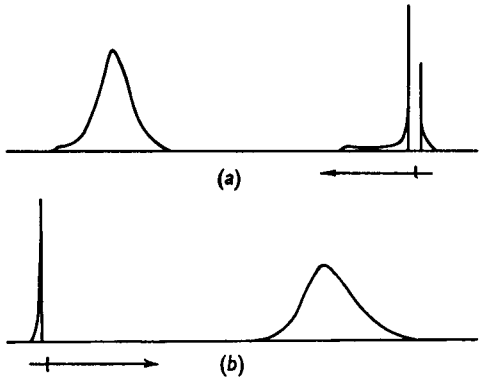

Fig. 3. Electrophoresis diagrams of *Past. pestis* lipopolysaccharide–'Shiga' conjugated-protein complex, 0·8% (w/v) at pH 7·0 (phosphate–NaCl, $I = 0{\cdot}2$); exposure at 10 hr.; potential gradient 1·83 v/cm.$^{-1}$. (*a*) Anode (ascending) limb; (*b*) cathode (descending) limb. Direction of migration shown by arrows; position of initial boundary shown by intercept on arrows.

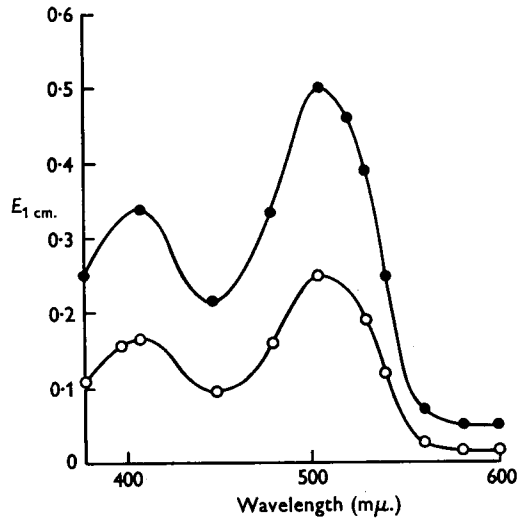

Fig. 4. Absorption spectra of *Past. pestis* polysaccharide fractions (250 μg.) after treatment with H_2SO_4–cysteine: readings at 22 hr. ○, Lipopolysaccharide; ●, degraded polysaccharide. The absorption maximum at 505 mμ. is due to aldoheptose sugar.

Chemical composition

Analytical figures obtained with fraction PR 1 were C, 45; H, 7·6; N, 1·6; total P, 2·2; S, nil; acetyl, nil. The material PR 1 *a* had $[\alpha]_D^{20} = +48° \pm 2°$ in water (*c*, 0·5%, w/v) and the viscosity of a 1% (w/v) aqueous solution at 20° was 4·0 relative to water at the same temperature. The reducing value (Somogyi), calculated as glucose, on hydrolysing with 0·5N-HCl rose to a maximum of 30% in 6 hr. but reached 26% (87% of the final figure) in 2 hr. Hexosamine was estimated to compose 15% of the material; the absorption spectrum of the pink chromogen in the Elson–Morgan reaction showed a maximum at 535 mμ. with a slight asymmetry at 550 mμ. which agreed with that found with an authentic sample of glucosamine.

Component sugars. When examined by paper chromatography, hydrolysates of fraction PR 1 gave two spots with butanol–pyridine–water (3:2:1·5) as solvent. One of these gave positive reactions with anisidine, ninhydrin and Ehrlich's reagent and corresponded in position and colour with glucosamine; it differed clearly from a standard galactosamine spot. The second spot ran in the position of glucose both in this solvent system and in phenol. The colour of this spot when sprayed with anisidine was not, however, typical of that given by glucose. A sample of lipopolysaccharide (250 μg.) was therefore examined by the H_2SO_4–cysteine reaction and a strong absorption was found with a maximum at 505 mμ. which is characteristic of aldoheptose sugars (Fig. 4).

