BACTERIOLOGICAL APPROACH TO THE PROBLEM OF INTERCONVERSION OF HEXOSES IN ALKALINE SOLUTIONS

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The demonstration by Lobry de Bruyn and van Ekenstein and succeeding workers of the interconversion of hexoses in alkaline solutions has often caused bacteriologists some apprehension. Just how much interconversion does occur in the mildly alkaline culture mediums containing simple sugars that are used in studying the fermentation reactions of bacteria? This possibility may be minimized because the bacteriological incubation periods are too short to permit much alteration of the sugar and because the chemical studies demonstrating interconversion have usually been carried out in much more alkaline solutions than are suitable for culture mediums.

A recent quantitative study (Wedum, 1936) of the fermentation reactions of bacteria which had been cultivated in alkaline sugar mediums for three months incidentally showed that no appreciable interconversion of hexoses had occurred. This was particularly striking in the case of *Bacterium proteus* and *Bacillus anthracis* in mannose mediums. Seven strains of *B. proteus* and 3 of *B. anthracis* were found to ferment glucose and fructose but not mannose. If the interconversion system glucose $\rightleftarrows$ fructose $\rightleftarrows$ mannose were operative, one would expect the organisms gradually to cause an appreciable diminution in the amount of reducing sugar in the medium, but such was not the case. It may be proved that the organisms are capable of metabolizing glucose or fructose during the long incubation period by adding to such old cultures 0.5 per cent glucose or fructose and an acid-indicator.

Consideration of the work of Spoehr and Strain (1929) indicates

that a more direct bacteriological attack upon this problem of interconversion may be undertaken. These authors showed that 33 per cent mannose in the presence of 25 per cent disodium phosphate at 37°C. was converted in the course of 166 days into 32 per cent ketose and 22 per cent aldose other than mannose. Table 1 is compiled from their results and is the basis of the present experiment.

The present work is founded upon the following assumption. If, as is indicated in table 1, 54 per cent of the mannose is converted into what is presumably chiefly glucose and fructose, then it should be possible to demonstrate evidences of fermentation

TABLE 1

Alteration of 33 per cent mannose at 37°C. in 25 per cent disodium phosphate

(From Spoehr and Strain)

TIME	pH	TOTAL REDUCING POWER	PERCENTAGE OF ORIGINAL MANNOSE				
			Hexose other than mannose	Ketose	Aldose	Glucose*	Mannose*
days							
(1 hour)	8.16	85.0	10	0	100	10	90
27	7.74	89.7	24	13	87	11	76
71	7.57	90.2	39	22	78	17	61
166	7.35	89.8	54	32	68	22	46

* Assuming that these are the only aldoses present.

by introducing *B. proteus* or *B. anthracis* with an acid indicator into such a solution. That is, the glucose or fructose should be selectively fermented with production of evident acid and gas while the mannose remains untouched because these bacteria ferment glucose and fructose but not mannose.

METHODS

Preparation of mannose solutions[1]

Solution 1. An aqueous solution of 44 per cent mannose[2] and one of 100 per cent $Na_2HPO_4 \cdot 12H_2O$ were autoclaved separately

[1] These procedures followed those of Spoehr and Strain except that (1) xylene was not added as a preservative, (2) the volume here was 1 cc. per tube instead of 333 cc. in a flask.

[2] C.p. Pfanstiehl. Melting point of phenyl hydrazone 195° to 200°C.

at 15 pounds for 20 minutes and then thoroughly mixed in such proportions as to provide a 33 per cent mannose 25 per cent phosphate solution. One cubic centimeter of the mixture was then placed with aseptic precautions in each of several sterile 12 by 100 mm. test tubes, the sterile cotton stopper replaced, cut short, singed, and a freshly paraffined cork inserted. The pH as determined colorimetrically was 8.4 or above.

Solution 2. 66 per cent mannose and 50 per cent $Na_2HPO_4 \cdot 12H_2O$ were similarly treated to provide a 33 per cent mannose 25 per cent phosphate solution which was similarly distributed. The pH was 8.8 (colorimetric).

Solution 3 contained 33 per cent mannose and 12.5 per cent $Na_2HPO_4 \cdot 12H_2O$. The pH was 8.7.

Solution 4 contained 33 per cent mannose and 25 per cent acid phosphate $NaH_2PO_4 \cdot H_2O$. The pH was 5.2.

The tubes were incubated at 37°C. for 27 or 166 days.

