LVII. BACTERIAL METABOLISM.
II. GLUCOSE BREAKDOWN BY PNEUMOCOCCUS VARIANTS AND THE EFFECT OF PHOSPHATE THEREON.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

(*Received February 27th, 1932.*)

It was found that the lactic acid formed in cultures of haemolytic streptococci accounted for about 78 % of the glucose which had disappeared [Hewitt, 1932] and this proportion appeared not to vary under a variety of cultural conditions. In these respects the carbohydrate metabolism of haemolytic streptococci would seem to be less complex than that of many bacteria, since with many micro-organisms the products of glucose breakdown are very diverse and vary both qualitatively and quantitatively with the cultural conditions. The first object of this investigation, therefore, was to compare the carbohydrate metabolism of pneumococci with that of the closely related haemolytic streptococci.

With the haemolytic streptococcus variants investigated most glucose breakdown occurred in growing cultures of the matt virulent, less with the matt attenuated and least with the glossy variants. In each case more glucose breakdown occurred in the cultures showing heavier growth and it is uncertain whether heavier growth occurred owing to the greater ability of certain variants to break down glucose, or whether the amount of glucose breakdown is a measure of the amount of bacterial growth which is itself conditioned by other factors in the growth requirements of the different variants. In any event the fact remains that the differing antigenic structures of haemolytic streptococci and their differing virulence are associated with differences in the gross amount of chemical change produced in growing cultures. The appearance of 24-hour cultures in the medium used was sufficient to differentiate the variants on casual inspection.

Pneumococci also differ in antigenic behaviour, chemical structure and virulence and Finkle [1931] has described differences in the aerobic glycolysis effected by the different types of pneumococcus. Finkle worked with washed suspensions of bacteria in Ringer solution and estimated the glycolysis indirectly. The opportunity has now been taken of investigating the glucose breakdown in growing cultures of the different types of pneumococcus.

Since the growth requirements of pneumococci are, in general, similar to, although rather more rigorous than, those of haemolytic streptococci, it was

anticipated that the culture media described in the previous communication might prove suitable in the present experiments.

Analytical methods.

Glucose and lactic acid were determined by the methods previously described and the Bell-Doisy method was used for the determination of phosphorus. It was not considered necessary to treat the media with trichloroacetic acid before determining inorganic phosphate since so little material was precipitated by this reagent in the media employed.

Culture media.

The culture media used were constituted as follows:

	Medium no.			Phosphate-free
	M 6	M 7	M 8	M 9 and M 10
Glucose	10 g.	5 g.	10 g.	10 g.
$(NH_4)_2SO_4$	5 g.	5 g.	5 g.	5 g.
$NaHCO_3$	10 g.	Nil.	8 g.	8 g.
$Na_2HPO_4,12H_2O$	Nil.	8 g.	4 g.	Nil.
KCl	1 g.	1 g.	1 g.	1 g.
$MgSO_4,7H_2O$	0·01 g.	0·01 g.	0·01 g.	0·01 g.
$FeCl_3$	Nil.	Nil.	Nil.	Trace
0·02 % phenol red	3 cc.	Nil.	3 cc.	3 cc.
Tap-water	900 cc.	900 cc.	900 cc.	900 cc. (Distilled water)
Broth	100 cc.	100 cc.	100 cc.	100 cc. (Phosphate-free)

Before the addition of 100 cc. of broth to M 6 and M 8, CO_2 was bubbled through the medium until a reaction of about p_H 7·0 was reached. All the media were sterilised by filtration through Pasteur-Chamberland F candles and in the case of the bicarbonate media the media tubes were provided with cellophane caps secured by rubber bands over the cotton-wool plugs in order to minimise diffusion of carbon dioxide. The broth added to these media was a horse-flesh infusion containing 2 % of proteose peptone and had been sterilised by filtration and not by autoclaving or steaming.

Media M 9 and M 10 were similar to the others except that a trace of $FeCl_3$ was added and that inorganic phosphates were excluded; distilled water was used in place of tap-water, and the broth added had been freed from inorganic phosphates by adding 6 to 7 cc. of hot 10 % baryta to 100 cc. of broth, centrifuging, adding 2·5 to 3·0 cc. of *N* H_2SO_4 to remove barium, and again centrifuging. The clear supernatant fluid did not contain more than 3×10^{-6} g. per cc. of inorganic phosphorus.

Media 9 B, 9 C, *etc.* and 10 B, *etc.* were prepared by adding known volumes of a sterile 4 % sodium phosphate solution to M 9 and M 10. The glucose contents (Hagedorn-Jensen) of the media were M 6 1·035 %, M 7 0·548 %, M 8 1·055 %, M 9 1·020 %, and M 10 1·060 %.

Experimental.

