THE BIOCHEMISTRY OF STREPTOCOCCUS HEMOLYTICUS

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I. THE ACIDS PRODUCED BY STREPTOCOCCUS HEMOLYTICUS¹

The cessation of activity in a glucose broth culture of *Streptococcus hemolyticus* comes when a fairly constant point of hydrogen-ion concentration is reached. This change is caused by a fermentation of the medium with a resultant formation of acid substances. The present section of this paper has to do with the chemical nature of these acid products.

A review of the literature reveals the fact that scant attention has been paid to the biochemistry of bacterial fermentations. Emmerling (1896), in 1896, carried out determinations of acids obtained from the putrefaction of certain proteins through the action of *Proteus* and *Staphylococcus pyogenes*. Tissier and Martelly (1902) in a study of the putrefaction of meat found that *Streptococcus pyogenes* could only split natural proteins after the latter had been peptonized. The same organism rapidly attacked glucose forming, chiefly, lactic acid. *Clostridium welchii* and *C. sporogenes* were found to produce acid and alkaline substances simultaneously. Later work by Wolf and Telfer (1917) upon these last-mentioned organisms has shown that a large proportion of the acid formed in their fermentations is volatile in character. The method of Dyer (1916) was employed by these investigators in determining the volatile acids. Forty per cent of the total acid produced by the organisms mentioned proved to be non-volatile. The exact chemical nature of this fraction was not determined.

¹ Miss Bernice Rhodes rendered valuable assistance in carrying out the experimental work described in this section of the paper.

In a study of the acid fermentation of xylose by Fred, Peterson, and Davenport (1919) the main products were found to be acetic acid and lactic acid. The proportion of volatile acid to non-volatile acid proved to be the same throughout the entire ten to twelve days of fermentation; namely, 40 per cent of acetic to 60 per cent of lactic acid. The two acids represented about 90 per cent of the sugar consumed.

Speakman (1920) investigated the biochemistry of acetone and butyl alcohol fermentation of starch and showed that acetic and butyric acids are formed as intermediate products. A reduction of these acids to the corresponding alcohol subsequently sets in.

Methods

Volatile acids. Volatile acids were determined by the steam distillation method of Duclaux (1900) as modified by Dyer (1916). This procedure is one in which the acid solution is distilled with steam at a constant volume. The distillate is collected in 10 cc. fractions until 100 cc. have passed over, after which the remainder is taken off in 100 cc. portions. These fractions are then titrated with $N/50$ alkali, using phenolphthalein as indicator, and the percentage of acid is calculated. The amount of acid in a given fraction, the "distilling constant" for the fraction, is plotted against the corresponding portion of distillate on logarithmic coordinate paper. Pure acids are graphically represented as straight lines and arrange themselves consecutively from the lower to the higher members of the series. With a mixture of two volatile acids the first part of the curve occupies a position intermediate to the lines representing the distilling rates of the higher and lower boiling acids. As the higher boiling acid is removed, the curve gradually becomes parallel with the line representing the lower boiling acid.

It follows that the distillate from an unknown acid mixture may be approximately determined by plotting a curve from the distilling constants and comparing this with the curves established for known acids.

The apparatus employed differed from that of Dyer (1916) in that the heating of the flasks was done with gas instead of electricity. By thoroughly insulating the flasks and connecting tubes with asbestos it was possible to maintain a constant volume throughout a long distillation.

To test the accuracy of the method several preliminary determinations of pure acids were first carried out. The results obtained for formic, acetic, and caproic acids are to be found in

TABLE I
Distilling constants (pure acids)

FRACTION	FORMIC ACID	ACETIC ACID	CAPROIC ACID
cc.			
10	2.0	4.66	24.55
10	3.8	9.45	43.85
10	5.5	14.17	58.83
10	7.5	18.93	70.25
10	9.8	23.57	78.30
10	12.0	28.16	83.90
10	14.0	32.59	88.16
10	16.4	36.91	91.46
10	19.0	41.19	93.90
10	21.2	45.24	95.74
100	35.6	71.89	99.22
100	52.4	89.60	99.95
100	63.5	99.49	

table 1. Graphical representation of the distilling rates has been made on logarithmic coordinate paper (see fig. 1). The curves were found to fall between those of Dyer and those of Wolf and Telfer. Attempts to use the color tests suggested by Dyer did not meet with success and accordingly they were abandoned.

