after 9 days and the purified orsellinic acid, penicillic acid and dried mycelium were assayed.

(ii) *With orsellinic acid derived from sodium* [$1-^{14}\text{C}$]-acetate. The orsellinic acid used was a sample prepared by addition of sodium [$1-^{14}\text{C}$]acetate (200 μC) to cultures of *P. madriti* and was thus labelled in the 1, 3, 5 and 7 positions. After 4 days' growth of the mould, the labelled orsellinic acid (58 mg.; 7.95 μC), dissolved in ethanol (1 ml.) plus water (9 ml.), was added to one flask and on the ninth day the products were extracted and assayed for activity. The results (Table 5) show no activity of the penicillic acid in (i), but considerable activity in (ii).

SUMMARY

1. *Penicillium madriti* G. Smith strain BB389A, when grown on Czapek-Dox glucose medium, produces orsellinic acid and penicillic acid.

2. When sodium [$1-^{14}\text{C}$]acetate was added to the culture medium the orsellinic acid produced was shown by degradation to be labelled equally on four of the eight carbon atoms, consistent with its production from four acetate units linked head-to-tail.

3. When sodium [$1-^{14}\text{C}$]butyrate was used as precursor, the resulting orsellinic acid was labelled. The carboxyl group had one-quarter of the total activity. This indicates breakdown of the butyrate to two C_2 units before incorporation and precludes utilization of the intact C_4 chain.

4. When labelled orsellinic acid was presented to the mould, the labelling of the penicillic acid was consistent with the direct conversion of orsellinic acid into penicillic acid with elimination of the carboxyl carbon.

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Distribution of Metabolic Activity, Phospholipid and Hyaluronidase between the Heads and Tails of Bull Spermatozoa

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Early investigations on the distribution of chemical components and metabolic activities between the heads and tails of spermatozoa were based on materials obtained by the mechanical disruption of spermatozoa by such means as ultrasonic disintegration, shaking with fine glass beads or grinding the frozen cells in a mortar (Henle, Henle & Chambers, 1938; Zittle & O'Dell, 1941*a, b*; Mann, 1949, 1951; Nelson, 1954). Procedures of this type are of limited value since motility is invariably abolished and some fragmentation of the sperm tails always occurs. However, a new approach to the problem was made possible by the

acquisition of an infertile bull of the Guernsey breed, the semen of which contains spermatozoa which are all in a 'decapitated' state, namely as free sperm heads and detached, yet perfectly motile, midpiece-tails. The production of decapitated spermatozoa is a congenital defect and is not of pathological origin. Using this semen Mann (1958*a, b*) separated the two cell components by differential centrifuging. He showed that rates of fructolysis and respiration were much higher in the midpiece-tails than in the heads, and that, though the midpiece-tails contained a high concentration of acid-soluble phosphorus compounds and phospholipid, nucleic acid was confined entirely to the tails.

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In the present paper we report on density-gradient centrifuging as a means of separating components of the decapitated spermatozoa. Using this method we have studied the distribution of phospholipid, plasmalogen and hyaluronidase, as well as respiration and fructolysis, in sperm fractions. In addition, we have investigated the release of hyaluronidase from normal bull spermatozoa into the seminal plasma.

MATERIALS AND METHODS

Abbreviation. The term 'tail' will be used to denote the combined midpiece-tail of the decapitated spermatozoon.

Semen and sperm fractions. A Guernsey bull at the Animal Research Station provided semen containing only decapitated spermatozoa. Normal semen was obtained from Friesian bulls at the Station. Semen was collected in an artificial vagina (Walton, 1945) and allowed to cool slowly to 20°. In ejaculates from the Guernsey bull the ratio heads:tails was always greater than 1 and had a mean value of 1.13 ± 0.029 (S.E.M. of 22 samples). Whole spermatozoa were separated from seminal plasma and washed with calcium-free 'Ringer solution for spermatozoa' as described by Mann (1946). To obtain mixtures of heads and tails the semen from the Guernsey bull was treated in the same way.

Separation of heads and tails. Solutions of polyvinylpyrrolidone (May and Baker Ltd.) in aqueous sucrose were used to prepare differential-density columns (Thomson & Klipfel, 1958). Centrifuge tubes (1 cm. diam. $\times$ 8 cm.) received a measured volume of 10% (w/w) polyvinylpyrrolidone in 0.25 M-sucrose over which was layered a measured volume of 2% (w/w) polyvinylpyrrolidone in 0.25 M-sucrose.