A number of synthetic heptose sugars were examined on paper chromatograms, but none of them corresponded in position to that of the unknown sugar in all of the solvent systems tried. The heptoses from which the unknown was thus shown to differ were: D-*glycero*-L-*galacto*-, D-*glycero*-D-*galacto*-, D-*glycero*-L-*gluco*-, D-*glycero*-D-*allo*-, D-*glycero*-D-*gulo*-, D-*glycero*-L-*manno*-, D-*glycero*-D-*ido*-, and D-*glycero*-L-*talo*-heptoses. The unidentified aldoheptose which would appear to be one not previously found in nature will be described in a later paper.

The absorption curve illustrated in Fig. 4 also shows a maximum at 415 mμ. which is not given by either pure aldoheptose sugars or hexosamines but is characteristic of hexoses. This is thought to be due to glucose, since no other hexose could be detected on paper chromatograms and this sugar would have been obscured by the larger amount of heptose present and occupying the same position. The amount of glucose which would account for this absorption is approx. 12% of the weight of the material.

Degradation of the lipopolysaccharide. A sample of PR 1 (1·6 g.) was heated for 4 hr. in 1% (v/v) acetic acid at 100° in an atmosphere of nitrogen gas (Morgan & Partridge, 1940). A brown flocculent precipitate was recovered by centrifuging, and was washed once with dilute acetic acid and once with water, and the washings were added to the original acid-soluble fraction. The insoluble fraction was freeze-dried and weighed, 735 mg. of it representing 46% of the weight of the starting material. A

small part of this dissolved in ether. The ether-insoluble part was extracted with chloroform and the chloroform solution was centrifuged to remove some brown sediment; from the supernatant 600 mg. of material was recovered, which contained N, 1·7 % and P, 2·2 %.

The acid-soluble fraction was centrifuged at 15 000 *g* for 30 min. and a small amount of insoluble material was removed. The supernatant solution was concentrated under reduced pressure at 16° to about 50 ml. and poured into 10 vol. of cold ethanol. The precipitated fraction was redissolved in water and precipitated by the addition of 6 vol. of cold ethanol, redissolved, extracted with chloroform, centrifuged at 20 000 *g* for 1 hr. and the supernatant solution freeze-dried. The degraded polysaccharide thus obtained weighed 595 mg. and contained N, 1 %, and P (organic), 0·3 %. When a sample of this polysaccharide was tested by the H_2SO_4–cysteine reaction the absorption at 505 mμ. indicated that the heptose content of the degraded material was twice that of the lipopolysaccharide from which it was derived (Fig. 4). The actual amount of heptose present could not be determined since different aldoheptose sugars give different amounts of absorption in the test; it appeared, however, that heptose accounted for more than half of the weight of the degraded polysaccharide.

The degraded polysaccharide failed to be precipitated when tested with sera known to contain antibodies corresponding to the lipopolysaccharide, and examination of a sample in the analytical ultracentrifuge showed the material to be of such low molecular weight that it failed to resolve as a peak from the meniscus on running for 2 hr. at 260 000 *g*. In order to determine whether failure to give specific precipitation was due to the low molecular weight or to a loss of some essential part of the molecule during hydrolysis, a further sample was hydrolysed for the minimum time necessary to separate the phospholipid and polysaccharide moieties. This sample was heated in 0·01 N-HCl for 30 min. and the products were recovered as previously described. The degraded polysaccharide had the same N and P contents as the original sample and had $[\alpha]_D^{20} = +58 \pm 2°$ in water (*c*, 1 %, w/v). Reduction (Somogyi) measured on samples of the polysaccharide without further hydrolysis gave a figure of 1·64 % calculated as glucose, from which a maximum molecular weight of the order of 11 000 can be deduced. If the reducing end-groups of the polysaccharide chains were glucosamine residues the molecular weight would be close to this figure; if heptose residues comprised the reducing end-groups the figure would be somewhat higher, since the heptoses which have been tested all give a lower reducing value than glucose under the conditions used.

This second sample of degraded polysaccharide failed to precipitate with a specific polysaccharide antiserum but its specific determinant groups had not been destroyed (see below).