Lactic acid. It was presumed that the non-volatile portion of the cultures consisted mainly of lactic acid; therefore the following quantitative method suggested by Fred, Peterson, and Davenport (1919) was employed for its determinations:

The residue from the distillation flask was carefully evaporated on a hot plate to a volume of about 40 cc. This was placed in a Soxhlet extractor and extracted with ether for fifty to sixty hours

to remove the lactic acid. About 30 cc. of water were added to the ether extract and the ether removed by distillation. The water extract remaining was then titrated with $N/10$ $Ba(OH)_2$ adding a 5 cc. excess of the base. The material was boiled for fifteen minutes to convert all of the lactic acid to the barium salt, after which the excess of $Ba(OH)_2$ was removed by neutralization with H_2SO_4 . This mixture was allowed to stand for several hours on a steam bath, filtered, and the filtrate and

FIG. 1

washings evaporated to dryness. After taking up the residue with water the material was again filtered to remove traces of organic matter or carbonates. The filtrate was then made up to a definite volume (50 or 100 cc.) and a 10 or 20 cc. aliquot taken for analysis. A test for succinic acid was made at this point by adding to the aliquot sufficient 95 per cent alcohol to bring the volume to 100 cc. Succinic acid, if present, gives a precipitate, and the material must be filtered. The filtrate was again evaporated to dryness and the residue taken up in 60 per

cent alcohol. This alcoholic solution was carefully evaporated to dryness in a tared dish and dried at 130°C. to a constant weight. An excess of H_2SO_4 was next added and the material converted to BaSO_4 by ignition. From the weight of BaSO_4 obtained, the corresponding weight of lactic acid was readily calculated. In case lactic is the only acid present the theoretical yield of BaSO_4 may be estimated from the weight of dried barium salt previously found.

Experiment I. The volatile and non-volatile acids produced in streptococcus fermentations

One hundred and fifty cubic centimeters of broth were inoculated with 6 cc. of a first-generation, glucose broth culture of *Streptococcus hemolyticus* and incubated for eighteen to twenty-four hours. Determinations of the volatile and non-volatile acids were made upon 100 cc. of this culture in the manner described. Control determinations were carried out upon a sample of uninoculated broth. The following results were obtained:

<i>Acids</i>	<i>Broth</i>
Volatile (as cc. of N/10 acid per 100 cc. broth).....	4.42
Lactic acid (as grams per 100 cc. broth).....	0.031

The values shown in the table were applied as corrections in the analyses of cultures.

Of the numerous estimations which have been made the results of but three will be presented: Culture (1), 1 per cent glucose broth; culture (2), 1 per cent glucose broth; culture (3), 1 per cent glucose, 5 per cent horse serum broth.

The curves plotted from the "distilling constants" (shown in table 2) proved to be so closely analogous that only one result will be shown. From the position of the line (fig. 1) representing the distilling rate of culture (1) it would appear that acetic acid, chiefly, is elaborated by the streptococcus during its growth in broth media containing either glucose or glucose plus horse serum. A trace of formic acid may also be present. This conclusion is the only one that may be drawn at present even though the

curve is not typical of a mixture of two volatile acids. No attempt has been made to re-fractionate the distillates, consequently the percentage of each acid in the volatile portion is not known. Reference to table 3 indicates that no close agreement exists between the per cents of volatile acid from culture (1) and culture (2) though the same lot of broth was used in each and the fermentation apparently progressed to the same point as the final P_H of the cultures was practically equal. Culture (3) gave a fraction of volatile acid still smaller than was noted in the other cases.

TABLE 2
Experiment I

FRACTION	CULTURE (1)	CULTURE (2)	CULTURE (3)
cc.			
10	4.50	5.32	3.45
10	9.00	9.06	7.13
10	13.81	12.07	10.58
10	18.68	15.71	13.32
10	23.83	18.50	16.01
10	28.08	21.82	18.69
10	32.63	24.61	21.8
10	35.84	27.62	24.07
10	39.26	30.10	27.05
10	42.14	32.36	30.26
100	61.19	59.40	60.06
100	77.32	77.52	76.56
100	93.45	91.17	88.26
100	99.89	100.90	99.96

The lactic acid estimations in the two glucose broth cultures do not correspond especially well and the value obtained in culture (3) is smaller than would be expected.