The general experimental procedure was that previously described. In many experiments 50 cc. of culture medium contained in 7 × 1 in. tubes were used in place of 90 cc. batches in 7 × 1½ in. tubes. The inocula were each 0·2 cc. of a 24-hour broth culture of pneumococci in peptone-infusion broth. The strains of organisms used and their descriptions were kindly supplied by Dr J. M. Alston, Dr F. Griffith, and Dr G. F. Petrie. The organisms are designated as S (smooth) and R (rough) and by the number of the type; thus R II refers to the rough pneumococcus derived from Type II organisms.

Incubation of the cultures was conducted at 37° for 24 hours in all experiments and the analyses recorded were carried out on the filtrates of the cultures.

Lactic acid production under different conditions.

The data on lactic acid production from glucose are summarised in Table I. The mean value for the percentage yield of the lactic acid formed from the glucose broken down is 77·5 % and the extreme values are 72·2 and 83·5 %. The deviation from the mean value is small and within the limits of experimental error. In the case of haemolytic streptococci the mean value for the yield of lactic acid was 78 % and the limits were 68 % and 85 %. The figures for pneumococci and haemolytic streptococci are therefore, for practical purposes, identical. The yield of lactic acid appears not to vary over a range of variations of cultural conditions. Alterations were effected in the oxygen supply, in the inorganic phosphorus content (from 0·004 to 0·038 %), in the amount of sugar decomposed (from 0·215 to 0·992 %) and in the type and condition of the bacteria. All these appeared to be without sensible effect on the lactic acid yield.

Table I. *Lactic acid production from glucose by pneumococci.*

(Results are expressed in g. per 100 cc. of culture.)

Exp. no.	Type of organism	Medium	Inorganic P content of medium %	Conditions	Glucose disappeared	Lactic acid formed	Yield of lactic acid formed from glucose %
P 31	S I	M 6	0·004	Aerobic	0·215	0·164	76·3
P 36	R III	,,	0·004	,,	0·992	0·779	78·5
P 41	R III	,,	0·004	Anaerobic	0·872	0·685	78·6
P 42	S I	M 7	0·073	,,	0·248	0·195	78·6
P 43	S II	,,	0·073	,,	0·245	0·203	82·0
P 45	S III	,,	0·073	,,	0·225	0·174	77·3
P 46	R III	,,	0·073	,,	0·245	0·190	77·6
P 48	S I	,,	0·073	Aerobic	0·273	0·221	80·9
P 52	R III	,,	0·073	,,	0·303	0·253	83·5
P 53	S I	M 8	0·038	,,	0·545	0·422	77·5
P 54	S II	,,	0·038	,,	0·930	0·710	76·3
P 55	R II	,,	0·038	,,	0·845	0·610	72·2
P 56	S III	,,	0·038	,,	0·425	0·308	72·5
P 57	R III	,,	0·038	,,	0·980	0·765	78·1
P 80	R III	M 10 D	0·007	,,	0·844	0·645	76·4
P 81	R III	M 10 E	0·036	,,	0·914	0·674	73·7

GLUCOSE BREAKDOWN BY DIFFERENT TYPES.

The ratio of lactic acid produced to glucose broken down is the same for the different forms of pneumococcus, as seen in Table I, but the actual amount of glucose broken down (or lactic acid formed) is different for the different types. In a typical experiment six 90 cc. tubes of bicarbonate medium M 6 were each inoculated with 0·5 cc. of a broth culture of a pneumococcus variant. After 24 hours' incubation there was very heavy growth in the R III culture, slight growth was visible in the S I culture and none of the others showed signs of growth. The R III culture was acid in reaction and effervescing freely (due to the liberation of CO_2 from the bicarbonate), whilst the others remained nearly neutral and showed no signs of liberation of gas bubbles. The differences were most striking and the results are summarised in Table II.

Table II. *24-hour cultures of pneumococcus in bicarbonate medium (M 6).*

Exp. no.	Type of organism	Growth in 24 hours	p_H	Gas liberation	Glucose disappeared %	Lactic acid formed %
P 31	S I	+	7·0	0	0·215	0·164
P 32	S II	0	7·2	0	0·065	—
P 33	R II	0	7·4	0	0·020	—
P 34	S III(*a*)	0	7·4	0	0·015	—
P 35	S III(*b*)	0	7·4	0	0·010	—
P 36	R III	+ + + + +	6·4	+ + +	0·992	0·779

Finkle [1931], working with washed pneumococcus suspensions, found that only the S I and R III forms effected aerobic glycolysis but whereas he found these two forms almost equal in activity the results above show that in growing cultures R III is much more active than S I.

