SET V.—0.07 MG. OF MALT AMYLASE NO. 111*a* AND 100 CC. OF NEUTRAL 2% SOLUTION OF STARCH NO. 6 USED IN EACH CASE.

Amount of sodium bromide added.	Concentration of sodium bromide.	Reduction in mg. of Cu_2O.	Power.
None	....	54.5	242
1.0 cc. of 4 Molar	0.04 Molar	33.6	148
2.5 cc. of 4 Molar	0.10 Molar	32.5	143
5.0 cc. of 4 Molar	0.20 Molar	60.1	263
10.0 cc. of 4 Molar	0.40 Molar	75.9	341
20.0 cc. of 4 Molar	0.80 Molar	60.0	263

SET VI.—EFFECT OF POTASSIUM BROMIDE. SAME ENZYME AND STARCH AS SET V. KBr USED INSTEAD OF NaBr.

None	....	54.0	236
1.0 cc. of 4 Molar	0.04 Molar	35.6	161
2.5 cc. of 4 Molar	0.10 Molar	37.5	165
5.0 cc. of 4 Molar	0.20 Molar	78.7	350
10.0 cc. of 4 Molar	0.40 Molar	111.1	496
20.0 cc. of 4 Molar	0.80 Molar	107.6	482

Note the initial drop in activity (from 0–0.1 Molar concentration of the bromide) in each case preceding the activation. The subjoined curves bring out the effect more strikingly.

An explanation for the above results is now being sought by further experiments with various salts, including the bromates and iodates.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA.]

THE INFLUENCE OF AVAILABLE CARBOHYDRATES UPON AMMONIA ACCUMULATION BY MICROÖRGANISMS.

BY SELMAN A. WAKSMAN.

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It is a well-established fact that in human metabolism the energy is derived from the fats and carbohydrates of the diet or from those accumulated in the body as reserve materials. In the absence of available carbohydrates the organism will utilize the proteins available as a source of energy; but in this case there will be a waste of materials that cannot be utilized by the organism and are thrown off as waste products.

A similar case has been observed in the metabolism of microörganisms. Kendall and his associates[1] have shown in a number of experiments that fermentation takes precedence over putrefaction; when bacteria are grown in media containing both carbohydrates and proteins, they derive their energy from utilizable carbohydrates in preference to the proteins, even

[1] A. J. Kendall, *J. Med. Res.*, **20**, 117 (1911); "Bacteriology, General, Pathological and Intestinal," Philadelphia, 1916; Kendall and Farmer, *J. Biol. Chem.*, **12**, 13, 19, 215, 219, 465, 469; **13**, 63 (1912); Kendall, Day and Walker, THIS JOURNAL, **35**, 1201 (1913).

if the latter can also be utilized. All the organisms which ferment dextrose produced less ammonia in the dextrose medium than in the corresponding sugar-free medium, although the number of living bacteria were found to be greater in the former than in the latter. The small quantity of ammonia in the dextrose broth was thought to be due largely to the nitrogenous waste incidental to the utilization of protein for structural purposes. In the media where sugar was absent, the large accumulation of ammonia was explained by the combination of the "structural waste" and the "deamidization" incidental to the utilization of protein for their energy requirement. Kendall, on the basis of his experiments, advanced the theory that "utilizable carbohydrates protect proteins from bacterial breakdown." Kendall has further stated that the carbohydrate molecule, which contains no nitrogen and in which the carbon is already partially oxidized, can be utilized for fuel purposes by most bacteria with less expenditure of energy, than can be done in the case of most amino acids, peptones, or proteins. The amount of material required to provide energy for the bacterial cell far exceeds the amount of material required to build up the bacterial cell.

Aubel and Colin[1] have shown that a slight concentration of glucose in the medium is sufficient to decrease and finally cause the disappearance of the blue color in the culture of *B. pyocyaneus*. The ammonia produced from the nitrogenous compounds in the medium keep it alkaline, but if into a medium containing peptone, glucose, or any other assimilable carbohydrate, is introduced, the production of ammonia is prevented and the medium becomes acid. Sugars, such as cane sugar which cannot be inverted by *B. pyocyaneus*, do not have any effect upon the production the pyocyanic color. Aubel and Colin[2] have further shown that a large number of ammonifying organisms are inverted in their action upon nitrogenous organic substances by the presence of assimilable carbohydrates. They attempt to explain by these results the fact that carbohydrates diminish in the soil the intensity of the phenomenon of nitrification; independently of their proper action upon the nitrite- and nitrate-forming organisms, the sugars and other analogous substances hinder the role of ammonifying bacteria and prevent the transformation of nitrogenous organic substances into ammonia.