Through a lack of time it has been impossible to carry the present phase of the investigation to a logical completion. As a consequence it will be inadvisable to draw other than very general conclusions from the data presented.

It may be concluded from results that are to be presented later that in a 1 per cent glucose broth culture of the streptococcus some 156 mgm. of glucose are utilized in the first twelve to eighteen hours. From the data shown in table 3 it is possible

to calculate the amount of glucose destroyed in the formation of the acids. Assuming for the moment that the volatile fraction consists entirely of acetic acid and the non-volatile fraction of lactic acid, a calculation indicates that the total acidity of this culture accounts for only 50 per cent of the glucose utilized. Fred, Peterson, and Davenport (1919) were able in this way to account for 90 per cent of the sugar utilized in their xylose fermentations. The large discrepancy in the present experiment may possibly be due to two factors, first to experimental error, and second to the fact that another unknown non-volatile acid is present in the fermentation mixtures.

TABLE 3
Experiment I

NUMBER	CULTURE	PH		VOLA- TILE ACID (N/10 ACID PER 100 cc.)	LACTIC ACID		VOLA- TILE ACID	LACTIC ACID
		Initial	Final		Per 100 cc.	N/10 acid per 100 cc.		
					cc.	grams	cc.	per cent
1	Glucose broth	7.4	5.3 (18 hrs.)	1.196	0.061	6.78	15.0	85.0
2	Glucose broth	7.4	5.4 (18 hrs.)	2.98	0.0725	8.05	27.1	72.9
3	Glucose serum broth	7.4	4.9 (24 hrs.)	2.68	0.0534	5.25	33.8	66.2

II. THE METABOLISM OF STREPTOCOCCUS HEMOLYTICUS

Within the past few years evidence has been increasing which indicates that bacterial metabolism and human cellular metabolism have certain fundamental characteristics in common. We are indebted principally to Kendall and his collaborators for our more definite knowledge of the chemical activities of unicellular organisms. Cellular metabolism consists of two distinct phases—(1) the anabolic or structural phase, (2) the katabolic, destructive or “fuel” phase. As in the case of man, bacteria obtain structural material from nitrogenous nutrients while the energy requirements are best satisfied by carbohydrate substances. The analogy may be extended farther to the well known physiological principle that “Carbohydrates spare body nitrogen.” In other words, those bacteria which are capable of utilizing both carbo-

hydrate and protein for katabolic purposes will attack the former in preference to the latter (Kendall and Farmer, 1912a, 1912b). This phenomenon, which has been established by Kendall and his associates as a fundamental principle of bacterial metabolism, may be expressed concisely, according to Kendall and Farmer (1912d) in the statement that, "Fermentation takes precedence over putrefaction." These authors define fermentation as, "The action of microorganisms upon carbohydrates, putrefaction as the action of microorganisms upon nitrogenous substances." They state further that "The products of proteolytic activity, which are only formed when bacteria are utilizing protein for fuel are alkaline nitrogenous substances; the products of fermentation, on the contrary, which are formed when bacteria are utilizing carbohydrates for fuel, are non-nitrogenous, acid products."

Inasmuch as nitrogen is the most important structural element entering into the composition of the cell, a quantitative measure of nitrogen degradation must form a very important step in the study of cellular metabolism. In man, nitrogenous waste is excreted from the body mainly as urea, but with bacteria, which are known to excrete nitrogen principally as ammonia, urea, if formed at all, would represent a product of intermediary metabolism. This theory is borne out by the fact that certain bacteria are able actually to utilize urea.

Kendall and his associates (1913) have concluded after many studies upon a variety of organisms that ammonia formation, representing the final step in the degradation of proteins and protein derivatives, is the best available index of proteolysis by bacteria. Ammonia formation is considered by Kendall and Walker (1915) to result from intracellular deamination of assimilated protein derivatives, incidental to their transformation into energy.