Fig. 1. Fractionation of sperm heads and tails by density-gradient centrifuging. The supernatant fraction contained motile tails; the sediments contained heads and immotile tails. 1, Before centrifuging; 2, after centrifuging. A, Semen; B, 2% polyvinylpyrrolidone in 0.25 M-sucrose; C, 10% polyvinylpyrrolidone in 0.25 M-sucrose; D, supernatant fluid; E, sediment.

Semen from the Guernsey bull (1–2 ml.) was added to form an uppermost layer. The total volume in the tube did not exceed 5 ml. The tubes were centrifuged for 30 min. at 70g and 20°. The conical head of the Servall SS1 centrifuge and the horizontal head of the Wifug X2 centrifuge gave similar degrees of separation. Fig. 1 illustrates the separation obtained with the latter machine. The cloudy part of the supernatant fluid, which contained only motile tails, was centrifuged at 1000g and the residue was washed twice in the centrifuge with Ringer solution.

Two sediments which appeared in the lower portion of the tubes (Fig. 1) each consisted of heads and immotile tails. For some respiration experiments the two sediments were combined and washed twice with Ringer solution. Otherwise the metabolic activity, or lipid content, of sperm heads was calculated from measurements on motile sperm tails and on the head-plus-tail mixture obtained from unfractionated semen. Thus if A is the oxygen uptake (or other parameter) of a mixture of H_A heads and T_A tails, and B the oxygen uptake of T_B tails, the oxygen uptake of x heads is given by $x[A - (BT_A/T_B)]/H_A$.

Respiration and fructolysis. Respiration was measured in Barcroft differential manometers at 37°. The manometer flasks received washed cells or cell fractions suspended in 3 ml. of Ringer solution and CO_2 was absorbed in the usual way in 0.3 ml. of 2.5 N-NaOH. Fructolysis was followed by adding 0.2 ml. of 0.25 M-phosphate buffer, pH 7.4 (3.55 g. of Na_2HPO_4 dissolved in 100 ml. of 0.05 N-HCl), to 0.4 ml. of either semen or a suspension of sperm tails in Ringer solution containing fructose. The mixtures were incubated anaerobically at 37°. At zero time and at hourly intervals, 0.1 ml. portions were withdrawn and diluted to 2 ml. with water. These solutions were deproteinized, and the fructose was estimated, according to Mann (1948). Activities of respiration and fructolysis are expressed on the basis of 10^9 heads, tails or spermatozoa.

Hyaluronidase. The enzyme was determined turbidimetrically as described by Tolksdorf (1954) except that the incubation time was reduced from 30 to 15 min. Turbidities were read on an EEL colorimeter with a blue filter. Results are expressed as turbidity-reducing units in 1 ml. of semen or in the material (heads, tails) obtainable from 1 ml. of semen. The hyaluronidase content of normal spermatozoa was calculated from measurements on whole semen and seminal plasma. Hyaluronic acid for these determinations was prepared from the vitreous humour of bovine eyes ['preparation A' of Meyer & Palmer (1934, 1936)]. From 88 eyes 215 mg. of hyaluronic acid was obtained.

Lipids. Lipids were extracted with chloroform-methanol (2:1, v/v). The extracts were washed with 3 mM-MgCl₂ soln., evaporated, and the residues dissolved in chloroform as described by Hartree & Mann (1961). Phosphorus was determined according to King (1932) and plasmalogen according to Hartree & Mann (1959). Results are expressed as $\mu\text{g. atoms}$ of lipid phosphorus, or of plasmalogen phosphorus, in 10^9 heads, tails or spermatozoa.

RESULTS

Efficiency of separation of heads and tails

The composition of the supernatant fractions from the semen of the Guernsey bull, obtained by the density-gradient method, are shown in Table 1.

Table 1. Recovery of heads and motile tails in the supernatant fluid after density-gradient centrifuging of semen containing decapitated spermatozoa

Semen was layered above solutions of polyvinylpyrrolidone (2% and 10%, w/w) in 0.25M-sucrose, as shown in Fig. 1, and centrifuged at 70g for 30 min.