The oxygen supply was restricted in the next series of experiments since Finkle found that all the types effected anaerobic glycolysis. With the bicarbonate media it is not possible to establish rigorously anaerobic conditions—the medium cannot be boiled to remove air since CO_2 is evolved and the medium becomes too alkaline. Cultures of the different types in bicarbonate medium were placed in a Bulloch's apparatus, and the air was replaced by passing hydrogen before incubation but the results were little different from those with aerobic cultures (Table III, col. 3). Phosphate medium (M 7) was therefore used, and in the anaerobic experiment the medium was heated in a boiling water-bath, cooled rapidly before inoculation and the culture incubated in a McIntosh and Fildes jar in the usual way.

Table III. *Glucose breakdown by different types of pneumococcus.*

	Bicarbonate medium M 6		Phosphate medium M 7	
Type	Aerobic %	Partly aerobic %	Aerobic %	Anaerobic %
S I	0·215	0·015	0·273	0·248
S II	0·065	0	0·065	0·245
R II	0·020	0	0·005	0·035
S III	0·015	0	0·158	0·225
R III	0·992	0·872	0·303	0·245

It will be seen in Table III that in anaerobic phosphate medium there is little difference in the amounts of glucose broken down by the different forms (except R II).

It should be remarked that in every case the amount of glucose breakdown has varied with the amount of growth of the organisms, as judged by the turbidity and deposit in the cultures.

Effect of inorganic phosphate.

The data in Table III suggested that inorganic phosphate might have an effect on glucose breakdown and Table IV gives the results of experiments conducted in two media both buffered with bicarbonate but one containing 0·4 % $Na_2HPO_4, 12H_2O$ as well.

Table IV. *Glucose breakdown in different media.*

Type	Bicarbonate medium M 6 (0·004 % inorganic P) %	Bicarbonate-phosphate medium M 8 (0·038 % inorganic P) %
S I	0·215	0·545
S II	0·065	0·930
R II	0·020	0·845
S III	0·015	0·425
R III	0·992	0·980

It will be seen that in every case there is more glucose breakdown in the media containing added inorganic phosphate except with R III where glucose breakdown was already maximal.

Experiments were next conducted with phosphate-free media (M 9 and M 10) from which the inorganic phosphates had been removed as described in a previous section. In cultures in media M 9 A and M 10 A the only inorganic phosphate present was derived from the culture used for inoculation. Varying amounts of inorganic phosphate were added to these basic media.

Table V. *Glucose breakdown in media containing varying amounts of inorganic phosphate.*

(Results expressed in g. per 100 cc.)

	Inorganic P content				
Type	A 0·0002 %	B 0·0006 %	C 0·0020 %	D 0·0072 %	E 0·0362 %
S I	0·095	0·129	0·240	0·269	0·260
S II	0·045	0·069	0·290	0·365	0·360
S III	0·065	0·189	0·175	0·240	0·272
R III	0·190	0·309	0·780	0·844	0·914
Mean	0·099	0·174	0·371	0·429	0·452

The amount of glucose broken down increases 3·7 times when the inorganic phosphorus content is increased from 0·0002 to 0·0020 %, and it increases 1·2 times when the inorganic phosphorus content is further increased from 0·0020

to 0·0362 %. As observed previously the amount of growth of the organisms as judged by the turbidity of the cultures appeared to be proportional to the sugar breakdown in each case. It should be emphasised that the media used in these experiments were adequately buffered with bicarbonate and the inorganic phosphate effect is not that of a buffer salt.

Discussion.

As with haemolytic streptococci so also with pneumococci glucose cleavage appears to be a relatively simple process, in contrast with the complexity of glucose metabolism in the case of many other bacteria. About 77·5 % of the glucose disappearing from cultures of either haemolytic streptococci or pneumococci may be recovered in the form of lactic acid, and this proportion seems not to vary over a considerable range of variation of cultural conditions. It is notable but not unexpected that glucose breakdown by pneumococci appears to be a similar process to that effected by haemolytic streptococci. This relatively simple method of glucose breakdown is probably accounted for in part by the absence of certain oxidising systems from the enzyme equipment of the organisms. Many other bacteria, for example, are able to oxidise lactic acid to carbon dioxide. But with the pneumococcus and haemolytic streptococcus the glucose molecule appears to break down to two molecules of lactic acid with a 78 % yield and the lactic acid formed appears not to be decomposed further in the cultures.