Kendall, Day, and Walker (1913a) have estimated that the amount of protein needed for structural purposes by the bacterial cell is in all probability exceeded by the amount wasted through excretion. The combined structural needs and structural waste are much less than the fuel needs and the fuel waste. Further, the fuel requirements only cease upon the death of the organism,

whereas the structural needs are practically complete when the cell attains its morphological maturity. Consequently the fuel requirement is one of comparatively long duration. According to the same authors, a rapid disintegration of fuel materials occurs in the case of saprophytic bacteria. In other words, such microorganisms must, in general, be considered more active chemically than are pathogenic bacteria.

In the experiments conducted with the streptococcus it has been noted that growth in vitro is always accompanied by elaboration of acid products through the fermentation of materials of carbohydrate nature. No medium has ever been used which does not respond to the fermentative activities of this organism. Kendall, Day, and Walker (1913b) state that when bacteria are metabolizing carbohydrate the nitrogen requirement is minimal, so that in glucose broth cultures of *Streptococcus hemolyticus* we would expect the katabolic or "fuel" phase to predominate over the anabolic or structural phase of metabolism.

Moreover, the presence of horse serum in broth was found to exercise a decided stimulatory effect upon growth rate and acid formation, and also proved effective in permitting growth throughout a wider range of hydrogen-ion concentration. From a consideration of the fundamental features of bacterial metabolism as outlined in the foregoing discussion, it would seem obvious that these phenomena represent a stimulated metabolism of the organisms brought about through some property of the serum. It was suggested previously that structural or growth-accessory substances are perhaps furnished by this material thus permitting the organisms to inaugurate their metabolic activities earlier with consequent reduction of lag. This theory would be in accord with the statement of Kendall, Day, and Walker (1913b) that the structural function always precedes the vegetative or fuel function chronologically, inasmuch as the cell must be formed before it can carry on its appropriate activities.

The following experiments represent an attempt to study the metabolism of the streptococcus in various culture media with an especial effort to determine whether correlation exists between the rates of acid formation and the rates of other metabolic processes in (1) glucose broth, and in (2) glucose-serum broth.

Methods

Ammonia was determined by the Folin air current method (1912) using 2 cc. of culture and collecting the gas in N/50 acid after which the residual acid was determined by back titration with N/50 base. Results are expressed as milligrams per 100 cc. of culture.

Amino acids were determined by the formol titration method of Sørensen previously described (see section I).

Glucose was determined by the method of Bertrand (Hawk, 1918). As the presence of peptone and protein material in the medium rendered the application of the method impossible, the following procedure, devised by Dr. Marjorie W. Cook, was employed to free the cultures from interfering substances:

Twenty cubic centimeters of culture was diluted to 100 cc. with distilled water and precipitated with 10 to 15 cc. of saturated tannic acid solution. After filtering, 5 to 7 grams of lead acetate were added to the filtrate to remove excess of tannic acid and this mixture was filtered. If the filtrate was turbid more lead acetate was added. To the filtrate from this treatment was added 2 to 3 grams of sodium oxalate. This removed the lead as $Pb(C_2O_4)$. The filtrate from this last treatment should be perfectly clear and colorless. It is important throughout the whole procedure to keep the containers and funnels covered thus minimizing evaporation and reducing the error from this source. Two 10 cc. portions of this liquid were now used for determinations of glucose.

Bacterial counts were made by the method of Wright and logarithms of the values so obtained were employed in plotting growth curves. It must be borne in mind that the method of Wright gives only approximate results and that the values represent the total number of organisms rather than the number of viable cells.

Titration of hemotoxin were made with sterile tubes, pipettes, etc., to maintain the purity of the streptococcus cultures. Rabbit corpuscles which had been washed three times in 0.85 per cent NaCl and made up in a 1 per cent suspension in beef infusion

broth were used in the tests. To 0.5 cc. of the corpuscular suspension were added amounts of culture varying from 0.005 cc. to 0.5 cc. The volume was then made up to a total of 1 cc. with broth, after which the mixture was incubated at 37° for two hours. During the first hour of incubation the tubes were frequently shaken to insure thorough mixing. At the end of the incubation period the degree of hemolysis was observed, and expressed as follows:

100 per cent of corpuscles hemolyzed ++++
 90 per cent of corpuscles hemolyzed +++±
 75 per cent of corpuscles hemolyzed +++
 50 per cent of corpuscles hemolyzed ++
 25 per cent of corpuscles hemolyzed +
 0 per cent of corpuscles hemolyzed -

Experiment II. The "protein sparing" action of Streptococcus hemolyticus

Bacto beef broth, P_H 7.2, served as the basis of the combinations used in the experiment. The inoculum for each 10 cc. of broth consisted of 0.4 cc. of an eighteen-hour, glucose broth culture. The ammonia determinations were made after seventy hours incubation. The results are incorporated in table 4.