2% Polyvinyl- pyrrolidone (ml.)	10% Polyvinyl- pyrrolidone (ml.)	Semen (ml.)	Supernatant removed (ml.)	Recovery in supernatant (%)	
				Heads	Tails
2.0	1.5	1.0	2.5	3	40
1.5	1.5	2.0	2.7	3	56
1.5	1.5	1.0	2.0	2	36
1.5	1.5	1.5	2.4	0	34

Table 2. Respiratory activity of normal bull spermatozoa, sperm heads and sperm tails as suspensions in Ringer solution at 37°

Activities are given as $\mu\text{l.}$ of O_2 uptake in 1 hr. by 10^9 spermatozoa, heads or tails, except in the second line where activities are those of 10^9 heads plus the accompanying tails (approx. 0.86×10^9) in the semen of the Guernsey bull. F, Friesian bull; G, Guernsey bull. The sediment contained some immotile tails.

	O_2 uptake		
	Mean	Range	No. of determinations
Normal spermatozoa (F)	142	131-156	3
Heads + tails (G)	157	126-188	5
Tails (G)	128	100-155	5
Heads (calculated) (G)	47	31-63	5
Heads (sediment) (G)	42	36-47	3

The conditions selected for experiments described in this paper are given in the last line of the Table. Under such conditions the supernatant fraction contained 30-40% of the total sperm tails, all of which were motile, but no sperm heads. A slightly simpler procedure in which 1.5 ml. of semen was layered above 3.0 ml. of 2% polyvinylpyrrolidone gave the same recovery of pure tails in the upper layer.

Under the other conditions in Table 1, and also at lower temperatures, the degree of separation was generally less reproducible.

Metabolic activity of heads and tails

Respiration. A comparison of the respiratory activities of tails and heads indicated that the former account for about 75% of the oxygen consumed by bull spermatozoa (Table 2). Measurements with mixtures of heads and immotile tails obtained from the pooled sediments (see Fig. 1) yielded activities similar to those calculated for an equal number of heads. Thus the respiratory activity of the immotile tails was negligible. The oxygen uptake of both heads and tails proceeded almost linearly for 3 hr.

Fructolysis. In Fig. 2 are given the results of an experiment in which three incubation mixtures were set up as follows: (1) 0.2 ml. of phosphate buffer plus 0.4 ml. of whole normal semen from a Friesian bull (5.6×10^8 cells); (2) 0.2 ml. of buffer plus 0.4 ml. of whole semen of the Guernsey bull

Fig. 2. Anaerobic fructolysis at 37° of normal spermatozoa, sperm tails and sperm heads. The curves are derived from three experiments involving measurements of fructolysis in the following mixtures: (1) 2 vol. of normal semen plus 1 vol. of phosphate buffer, pH 7.4; (2) 2 vol. of 'decapitated' semen plus 1 vol. of buffer; (3) 2 vol. of suspension of tails in Ringer solution plus 1 vol. of buffer. ●, Normal semen (Expt. 1); ■, tails (Expt. 3); ▲, activity of heads calculated from Expt. 2 (heads plus tails) and Expt. 3.

(1.88×10^8 heads and 1.80×10^8 tails); (3) 0.2 ml. of buffer plus 0.4 ml. of tails in Ringer solution (1.00×10^8 tails). The concentrations of seminal

Table 3. *Distribution of lipid phosphorus and plasmalogen phosphorus in normal spermatozoa, sperm heads and sperm tails*

Results are expressed as $\mu\text{g. atoms of phosphorus}/10^9$ spermatozoa, heads or tails. In the semen from the Guernsey bull the tail count was lower than the head count (see Table 2). F, Friesian bull; G, Guernsey bull.

	Sperm count (millions/ $\mu\text{l.}$)	Head count (millions/ $\mu\text{l.}$)	Lipid phosphorus		Plasmalogen phosphorus	
			Heads	Tails	Heads	Tails
F	1.00	—	1.18		0.94	
F	1.45	—	0.98		0.74	
G	—	0.27	0.60	1.37	0.31	0.68
G	—	0.40	0.56	1.60	0.25	0.76
G	—	0.50	—	—	0.21	0.73
G	—	0.54	0.58	1.15	0.37	0.73

Table 4. *Distribution of hyaluronidase in five samples of semen from the Guernsey bull, containing decapitated spermatozoa*

Determinations were made 30 min. after ejaculation. Hyaluronidase activity is expressed as turbidity-reducing units/ml. of semen or of components present in 1 ml. of semen.