Lipopolysaccharide in different Pasteurella pestis *strains*

Two different rough variants of *Past. pestis* strain TS were described by Schütze (1939) which he called TSR and TRU; these gave large and small rough colonies respectively. The antigenic differences between these two strains and between these and the smooth parent strain (TS) are described by Crumpton & Davies (1956). The nature of these differences suggested that neither of the rough forms was analogous to the rough forms found among species of the Enterobacterioaceae. In order to obtain more information on this point batches of acetone-dried cells of strains TSR and TRU were extracted with saline and subsequently with phenol as described above, and in each case a lipopolysaccharide was obtained which could not be distinguished from the product of the smooth form.

Biological properties

Antigenicity. It has already been stated that when ten injections of purified lipopolysaccharide, each of 250 μg., were given to rabbits no antibodies (agglutinins or precipitins) could be detected in the sera obtained; thus it is clear that the lipopolysaccharide is a hapten. When combined with 'Shiga' conjugated protein, however, polysaccharide specific antibodies could readily be obtained. When such antisera were tested against conjugated protein alone, either on diffusion plates or by the tube precipitation method with a series of dilutions of the conjugated protein, no reaction could be detected.

Serological reactions. Specific anti-polysaccharide sera failed to agglutinate *Past. pestis* cells of strain TS grown at 20° or 37°, neither did they agglutinate cells of *Past. pseudotuberculosis*.

The lipopolysaccharide precipitated at a dilution of 1 in 5 million when tested against sera prepared by immunizing rabbits with the artificial antigenic complex, with *Past. pestis* cells of strain TS grown at 20° or with cells of strain TRU grown at 37°. A weaker reaction (titre 1 in 200 000) was obtained, using sera prepared by immunizing with TS cells grown at 37°. No reaction occurred with an anti-*Past. pseudotuberculosis* serum, nor did a specific polysaccharide from a strain of this species (to be described in a later communication) precipitate with any *Past. pestis* antisera.

The degraded polysaccharide obtained by mild acid hydrolysis of the lipopolysaccharide was not precipitated when tested against the specific anti-

polysaccharide sera. A test to determine whether the specific determinant groups had been destroyed during the hydrolysis was therefore carried out. Degraded polysaccharide (5 mg.) was dissolved in 2 ml. of specific anti-polysaccharide serum and allowed to stand at 20° for 2 hr.; this mixture and a sample of the original serum were tested against dilutions of lipopolysaccharide, the tubes being kept at 20° for 24 hr. and at 0° thereafter for 2 days. The lipopolysaccharide precipitated at a dilution of 1 in 5 million with the original serum, but complete inhibition was found in the serum to which degraded polysaccharide had been added, there being no precipitate in any tube over the range tested (1 in 2000 to 1 in 20 million).

Serum samples from four rabbits taken after each of six injections of the antigenic complex were tested for the presence of Forssman heterophile antibodies (sheep-cell haemolysins). In none of these serum samples could Forssman antibodies be detected.

Toxicity. Unlike the lipopolysaccharides obtained from species of the Enterobacteriaceae which have a toxicity in mice of the order of $LD_{50} = 100\,\mu g.$, and which are generally even more toxic for rabbits, the *Past. pestis* product killed mice only when 5–10 mg. was injected. The LD_{50} value in guinea pigs was approx. 2 mg. and in the rabbit approx. 5 mg.

Pyrogenicity. The lipopolysaccharide, although relatively non-toxic, was, like the lipopolysaccharides and 'O' somatic antigens of the Enterobacteriaceae (Westphal, Lüderitz, Eichenberger & Keiderling, 1952*b*), strongly pyrogenic. The body temperature of control rabbits observed during these tests varied only between the limits of 39° and 39·5°. As little as 7 μmg./kg. of rabbit wt. injected intravenously gave a marked response, the temperature rising to 40·6° in 2 hr. Larger doses only resulted in pyrexia being maintained for longer periods. Some typical results are shown in Fig. 5. When 500 μg./kg. of rabbit wt. was administered the body temperature remained over 40·5° for over 24 hr., fell to 40° during the second 24 hr. and returned gradually to normal during the third day. The injection of a lethal dose (10 mg.) resulted in a sustained high temperature for 24 hr. and a steady fall over the following 12 hr. to the subnormal value of 36·5° when the animal died.