TABLE 4
Experiment II

NUMBER	SERUM	GLUCOSE	FREE NH ₃ AS MGM. N PER 100 CC. (CULTURE)	FREE NH ₃ AS MGM. N PER 100 CC. (CONTROL)
	<i>per cent</i>	<i>per cent</i>		
1	—	—	9.80	9.73
2	5	—	13.02	8.55
3	—	1.0	9.25	7.85
4	—	0.1	10.22	7.00
5	—	0.3	9.95	8.00
6	5	1.0	9.95	8.68

The results show that in the presence of little or no free carbohydrate, as in (2) and (4) of the table, the ammonia output is slightly increased over that found in the presence of 1 per cent glucose. If protein material in the form of horse serum be present the ammonia output is distinctly higher. This is shown

in (2). When the 5 per cent horse serum is in the presence of 1 per cent glucose, however, no increase in NH_3 is manifest ((6) in the table) showing that the sugar shields the protein from attack in this instance.

Experiment III. The protein and carbohydrate metabolism of Streptococcus hemolyticus in broth containing (1) glucose and (2) glucose plus horse-serum²

Three hundred cubic centimeter lots of media of the following composition were prepared from beef infusion broth, P_H 7.3: (1) 1 per cent glucose broth; (2) 1 per cent glucose, 5 per cent horse serum broth; (3) same as (2).

(1) and (2) were inoculated with 12 cc. of an eighteen-hour, second-generation culture from pleural fluid no. 198 in 1 per cent glucose-broth. (3) was inoculated with an equal amount of an eighteen-hour, first generation culture of the so-called laboratory strain. This culture differs from the pleural fluid culture in that it has been repeatedly transplanted upon artificial culture media since the original isolation, whereas the latter has been passed many times through rabbits. Both cultures were carried in this experiment in order to determine if repeated transplantation upon artificial culture media had brought about changes in the strain which might appear as an alteration of some phase of its metabolism.

The following determinations were carried out upon samples removed with aseptic precautions at intervals of three hours: (1) Bacterial counts; (2) P_H ; (3) glucose; (4) ammonia; (5) amino acids; (6) hemotoxin.

The experiment extended through a period of twelve hours. Table 5 contains the experimental data.

The curves of growth, acid³ formation and glucose utilization in the three cultures are found in figures 2, 3, and 4. It will be

² The writer desires to express his appreciation for the assistance rendered by Dr. Marjorie W. Cook and Miss Bernice Rhodes in carrying out this experiment.

³ The term acid is used to express true acidity in terms of P_H .

noted that although a rough parallelism between these factors is shown in the three curves of each culture it is most striking in the pleural fluid culture (fig. 3). Here, particularly in the maximum period, may be seen a close relationship between the curves of growth, acid formation, and glucose utilization. A correspondence of the three factors in the maximum period