Head count (millions/ $\mu\text{l.}$)	Hyaluronidase activity			
	Semen	Heads	Tails	Plasma (by difference)
0.35	720	90	20	610
0.62	850	210	50	590
0.66	870	200	70	600
0.89	830	250	40	540
0.91	990	490		500

Table 5. *Distribution of hyaluronidase in normal semen from seven Friesian bulls (A-G)*

Determinations were made 30 min. after collection. Hyaluronidase activity is expressed as turbidity-reducing units/ml. of semen.

Bull	Sperm count (millions/ $\mu\text{l.}$)	Hyaluronidase activity	
		Semen	Spermatozoa
A	0.34	650	—
	0.34	500	380
B	0.55	860	560
	0.68	1100	860
C	0.93	860	570
	1.33	1060	920
D	1.50	700	440
E	1.64	950	720
F	1.89	1000	770
G	2.07	950	760

Phospholipids in heads and tails

Phospholipid analyses were carried out on four semen samples from the sterile Guernsey bull and one from each of two fertile Friesian bulls (Table 3). In the semen from the Guernsey bull the concentration of cellular plasmalogen, as well as the distribution of plasmalogen between heads and tails, showed only minor variations. The corresponding phospholipid concentrations were more variable. About 70 % of the plasmalogen and of total phospholipid was present in the tails. Though the plasmalogen content of the normal spermatozoa was similar to that of the decapitated cells in the defective semen, the total phospholipid was significantly lower.

Distribution of hyaluronidase in bull semen

fructose in Expts. 1 and 2 were 34.5 and 19.5 mm respectively; 20 mm-fructose was added to Expt. 3. Utilization of fructose was measured over a period of 3 hr. and the fructolytic activity of sperm heads was calculated from Expts. 2 and 3. The activities of the normal and abnormal semens were approximately equal (Fig. 2). In the abnormal semen most of the fructolysis took place in the tails.

Experiments with semen of the Guernsey bull indicated that sperm heads contain far more hyaluronidase than sperm tails. However, the significance of this finding is uncertain in view of the release of hyaluronidase from the spermatozoa into the seminal plasma that occurs when bull semen is left standing at room temperature. It was not possible to reduce below 30 min. the interval between semen collection and determination of

Table 6. *Changes in hyaluronidase activity of spermatozoa during storage at 20° of normal semen from four Friesian bulls and of semen containing decapitated spermatozoa from a Guernsey bull*

Hyaluronidase activity is expressed as turbidity-reducing units/ml. of semen or of components present in 1 ml. of semen.

Bull	Sperm (or head) count (millions/ μ l.)	Fresh whole semen	Hyaluronidase activity					
			Cells separated from semen after:					
			0.5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.
Friesian								
A	0.34	500	—	320	150	90	—	—
B	0.55	860	560	—	170	—	90	60
E	1.64	940	—	620	300	180	—	—
F	1.89	1000	—	600	380	310	—	—
Guernsey	0.89	830	290	—	200	—	130	—

hyaluronidase in the semen components. Thus no estimates of 'zero-time' distribution of hyaluronidase are possible other than by extrapolation.

Table 4 shows the distribution of hyaluronidase between the seminal plasma, heads and tails of semen from the Guernsey bull. Comparable determinations of enzyme in the plasma and intact spermatozoa of semen from Friesian bulls are listed in Table 5. In both sets of experiments analyses were carried out 30 min. after semen collection. For the Guernsey bull the concentration of enzyme in the seminal plasma varied only slightly in the five ejaculates that were examined. Cellular hyaluronidase, however, was approximately proportional to cell count. The samples of semen from seven normally fertile Friesian bulls understandably failed to show the same degree of regularity in hyaluronidase distribution. However, whereas seminal plasma of the Guernsey bull always contained at least 50% of the seminal hyaluronidase, the maximum figure for the Friesian bulls was 37%.

The loss of hyaluronidase from both the intact and decapitated spermatozoa which occurs when semen is stored at 20° is shown in Table 6. With semen containing decapitated spermatozoa about two-thirds of the enzyme was found in the plasma after 30 min. With normal semen, however, a similar distribution between cells and plasma was only reached after 1½–2 hr.