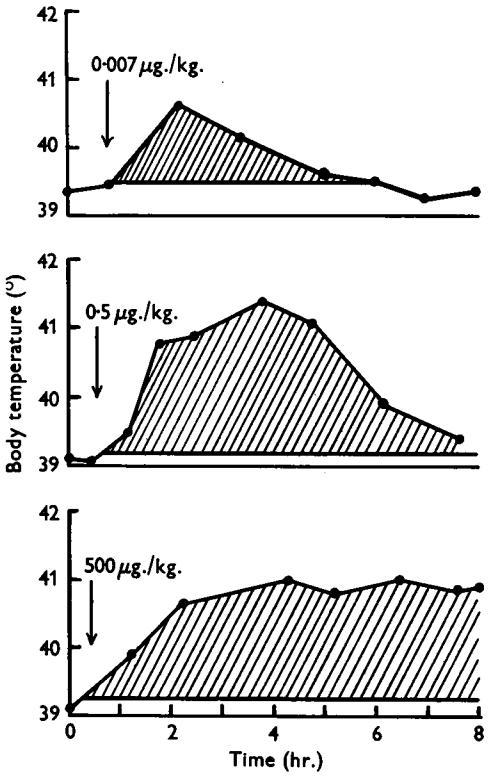

Fig. 5. Effect of intravenous injections of *Past. pestis* lipopolysaccharide (PR1) on the body temperature of rabbits.

Protection tests. No protection against plague infection was afforded by immunization of either mice or guinea pigs with *Past. pestis* lipopolysaccharide–'Shiga' conjugated-protein antigen, neither was any passive protection imparted to these animals by injecting rabbit anti-*Past. pestis* polysaccharide sera of high titre.

DISCUSSION

Possibly the reason why no *Past. pestis* specific polysaccharide has previously been purified and characterized is that this organism surrounds itself with unusually large amounts of protein. Saline extraction removes nearly 30 % of the bacterial dry weight without lysing the cells, and from the analytical figures for these extracts and the finding that they contain no significant amount of nucleic acid it is clear that they are largely protein in nature. This protein, much of which is related to the envelope, is present not only in the smooth strains but also in some of the rough forms (strains of the TSR type). It is now clear why some investigators have failed to extract anything with the protein precipitant trichloroacetic acid. The phenol method of extraction (Westphal *et al.* 1952*a*) is valuable under these conditions because protein passes into solution in the phenol phase. It is still advisable, however, to remove the bulk of the protein by saline extraction to facilitate the separation of phenol and water phases, although some polysaccharide may be lost in the saline extracts.

The poor solubility of the material extracted is due to a very high lipid content. Because of this and for purposes of antibody production it is an advantage to extract this type of material with some protein attached, as has been done with many Gram-negative species. In *Past. pestis* this has not been achieved. The primary extraction product in the phenol-saturated water phase is soluble, but after precipitation with organic solvents and more especially after freeze-drying this solubility is lost. This lack of solubility makes examination for physical homogeneity extremely difficult, though a fraction has been obtained which, when examined in the analytical ultracentrifuge in salt-free water, showed no evidence of inhomogeneity. A soluble antigen could readily be obtained by combining the lipopolysaccharide with 'Shiga' conjugated protein, but although the artificial antigen was shown to be immunologically homogeneous, the physical examination revealed a complex pattern which could not be interpreted. It could be deduced from the known properties of the reactants that neither of them was responsible for any of the three components shown by ultracentrifugal analysis to be present in the antigenic complex.

The lipopolysaccharide was free from protein and nucleic acid so far as could be shown by chemical methods. Degraded polysaccharide, which itself showed no evidence of inhomogeneity, and chloroform-soluble phospholipid accounted for nearly all of the weight of the material. Acid hydrolysis results in a degraded polysaccharide of rather low molecular weight which probably accounts for the failure of the degraded material to give specific precipitation; its ability to inhibit precipitation of the lipopolysaccharide shows that its specific determinant structure has not been lost.

The aldoheptose sugar which appears to account for most of the weight of the degraded polysaccharide is an unusual feature. However, since simple methods have been developed by which heptose sugars can be recognized, several heptoses have been detected as components of bacterial polysaccharides (Jesiatis & Goebel, 1952; Slein & Schnell, 1953; Weidel, Koch & Bobosch, 1954; Davies, 1955).