Bacteriological examination of the solutions

At 27 and 166 days certain of the tubes were opened and under aseptic precautions (1) the pH was determined colorimetrically, (2) a loopful of material was transferred to Andrade mannose or maltose agar plates or to Andrade mannose broth tubes as a test for sterility, (3) acid or alkali was added to reduce the buffering effect of the phosphates (0.1 cc. normal H_2SO_4 to the alkaline solutions 1, 2, 3 and 0.5 to 1.0 cc. normal NaOH to the acid solution 4), (4) 3 cc. of a 24 hour 1 per cent Andrade indicator, sugar-free, beef-heart-infusion broth culture of *B. proteus* or *B. Anthracis* was added, (5) cultures were then incubated at 37°C. and observed for acid and gas at intervals of not greater than 12 hours for a period of 48 hours, (6) after 48 hours in order to determine just how much known glucose or fructose would permit the production of evident acid and gas, 0.2 or 0.4 cc. of sterile, (Berkefeld filtered) 10 per cent glucose or fructose was added, (7) cultures were again observed at frequent intervals for acid and gas.

RESULTS

Table 2 indicates that after 166 days incubation at 37°C. the mixture of 33 per cent mannose 25 per cent phosphate gave no indication of the presence of glucose or fructose when examined bacteriologically, although small amounts of added known glucose or fructose caused the formation of evident acid or gas.

TABLE 2

Bacteriological examination of 33 per cent mannose 25 per cent phosphate (solution 1) after 166 days at 37°C.

TUBE	pH	3 CC. 24-HOUR ANDRADE BROTH CULTURE ADDED	C.C. NORMAL H_2SO_4 ADDED	SUBSEQUENT COURSE OF FERMENTATION	KNOWN 10 PER CENT SUGAR SOLUTION ADDED AT 49TH HOUR	SUBSEQUENT COURSE OF FERMENTATION		
						16 hours	24 hours	48 hours
1	7.5	Anthrax 28	0.1	Abundant growth	0.4 cc. fructose	0	sl A	sl A
2	7.5	Proteus 78	0.1	but no acid or gas	0.2 cc. glucose	sl G	AG	AG
3	7.5	Proteus 226	0.1	in 48 hours	0.4 cc. glucose	G	G	
4	7.6	Coli I	0.1	AG	None			

sl = slight; A = acid; G = gas.

TABLE 3

Theoretical concentrations of converted hexoses in the test cultures

	CONCENTRATION OF CONVERTED HEXOSE IN THE TEST CULTURE*
	per cent
If all of the converted 54 per cent mannose were glucose	4.3
If all of the converted 54 per cent mannose were fructose	4.3
If all of the converted 22 per cent aldose were glucose	1.8
If all of the converted 32 per cent ketose were fructose	2.6

* One cubic centimeter mannose-phosphate, 3 cc. broth culture, 0.1 cc. normal sulphuric acid.

All sugar solutions before the addition of the bacteria were sterile as evidenced by transfer to plates or broth. Cultures were examined for acid and gas at 4, 8, 18, 24, 30, 42, 48 hours. *Escherichia coli* was included as a control because it ferments glucose, fructose, and also mannose.

Similar results were obtained at 166 days with all of 5 tubes of

solution 2, all of 6 tubes of solution 3, and all of 3 tubes of solution 4. Solutions 2 and 4 were tested with an additional organism, Proteus X 19. The acid or gas present after addition of known glucose or fructose was most marked in tubes of solution 3. Corresponding results had been previously obtained in other tubes tested after 27 days incubation of the 4 mannose-phosphate solutions.

Table 3 shows how much converted hexose, theoretically, should be present in the bacteriological test cultures of table 2. provided the conversion proceeded according to table 1.

TABLE 4

*Known glucose or fructose concentrations showing acid and gas within 48 hours**

MANNOSE-PHOSPHATE SOLUTION	GLUCOSE				FRUCTOSE			
	0.88 per cent tubes showing		0.47 per cent tubes showing		0.88 per cent tubes showing		0.47 per cent tubes showing	
	AG	None	AG	None	AG	None	AG	None
1	2	0	1	0	3	1		
2	5	0	2	0	0	1	0	1
3	2	0	4	0	1	1	2	0
4	2	2	1	0				

AG = acid and/or gas.

* In a culture consisting of 1 cc. mannose-phosphate, 3 cc. Andrade broth culture, 0.1 cc. normal sulphuric acid (sodium hydroxide in solution 4), 0.2 or 0.4 cc. 10 per cent glucose or fructose.