DISCUSSION

Our findings that the tails of bull spermatozoa are more active than the heads in terms of respiration and fructolysis, and also contain the greater part of the sperm phospholipid, confirm the results of Mann (1958*a, b*). The tail suspensions that we have used were free from heads, as well as from damaged or immotile tails, and they were, in our opinion, probably the purest motile-tail fractions that have yet been described. Though we were not

able to obtain a head fraction free from immotile tails the latter appeared to have negligible respiratory activity. Nevertheless, such immotile tails are a potential source of substrates and cofactors and are undesirable contaminants in metabolic studies. For this reason we have preferred to calculate activities of heads from those of tails and unfractionated head-tail mixtures. We have not attempted to differentiate the two head fractions which separated in the density gradient (Fig. 1). The specific gravity of bull spermatozoa increases considerably during maturation (Lindahl & Kihlström, 1952), and the separation that we observed is no doubt due to the presence of cells at different stages of maturation.

The respiratory and fructolytic activities of the head-tail mixtures obtained from the Guernsey bull come within the range of activities of normal spermatozoa. Similarly, the respiration of heads and tails measured separately was, within experimental error, equal to that of the head-tail mixture. We can thus infer that, as far as sugar metabolism is concerned, the semen from the Guernsey bull behaves just as normal semen and that the separation procedure has no deleterious effect on the heads and tails. It is surprising that the heads, being essentially cell nuclei, should exhibit a marked respiratory activity, but until heads can be obtained free from immotile tails and also from a significant number of small granules which are probably derived from the midpiece, the exact magnitude of their respiratory activity will remain in doubt. Sucrose, in the low concentrations present in fractions obtained by the differential density technique, was not metabolized by spermatozoa and did not interfere in the resorcinol-hydrochloric acid method used to measure changes in fructose concentration.

It has already been established that plasmalogen of ram spermatozoa can act as a metabolic reserve, to maintain sperm motility, when fructose is not available (Hartree & Mann, 1959, 1961). Although

motility is characteristic of the tails and not the heads, the plasmalogen:phospholipid ratio is approximately as high in heads as in tails.

The main function of sperm hyaluronidase in certain species, such as the rabbit, is believed to depend on the ability of this enzyme to disperse the cumulus oophorus and thus to enable the spermatozoon to reach the egg. It has been postulated that the reason why a large number of spermatozoa must be in the vicinity of the egg before one spermatozoon can effect penetration is that only in this way could an adequate hyaluronidase concentration be established near the egg. This hypothesis has been opposed on the grounds that, in the rat and mouse, spermatozoa can penetrate ova while the cumulus is still attached (Austin, 1948; Wada, Takehara & Yuhara, 1955). Such experiments do not, however, eliminate the possibility that the cumulus can be penetrated only after it has been modified in some way by hyaluronidase but not necessarily liquefied or completely dispersed.

The function of hyaluronidase in bull semen is even more obscure since in the cow the cumulus disappears soon after ovulation. In the bull (as in man, rabbit and boar) the concentration of enzyme in semen is approximately proportional to sperm density (Swyer, 1947) even though some enzyme is always found in the plasma (Johnston & Mixner, 1947). Our results with normal semen from Friesian bulls are consistent with the enzyme being present entirely in the spermatozoa at the time of ejaculation. Thus with sample B (Table 6) the release of hyaluronidase proceeds as a first-order reaction if the activity of fresh whole semen (860) is taken as the zero-time activity of the spermatozoa. The less complete records of samples A and E are also consistent with a first-order reaction. The result obtained with the semen of the Guernsey bull (Table 6) has two possible explanations. Either a rapid release of enzyme during the first 30 min. was followed by a much slower release, or a considerable portion of the enzyme was present in the plasma at zero time. The former explanation requires that the concentration of hyaluronidase in plasma at 0.5 hr. should be proportional to cell count whereas it is in fact sensibly constant (Table 4). The latter explanation is thus the more feasible one. If it is assumed that about 500 out of 830 units were initially present in the plasma, the subsequent release of enzyme into the plasma is a first-order process.

Vasectomy leads to disappearance of hyaluronidase from the semen of the rabbit (Chang, 1947) and of man (Bergental & Scott, 1948), and azoospermic human semen is also devoid of the enzyme (Swyer, 1947). Corresponding data on the bull are not available, but our experiments suggest that secretions of the accessory organs of reproduction

contribute no hyaluronidase to bull semen. We therefore suggest that in the Guernsey bull extracellular hyaluronidase is present at the time of ejaculation and that this portion of the semen hyaluronidase must be of testicular or epididymal origin. Although the predominance of hyaluronidase in the heads of decapitated spermatozoa may not necessarily be characteristic of normal spermatozoa, such a distribution is consistent with the suggested role of the enzyme.