With the agar-diffusion precipitin technique of Ouchterlony (1948, 1953) the line of precipitation due to the specific *Past. pestis* polysaccharide is difficult to detect underlying the complex line pattern produced when most strains are tested against *Past. pestis* antisera. It can be detected readily, however, when certain strains (e.g. TRU) which contain fewer antigens are tested (Crumpton & Davies, 1956). This technique is valuable as a test for immunological homogeneity and also as a method for following the purification of antigens, more especially when these are proteins.

It is known (Davies *et al.* 1954) that as little as 1 μg. of a somatic 'O' antigen (protein–polysaccharide–phospholipid complex) is sufficient to induce an antibody response in rabbits. From the amount of *Past. pestis* lipopolysaccharide which was injected into rabbits ($10 \times 250\,\mu$g.) without inducing any response it is clear that the material is not antigenic. Anti-polysaccharide sera could readily be produced by using the artificial complex with 'Shiga' conjugated protein.

Specific anti-polysaccharide sera failed to agglutinate cells of any smooth *Past. pestis* strains, no doubt because of the protein present on the cell surface. Rough strains could not be tested because of their instability when suspended in normal salt solution. When the lipopolysaccharide was tested by precipitation with various *Past. pestis* antisera, those sera prepared by immunization with cells grown at 20° gave a higher titre than when cells grown at 37° had been used. This can be accounted for by the fact that the envelope of *Past. pestis* is not formed in significant amounts when the cells are grown at 20°, thus making the underlying polysaccharide more readily available to the rabbit antibody-forming system. When tested with anti-TRU (grown at 37°) sera, however, a high precipitin titre was found because this strain possesses no envelope even when grown at 37°.

Since the specific polysaccharide could be extracted from rough and smooth strains, it can be analogous to neither the smooth 'O' somatic antigens which have been extracted from species of the Enterobacteriaceae nor the lipopolysaccharides derived from these 'O' antigens. This agrees with the deduction of Schütze (1932) that *Past. pestis* is basically a rough organism, although many strains are smooth in appearance.

The lipopolysaccharide is unusual in being relatively non-toxic, but a non-toxic 'antigène glucido lipidique' was obtained from *Past. tularensis* by Girard & Gallut (1951). A separation of toxic (killing) and pyrogenic activities has not previously been observed with this type of material. Westphal, Lüderitz, Eichenberger & Keiderling (1952*b*) who used a lipopolysaccharide pyrogen obtained from *Escherichia coli* found a sharp drop in body temperature on the injection of 10 μg. or more of their material into rabbits; this drop was not preceded by a temperature rise. At lower doses a powerful pyrogenic action was produced. The temperature fall found on approaching the lethal dose (approx. 20 μg.) appeared to be closely associated with the cause of death. The *Past. pestis* lipopolysaccharide could be injected in amounts up to 5 mg. but no temperature fall occurred. When a lethal dose (10 mg.) was injected the body temperature rose, but gradually fell thereafter as the animal died; this fall may have been an effect

of approaching death rather than a cause of it. In view of this the material is clearly not analogous to the endotoxins.

The polysaccharide preparation obtained by Seal (1951) would appear to differ from the one now described because it could be extracted only from protective strains (those which produced envelope material) and appeared to contain arabinose. Strain TRU from which lipopolysaccharide has now been obtained is non-protective and arabinose has not been found.

The polysaccharide detected by Chen & Meyer (1954) in their extracts was precipitated with sera prepared by immunization of rabbits with strain TRU. This observation suggests that theirs may have been the same polysaccharide, although strain TRU induces antibodies against at least four other antigens (Crumpton & Davies, 1956).

The polysaccharide does not appear to play any part in the protection of mice or guinea pigs against plague infection.

SUMMARY

1. Acetone-dried cells of *Pasteurella pestis* have been extracted with aqueous phenol (45%, w/v), after removal of proteins by saline extraction, and a specific lipopolysaccharide has been obtained. This was purified by ethanol fractionation and ultracentrifuging.

2. The purified material is poorly soluble in water and insoluble in salt solutions. Analysis gave 1·6% N, 2·2% P; $[\alpha]_D^{20}$ was +48°; relative viscosity at 1% (w/v) in water at 20° was 4·0.