TABLE 5
Experiment III

TIME	BACTERIAL COUNT		P _H	GLUCOSE		NH ₃ , MGM. N PER 100 cc.	AMINO ACIDS, MGM. N PER 100 cc.	HEMOLYSIS
	Per cu. mm.	Log		Per 100 cc.	Utilized			
(1) 1 per cent glucose broth (pleural fluid no. 198)								
0	16,400	4.21	7.30	grams 1.09	mgm. 0	6.01	18.87	0
3	50,700	4.71	7.20	1.172	0	5.73	19.07	0
6	960,000	5.98	6.40	1.007	89	6.57	17.73	purple
9	1,140,000	6.06	5.75	0.955	141	9.93	17.57	+++
12	1,700,000	6.23	5.55	0.935	161	9.79	19.51	0
(2) 1 per cent glucose, 5 per cent horse-serum broth (pleural fluid no. 198)								
0	23,800	4.38	7.35	1.096	0	5.03	19.77	0
3	90,200	4.96	7.20	1.068	28	6.29	18.21	0
6	2,000,000	6.30	5.80	0.941	155	9.93	16.87	++++
9	3,500,000	6.54	5.10	0.877	219	10.49	19.41	++++
12	4,560,000	6.65	4.90	0.847	249	10.07	20.73	0
(3) 1 per cent gucose 5 per cent horse-serum (Laboratory strain)								
0	40,000	4.60	7.35	0.992	0	6.43	19.17	0
3	90,400	4.96	7.20	1.001	0	5.45	20.95	0
6	1,456,000	6.16	5.90	0.862	130	8.67	18.03	++++
9	2,860,000	6.46	4.95	0.722	270	8.25	20.55	++++
12	4,700,000	6.67	4.90	0.728	270	7.69	20.61	0

appears in the other two cultures as well, though it is less striking. In each case it will be observed that a rise in the acidity curve is preceded by a rise in the growth curve. Attention should be directed to the fact that while acid production proceeds most rapidly during the time when the organisms are multiplying at a maximum rate, nevertheless, a considerable lowering of

P_H occurs during the succeeding period when the cells are increasing at a diminishing rate. Stated differently, the curves of growth in each culture depress more sharply from the maximum period than do the curves of glucose utilization and acid formation. Reference to table 5 shows that hemotoxin production commenced in the serum cultures by the sixth hour and persisted

FIG. 2. EXPERIMENT III. CULTURE (1); 1 PER CENT GLUCOSE BROTH

for at least three hours. In the glucose culture however, no definite appearance of hemotoxin was evident until the ninth hour. In each case hemotoxin appears in the period characterized by growth and acid formation at decreasing rates.

Reference to figures 5, 6, and 7 indicates that ammonia production was undergoing a definite increase by the third hour in each of the cultures. This increase in the serum cultures (figs.

6 and 7) was greatest between the third and the sixth hours while in the glucose culture (fig. 5) it was largest between the sixth and the ninth hours. The increase in ammonia output in a general way parallels growth and acid formation during the maximum period in each case. Associated with this increase

FIG. 3. EXPERIMENT III. CULTURE (2); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (PLEURAL FLUID STRAIN)

in the output of ammonia a coincident decrease in amino acid production is evident. In the serum cultures the curves of amino acid output rise sharply at the sixth hour while in the glucose culture the rise is delayed until the ninth hour. The initiation of this rise appears to be in direct correlation with a high point of the ammonia curve.

From the results of this experiment it seems evident that acid formation is closely associated chronologically with growth and active metabolism of the streptococcus. In each of the three cultures we find the maximum period of acid production correlated with maximum rates of growth, and of glucose utilization. These results are not in accordance with the findings on pneu-

FIG. 4. EXPERIMENT III. CULTURE (3); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (LABORATORY STRAIN)

mococci by H. M. Jones (1920) who reported a maximum period of growth correlated with slow acid formation, whereas the maximum period of acid production occurred during the time when the organisms were multiplying at a diminishing rate.

In a study of the nitrogen metabolism of actinomycetes, Waksman (1920) concluded that the production of amino acids

is not a waste process resulting from growth but that it represents a definite step in the metabolism of the organisms. In his experiments amino acids did not accumulate in the medium until after the organism had made its growth. In explanation Waksman suggests two possibilities; either (1) the growing cells utilized the amino acids as rapidly as the latter were formed,

FIG. 5. EXPERIMENT III; CULTURE (1); 1 PER CENT GLUCOSE BROTH

or (2) the proteolytic enzyme necessary for their elaboration appeared only in the later stages of growth. Attention has been called to the fact that the curves of amino acid formation in experiment III exhibit a rise at the sixth or the ninth hour which would correspond to the findings of Waksman on the actinomycetes. Examination of the growth curves (figs. 2, 3, 4) at this point shows that the maximum period has just been passed and that the organisms are now multiplying at a diminishing

rate. As a consequence the amino acid intake of the cells is reduced to a low level. This would account for the increased output in the medium. It has been pointed out that the rise in ammonia production in each culture, starting at the third hour, is correlated with a drop in the amino acid curve (figs. 5, 6, 7). The most probable explanation of this finding rests