There are very marked and characteristic differences between the behaviours of the different types of pneumococcus. As would be expected from their different antigenic behaviour, chemical structure and virulence, their metabolic activities also differ. The yield of lactic acid from each molecule of glucose broken down in the cultures is the same with each type, but the actual amount of glucose broken down by the different types varies greatly. In aerobic cultures there was very much more glucose breakdown with R III pneumococci than with any other variant, S I came next in order of activity and the glycolytic activities of the other variants were much less. In anaerobic cultures the differences in glucose breakdown were much smaller. Finkle [1931] using washed suspensions of pneumococci in Ringer solution observed aerobic glycolysis with the R III and S I forms only, S I being only slightly less active than R III. Marked anaerobic glycolysis was observed with all the forms. In his experiments Finkle was presumably measuring the glucose activation of non-proliferating bacteria whereas in the present experiments glucose breakdown cannot proceed to any great extent unless bacterial growth occurs. In the present experiments the amount of glucose breakdown appeared to go *pari passu* with the amount of bacterial growth. In so far that the present results agree with those of Finkle it would seem that the growth of the pneumococci in the media used was dependent on glucose breakdown, but the discrepancy in the results, namely that the S I organisms were relatively much less active glycolytic agents in growing cultures, suggests that there may be a difference

in the growth requirements of the different forms apart from that of glucose breakdown. Whatever proves to be the explanation of the phenomenon the empirical fact remains that, in the glucose medium used, variants of both pneumococci and haemolytic streptococci may be differentiated by the amounts of glucose broken down in their cultures.

In the bicarbonate-phenol red-glucose medium used in certain experiments the difference in appearance of 24-hour broth cultures of the different types was very striking; the R III culture was milky in appearance with a heavy white deposit of bacteria, the phenol red was completely yellow and CO_2 was evolved with frothing, the other cultures were much less turbid, retained some of the pink colour of the indicator and showed no signs of effervescence or frothing.

The acidity produced is occasionally the limiting factor in the amount of glucose breakdown, and hence of bacterial growth, in pneumococcus cultures as with haemolytic streptococci. The superiority of bicarbonate over phosphate as a buffer in the cognate p_H range (say p_H 6·0 to 7·4) accounts for the increased glucose breakdown in the more actively glycolytic cultures when they are

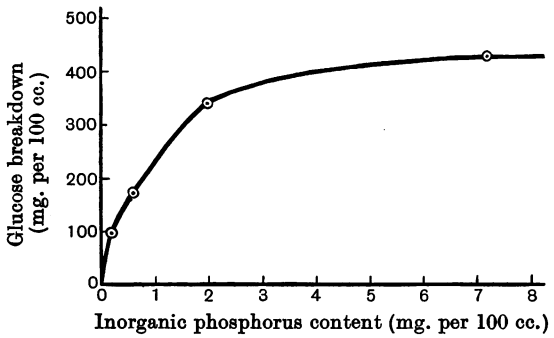

Fig. 1. Glucose breakdown in pneumococcus cultures containing varying amounts of inorganic phosphates.

buffered with bicarbonate rather than with phosphate. When, however, the inorganic phosphate concentration of the medium is too restricted the amounts of glucose breakdown and bacterial growth are very slight, but they are both stimulated when sodium phosphate is added. Thus in Fig. 1 are plotted the average figures obtained from Table V. By extrapolation it may be concluded that no growth of pneumococcus and no glucose breakdown would occur in this medium in the complete absence of inorganic phosphate. Friedlein [1928] found that the presence of inorganic phosphate was necessary for the growth of *B. coli* and *B. paratyphosus* B.

With regard to the mechanism of the effect of phosphate no data are yet presented but the analogies of the function of phosphates in glucose fermentation by yeast preparations [Harden and Young, 1906] and in the glucose metabolism of muscle suggest themselves. Virtanen [1924] sought for, but did not find, evidence of the formation of "zymophosphate" in the presence of living

Streptococcus lactis or of dried preparations, but in the case of dried preparations of *B. casei* ϵ, he noted a disappearance of inorganic phosphate during glycolysis and postulated the formation of "zymophosphate."

It should be noted that the beneficial effects of inorganic phosphate on the growth and glucose breakdown by pneumococci were observed in media sterilised by filtration. Deleterious effects may follow the addition of phosphate when the medium is afterwards autoclaved.

Summary.

1. Of the glucose disappearing from cultures of pneumococci about 78 % was recovered in the form of lactic acid.

2. The ratio of lactic acid formed to glucose broken down was unaffected by altering a variety of cultural conditions, and was the same as that observed in the case of haemolytic streptococci.

3. From the above it is concluded that glucose breakdown by pneumococci and haemolytic streptococci is a less complex process than with many other organisms.

4. There was more glucose breakdown in growing cultures with certain forms of pneumococcus than with others.

5. The presence of inorganic phosphate appears to play an essential part in bacterial growth and glucose breakdown, and not merely that of a buffer salt.

The author is indebted to Dr R. G. White and Dr E. W. Todd for their encouraging interest in the work.

REFERENCES.

Finkle (1931). *J. Exp. Med.* **53**, 661.
Friedlein (1928). *Biochem. Z.* **194**, 273.
Harden and Young (1906). *Proc. Roy. Soc. Lond.* B **77**, 405.
Hewitt (1932). *Biochem. J.* **26**, 208.
Virtanen (1924). *Z. physiol. Chem.* **134**, 300; **138**, 136.