Jones[3] came to the conclusion that the absence of proteolytic changes in sugar containing media is due to the fact that the enzyme causing these changes does not appear in cultures containing a utilizable carbohydrate. Kligler[4] stated that the concentration of peptone is an appreciable factor

[1] *Compt. rend. soc. biol.*, **75**, 25 (1913).
[2] *Ibid.*, **76**, 835 (1914).
[3] *J. Infect. Dis.*, **19**, 1 (1916).
[4] *J. Bact.*, **1**, 663 (1916).

controlling bacterial nutrition. With a moderate amount of glucose present the higher the concentration of peptone the greater was the amount of ammonia produced. The amount of ammonia was in no case as great as in the sugar-control, indicating a distinct sparing effect of the carbohydrates.

This protein sparing action of carbohydrates is of great importance in the study of soil fertility. It is a well-established fact that the nitrogenous organic substances present in the soil or added in the form of different manures have to be broken down into simpler compounds before they can be utilized by the higher plants. It is assumed that ammonia, the final stage in the decomposition of proteins, has to be first formed; this is further oxidized to nitrites and nitrates, and only in the last stage is the nitrogen assimilated by most cultivated plants, although there are indications that plants are able to assimilate more complex nitrogenous compounds, in the absence of nitrates, such as different amino acids and creatinine, as was shown by the work of Skinner.[1] The attention of the soil biologist was therefore directed primarily to the processes of ammonification and nitrification in the soil. It was observed that also in soil processes the presence of easily available carbohydrates acts injuriously upon the formation of ammonia. Lipman and his associates[2] have shown that in the presence of dextrose, saccharose, lactose, maltose, and mannite less ammonia was recovered from the dried blood added to the soil than where the latter was used without sugar. They attempted to explain this by the fact that either ammonification is in some way retarded by the presence of sugar, or the ammonia formed is converted through some unknown agency into insoluble forms, and therefore cannot be distilled over. It was also shown that small quantities of available carbohydrates appear to have a slight stimulating effect on ammonification; with somewhat larger quantities the depression in ammonification is noticed during the first few days of incubation, but the checking effect is later overcome; where large quantities of sugar were used, the check in ammonification was found even after nine days' incubation, although on the seventh day a gradual increase was already observed. An attempt was made to explain the lack of accumulation of ammonia in the presence of available carbohydrates by the competition taking place in the soil between the bacteria on the one hand and yeasts and molds on the other, the latter developing more readily in the presence of available carbohydrates and thus reducing the amount of available ammonia. This statement cannot be justified in the light of recent investigation by the writer,[3] as well as

[1] Skinner, *Botan. Gaz.*, **54**, 152 (1912); Skinner and Beattie, *Bull. Torrey Botan. Club*, **39**, 429 (1912).

[2] Lipman, Blair, Owen and McLean, *N. J. Sta. Bull.*, **247** (1912).

[3] *Soil Sci.*, **2**, 103 (1916).

by others, that the molds of the soil are as strong and even stronger ammonifying organisms than the soil bacteria.

In another place Lipman and Brown[1] stated that the addition of dextrose and sodium citrate to the soil caused an enormous increase in the numbers of bacteria in the soil, the numbers increasing with the increase of the amount of carbohydrate applied. The available carbohydrates checked at first the accumulation of nitrates, but later these began to accumulate also in the soils which received an application of carbohydrates. Hutchinson and Marr[2] found that, when starch was applied at the rate of one ton per acre, there was a decrease in the yield of barley of 25.7% on unmanured plots and 9.2% on manured plots; cane sugar gave a corresponding decrease of 39.3% and 22.9%. An examination of the soil for the bacterial content has shown a great increase in bacterial numbers. Hutchinson tried to explain this injurious effect of available carbohydrates by the temperature prevailing when the starch or sugar was applied, these temperatures stimulating putrefaction rather than the action of nitrogen-fixing bacteria.

Experimental.

To study the influence of carbohydrates upon ammonia and amino-nitrogen accumulation by microörganisms, pure cultures of fungi were selected for several reasons: first their action on carbohydrates and the accumulation of ammonia were found to be much greater than in cultures of bacteria; the fungus mycelium could easily be filtered and weighed, while the filtering of bacteria is attended with difficulties; general conditions of growth could be observed much better with the fungi than with bacteria. The organisms studied in this work were *Aspergillus niger* Van Tieghem and *Citromyces glaber* Wehmer, which were isolated from the soil by the writer.

Four liters of a medium were made up, of the following composition: peptone, 20.0 g.; K_2HPO_2, 1.0 g.; $MgSO_4$, 0.5 g.; KCl, 0.5 g.; $FeSO_4$, 0.01 g.; and distilled water, 1000 cc. 3% cane sugar was added to two liters of medium, and the other two liters were left without any sugar. The nutrient solutions were distributed in 100 cc. quantities in 200 cc. Erlenmeyer flasks, plugged with cotton and sterilized for 15 minutes at 15 lbs. pressure. The flasks were inoculated with approximately equal numbers of spores and incubated at 28°. At the end of every 24 hrs. a flask was taken from each set; the medium was filtered through filter paper, and ammonia nitrogen determined in a portion of the filtrate by the aeration method of Folin[3] for three hours. The ammonia was collected in 0.1 *N* sulfuric acid, and the excess titrated as usual, using alizarine sul-

[1] *Seventh Intern. Congr. Appl. Chem.* (London), 7, 35 (1909).