3. Mild acid hydrolysis released in about equal amounts a chloroform-soluble phospholipid and a degraded polysaccharide having 1% N, 1% P, $[\alpha]_D^{20}$ +58°.

4. The lipopolysaccharide is of very large particle size and sediments as one component in the analytical ultracentrifuge. The degraded polysaccharide has quite a low molecular weight, probably of the order of 10 000–15 000.

5. The lipopolysaccharide contains glucose, glucosamine and an unidentified aldoheptose sugar which composes the greater part of the polysaccharide moiety.

6. Immunological homogeneity was demonstrated by the agar-diffusion-precipitin technique.

7. The lipopolysaccharide is a hapten. It can be made antigenic by combination with the conjugated-protein component of the somatic antigen of *Shigella dysenteriae*. The degraded polysaccharide does not precipitate with specific polysaccharide antisera but inhibits the precipitation of the lipopolysaccharide with such sera.

8. The material can be extracted from rough and smooth strains, it is non-protective, relatively non-toxic, but strongly pyrogenic.

I wish to thank Professor N. K. Richtmyer for generous gifts of aldoheptose sugars. Ultracentrifugal and electrophoretic analyses were kindly carried out by Dr B. R. Record and Mr K. A. Cammack. I am much indebted to Dr B. C. Whaler for examining the pyrogenic action of materials in rabbits. The technical assistance of Mr V. S. G. Baugh is gratefully acknowledged.

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Thetin–Homocysteine Transmethylase

A PRELIMINARY MANOMETRIC STUDY OF THE ENZYME FROM RAT LIVER

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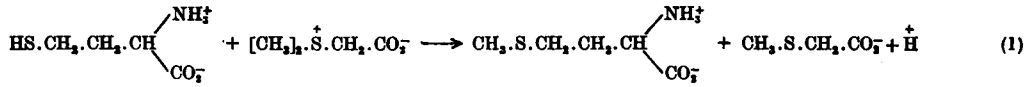
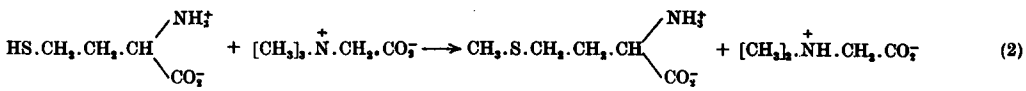
Transmethylation reactions involving the formation of methionine from homocysteine have been shown to take place in the presence of slices and suspensions of liver and kidney from the rat, pig and guinea pig (Borsook & Dubnoff, 1947; Dubnoff & Borsook, 1948). These tissues contain two distinct enzyme systems capable of catalysing the transfer of methyl groups to homocysteine, one requiring dimethylthetin or dimethyl-β-propiothetin, and the other requiring glycine betaine, as the specific methyl donor. Dubnoff & Borsook (1948) referred to these enzymes as dimethylthetin transmethylase and betaine transmethylase respectively, and showed that they could be distinguished from one another by their different stabilities in acid solution. They were both found to possess a pH optimum in the region of 7·8 and to function in anaerobic conditions. The thetin transmethylase was able to bring about transfer of one methyl group only, per molecule of dimethylthetin. The action of both enzymes was followed at pH 7·4 by colorimetric estimation of the methionine formed. More recently, Ericson, Williams & Elvehjem (1955) have made a further study of the betaine transmethylase.

If the overall chemical changes involved in the reactions catalysed by these two enzymes are examined, it is evident that, in both cases, the methylation of the sulphydryl group of homocysteine under physiological conditions must result in the liberation of a proton, since according to the data of Ryklan & Schmidt (1944) the magnitude of the pK attributable to the sulphydryl group is such that the ionization of the group is small:



(1)



(2)

In the reaction involving dimethylthetin (reaction 1) there are no other changes in the number of acid or basic groups initially present. The net result is the production of acid, and if the reaction be carried out in a bicarbonate medium it should be possible to follow it manometrically by the amount of CO_2 evolved. Smythe (1936) has studied the non-enzymic alkylation by iodoacetate of cysteine and other thiols in a bicarbonate buffer by such a procedure.