FIG. 6. EXPERIMENT III. CULTURE (2); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (PLEURAL FLUID STRAIN)

upon the supposition that during the early life of the culture amino acids are utilized by the cells for structural purposes thus reducing their concentration in the medium. As more amino acid nitrogen is assimilated, a larger amount of ammonia is split off intracellularly. This is evidenced by a rise in the curves of ammonia production during this period. Such an hypothesis is in accord with the theory of Kendall and Walker (1915) that

ammonia formation is the result of intracellular deamination of assimilated protein material.

Wolf and Harris (1917b) in their study of the biochemistry of *Clostridium welchii* and *C. sporogenes* noted in cultures grown in a medium of high amino acid content that at the close of the experiment the concentration of amino acids was less than at the begin-

FIG. 7. EXPERIMENT III. CULTURE (3); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (LABORATORY STRAIN)

ning. This indicates that these substances were assimilated by the growing organisms and destroyed through deamination.

Reference to figures 6 and 7 reveals a very decided difference in nitrogen metabolism in the two serum cultures during the initial three-hour period. The pleural fluid culture exhibited a definite decrease in amino acid output correlated with an increased

ammonia formation, whereas the laboratory culture showed a decided increase in amino acid output coupled with a slight decrease in ammonia excretion. Whether or not this deviation represents a permanently altered aspect of metabolism on the part of the laboratory strain must for the present remain undecided. It may be that through continued cultivation upon artificial media the organism has gained the ability to inaugurate proteolysis earlier. Such a conclusion would be opposed to the finding of Rosenthal and Patai (1914) that avirulent streptococci were less strongly proteolytic than cultures of the same strain the virulence of which had been increased by animal passage. In the present experiment the curves of nitrogen metabolism (figs. 6 and 7) show the same general features from the third hour to the end of the period of observation. No differences in the growth, acid formation, or glucose utilization were evident in the two cultures throughout the entire period of the experiment.

SUMMARY AND CONCLUSIONS

1. Lactic acid appears to be the principal acid formed by *Streptococcus hemolyticus* in its fermentation of glucose broth. A smaller proportion of volatile acids is formed. This fraction is made up chiefly of acetic, with perhaps a trace of formic acid.

2. A quantitative study of the ammonia excretion of the streptococcus indicates that a "protein sparing" action occurs in media containing available sugar to meet the energy requirement of the developing cells.

3. The maximum periods of glucose utilization and acid formation in glucose and in glucose-serum broth are correlated with growth at a maximum rate, though a considerable lowering of P_H occurs during the period when growth proceeds at a diminishing rate.

4. The greatest increase in output of ammonia is correlated in a general way with the maximum periods of growth, glucose utilization, and acid formation. Associated with this increased output of ammonia a corresponding decrease in amino acids is evident. This condition seems to be associated with the interval

in which the organisms are making their growth. During this period, in which it may be presumed that anabolic processes are actively under way, the organisms are utilizing amino acids for structural purposes. This would cause the latter to decrease in concentration in the medium and furthermore would produce an increased excretion of ammonia through the katabolism of a part of the absorbed amino acids.

5. Subsequent to the period in which the organisms have made their growth a rise in the curves of amino acid formation is manifest. In the cultures containing horse serum this rise is initiated by the sixth hour; in the glucose culture it appears by the ninth hour. A decrease in ammonia output, in general, accompanies the rise in amino acid formation. These findings indicate that a decreased utilization of nitrogenous materials ensues after the organism has passed its maximum period of growth, despite the fact that proteolysis continues.

6. A marked difference in nitrogen metabolism between a passage strain and a laboratory strain of *Streptococcus hemolyticus* is noted during the first three hours of incubation in glucose serum broth. Whereas the passage strain shows a definite decrease in amino acid output coupled with an increased ammonia excretion, the laboratory strain exhibits a decided increase in amino acid output coincident with a slight decrease in ammonia formation. Whether or not this represents a permanent deviation in metabolism resulting from continued transplantation upon artificial culture media is a question that for the present must remain undecided.

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