B. anthracis forms no gas.

Table 4 summarizes the experiments designed to determine just how much glucose or fructose was actually necessary in order to permit the production of evident acid and gas in the mannose-phosphate culture mixtures. The hexose concentrations should be compared with those in table 3. The table includes all tests made at the 49th hour (cf., e.g., table 2) in all 4 solutions at both 27 and 166 days with the 3 strains of *B. proteus* and 1 strain of *B. anthracis*. The presence of acid and gas in some tubes and absence in others (e.g., solution 4) merely represents differences in the fermentative potency of various strains of the bacteria.

Tables 3 and 4 emphasize the fact that if either one fifth of the

theoretical amount of converted fructose had been present, or one third of the theoretical glucose, acid and gas formation would have been clearly evident. One might well conclude therefore that either there was no such conversion or that the conversion products were not in a fermentable form.

SUPPLEMENTARY CONTROL EXPERIMENTS

Various experiments were performed to supplement the findings of table 4 regarding the amount of glucose or fructose necessary for *B. proteus* or *B. anthracis* to produce evident acid in the presence of phosphate. These tests consisted in the observation of the action of the bacteria in varying amounts of phosphate, glucose, and fructose with or without a 33 per cent concentration of non-fermentable sugar (lactose), and with or without the addition of small amounts of neutralizing sulphuric acid. It does not seem worth while to record these in detail. In general, they corroborated table 4 in the finding that 0.5 to 1 per cent glucose could practically always be detected within 48 hours by acid formation despite a phosphate concentration of 6 per cent;[3] while about 1 per cent fructose was required under similar conditions.

Other sets of experiments were designed to test further the inability of *B. proteus* and *B. anthracis* to ferment mannose.[4] The methods were much the same as those previously described (Wedum, 1936). In all, 75 cultures were made in beef broth and peptone mediums containing 0.5 per cent mannose and 0.5, 1, 2, or 4 per cent disodium phosphate (pH 7.0 to 7.7), or 1 per cent monosodium phosphate (pH 5.6 to 6.1), or sodium hydroxide or sodium carbonate (pH 7.8 to 8.0). Incubation was for 10 to 13 weeks. Quantitative analyses for reducing sugar were made in 10 weeks or at 1, 4, 10 or 1, 4, 13 weeks. The terminal pH in the alkaline media was usually about 8.4. Uninoculated media and *Bacterium alcaligenes* cultures served as controls. With the exception of one inexplainable and irreproduceable set of 6 cul-

[3] The mannose—25 per cent phosphate solutions were reduced to 6 per cent on the addition of 3 cc. broth culture, 0.1 cc. acid, 0.2 to 0.4 cc. glucose or fructose.

[4] Some of the strains of *B. proteus* and *B. anthracis* in the American Type Culture Collection do ferment mannose however.

tures[5] there was no evidence of clear-cut utilization of mannose by 2 strains of *B. anthracis* and 4 strains of *B. proteus*.

DISCUSSION

The failure to detect glucose or fructose in these mannose-phosphate solutions may be explained by the assumption that the glucose or fructose is present in a non-fermentable form. However, this non-fermentable form is sufficiently close to the ordinary fermentable form so that the chemical manipulations employed in the usual interconversion studies either convert it to or cause it to be identified as fermentable glucose or fructose.

Most interesting investigations could be carried out if additional organisms could be secured which would ferment only one or two of the hexoses, glucose, fructose, mannose. Several of these have been reported but the reports have been based upon the use of acid indicators whereas the frequent absence of evident acid production by such atypical organisms makes necessary the employment of a quantitative method of carbohydrate analysis. An extensive search to find such organisms is at present under way.

SUMMARY

According to Spoehr and Strain, 54 per cent of a 33 per cent solution of mannose in 25 per cent disodium phosphate which has been held at 37°C. for 166 days is converted into other aldoses and ketoses, presumably chiefly glucose and fructose.

When *Bacterium proteus* or *Bacillus anthracis*, which ferment glucose and fructose but not mannose, are introduced into such solutions on the 167th day there is no fermentative evidence of the presence of glucose or fructose.

It is shown that under the conditions of the experiment if either one fifth of the theoretical amount of converted fructose were present, or one third of the theoretical glucose, fermentative evidence of their presence would have been elicited.

[5] No evident acid was produced upon transfer to mannose indicator plates or to mannose indicator broth. Nevertheless the possibility of the presence of slow-fermenting mannose variants must be considered.

It is suggested (1) that the glucose or fructose is present in a non-fermentable form and (2) that this non-fermentable form is sufficiently close to the fermentable form so that the chemical manipulations employed in the usual interconversion studies either convert it to or cause it to be identified as fermentable glucose or fructose.

REFERENCES

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