SUMMARY

1. Semen from a Guernsey bull containing decapitated spermatozoa (i.e. heads detached from motile midpiece-tails) has been subjected to density-gradient centrifuging to separate midpiece-tails from heads. Metabolic activities, phospholipid contents and hyaluronidase activities of these fractions have been compared with those of normal spermatozoa from Friesian bulls.

2. Respiratory and fructolytic activities of the midpiece-tails were much higher than those of heads. Activities of heads plus midpiece-tails were similar to those of normal spermatozoa.

3. The cellular components of decapitated spermatozoa contained more lipid phosphorus than did normal spermatozoa. However, the plasmalogen contents were approximately equal.

4. When normal bull semen was stored at 20° hyaluronidase diffused from the cells to the seminal plasma. The enzyme appears to be present entirely in the cells at the time of ejaculation.

5. In the semen from the Guernsey bull most of the hyaluronidase was present in the seminal plasma at the time of ejaculation. The cellular enzyme was predominantly in the heads.

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The Glucosidic Linkages of the *Shigella flexneri* Polysaccharides

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A recent review of the specific polysaccharides of the Gram-negative bacilli (Davies, 1960) shows that the monosaccharide constituents of many of these substances have now been identified and the taxonomic significance of different groups of sugars defined. Comparatively little progress has been made, however, in determining the structure of these complex polysaccharides, so that in most instances it is not yet possible to interpret their serological activity in terms of molecular configuration. Nevertheless, significant advances have been made in the assignment of molecular structures to a few determinant groups of the *Salmonella* O somatic antigens, principally by Staub, Tinelli, Lüderitz & Westphal (1959) and Staub (1960) who used the specific serological-inhibition techniques so successfully employed in related fields by McCarty (1956) and Watkins & Morgan (1952). With the *Shigella flexneri* polysaccharides that form the subject of this paper, the nature of the glucosidic linkages between the constituent hexose units is unknown. Once this information is available, chemical interpretations of type specificity and cross-reactivity in this serologically heterogeneous group of organisms may become possible. The object of the present investigation was to define the type of glucosidic linkages which occur in the specific polysaccharides of the eight common *Sh. flexneri* serotypes. This was achieved by means of infrared spectroscopy and by specifically inhibiting the activity of high-titre *Sh. flexneri* antisera with glucose polymers of known chemical structure.

A necessary prerequisite to a study of the molecular structure of any polysaccharide is the identification of its monosaccharide constituents. Goebel, Binkley & Perlman (1945) identified the amino-

hexose in electrophoretically homogeneous polysaccharides from *Sh. flexneri* Types 1a, 2a, 3 and 6 as glucosamine, and estimated the percentage of this monosaccharide and of the other reducing sugars in hydrolysates of these substances. Slein & Schnell (1952, 1953) and Slein (1952) identified the main hexose constituents of *Sh. flexneri* Type 3 polysaccharide as glucosamine, glucose and rhamnose, but this material also contained 1% (w/w) of organically bound phosphorus in the form of a hexose 6-phosphate and an aldoheptose phosphate. Simmons (1957) demonstrated that the specific polysaccharides from *Sh. flexneri* Types 1a, 2a, 3, 4a, 5 and 6 and Variants x and y all contained glucosamine, glucose and rhamnose. The occurrence of the same hexoses in the polysaccharides of this serologically heterogeneous group is a possible chemical basis for their high degree of cross-reactivity (Simmons, 1958).

MATERIALS AND METHODS

Organisms investigated. The strains of *Sh. flexneri* used in this investigation were as follows, the numbers in parentheses being the N.C.T.C. numbers: Type 1a (3); Type 2a (4833); Type 3 (8205); Type 4a (8206); Type 5 (8201); Type 6 (8207); Variant x (2245); Variant y (4839).

Preparation of mass cultures. The purity of each organism was checked by morphological, biochemical and serological tests and its smoothness confirmed by colonial appearance and salt stability before mass culture was initiated. The organisms were grown, by courtesy of Dr D. W. Henderson, at the Microbiological Research Establishment, Porton, on C.C.Y. agar (Gladstone & Fildes, 1940), containing 0.5% (w/v) of lactose, for 18 hr. at 37°. The harvested material was homogenized, filtered and acetone-dried. The amount in terms of dry weight of bacteria varied from 93 g. with *Sh. flexneri* Variant x to

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