[2] *Ibid.*, 7, 37 (1909).

[3] *Z. physiol. Chem.*, 37, 161 (1902).

fonate as an indicator. After the solution was free from ammonia, 2 cc. of the filtrate were neutralized with acetic acid, and the amino-nitrogen content determined by the method of Van Slyke.[1] The fungus body was washed on the filter paper with absolute alcohol, absolute ether, dried over concentrated sulfuric acid, and weighed. All determinations are calculated back to the total amount of the filtrate in each flask, originally containing 100 cc. of 2% peptone solution.

TABLE I.—GROWTH, AMINO-NITROGEN, AND AMMONIA ACCUMULATION BY *Aspergillus niger* FROM PEPTONE IN THE PRESENCE AND ABSENCE OF SUGAR.

Period of incubation in days.	3% Sugar present.			Sugar absent.		
	NH_2-N. in mg.	NH_3-N. in mg.	Weight of mycelium in g.	NH_2-N. in mg.	NH_3-N. in mg.	Weight of mycelium in g.
0.....	40.60	0	0	40.60	0	0
1.....	37.74	1.00	0.036	36.01	0.84	0.009
2.....	31.16	1.68	0.220	32.89	1.12	0.048
3.....	28.60	1.68	0.611	30.03	9.10	0.083
4.....	27.84	2.24	1.874	32.48	11.48	0.108
5.....	24.94	7.00	1.776	30.16	34.72	0.193
6.....	25.17	12.18	2.421	33.18	49.84	0.200
7.....	23.45	18.62	2.846	40.04	53.90	0.204
8.....	22.54	28.74	2.736	32.60	70.00	0.220
9.....	22.27	35.60	2.506	31.98	73.62	0.260
10.....	19.41	48.02	2.012	30.26	76.02	0.300
11.....	21.13	57.24	1.580	31.68	79.94	0.310
12.....	20.55	71.12	1.386	36.54	87.36	0.450
13.....	19.18	75.60	1.380	27.98	96.04	0.490
14.....	17.13	78.02	1.310	29.69	100.80	0.460
15.....	15.98	84.00	1.320	28.55	116.06	0.470
16.....	18.26	88.32	1.330	21.00	129.22	0.450
17.....	16.54	93.28	1.320	29.50	128.94	0.445
18.....	...	...	...	21.50	129.64	0.460
19.....	...	...	...	22.34	129.92	0.455

As was seen from Table I, the effect of sugar on the accumulation of ammonia by *A. niger* is marked. Where the sugar was absent, the organism made a rather slow growth, as shown by the weight of the mycelium, but the ammonia accumulated in large quantities, from the third till the sixteenth day, the amount increasing rapidly, so that on the sixteenth day about a half of the total nitrogen of the medium was in the form of ammonia. Where the sugar was present, the ammonia accumulated only in very small quantities, while the weight of the mycelium increased rapidly till the seventh day, when autolysis set in and the weight of the fungus body began to decrease. The amount of ammonia accumulated was small when the organism grew rapidly; but as the maximum of growth was reached, which was also accompanied by the utilization of all the sugar in the medium, the ammonia began to accumulate very rapidly. On the

[1] *J. Biol. Chem.*, 16, 121 (1913).

sixth and seventh days, when the weight of the fungus body reached its maximum, being 12 to 14 times that of the mycelium in the corresponding cultures, where sugar was absent, the amount of ammonia was one-fifth and one-fourth of that accumulated in the sugar-free culture. On the sixteenth day the weight of the mycelium in the sugar containing medium was nearly three times and the amount of ammonia about three-quarters of that of the corresponding sugar-free culture.

It is thus seen that where sugar was absent, the growth of the organism was much smaller than in the cultures containing sugar, while the ammonia content was larger particularly at the early stage of growth. We might reason from that that in the sugar-free media the organism has to decompose the peptone for its supply of carbon; the nitrogen present in the peptone molecule is in quantities much greater than that needed for the growth of the organism; the excess of nitrogen is then liberated as waste material into the medium in the form of ammonia. In the case where sugar was present, the latter was utilized for the fuel requirements of the organism and the peptone was decomposed only in so far as it was needed for the nitrogen metabolism of the organism, slight quantities of ammonia being given up to the medium as waste material. But when all the sugar was used up, the organism had to attack the peptone molecule for its carbon supply, and large quantities of ammonia were then produced as in the case of the sugar-free media. Some of the ammonia might have come in this case also from the body of the organism which was undergoing autolysis much more rapidly than in the case of the organism grown on the sugar-free media, as seen from the corresponding weights of the mycelium.

Looking through the amino-nitrogen columns, we see that a great deal more amino-nitrogen has been used up in the cultures in which sugar was present, this tending to confirm the idea that the organism uses, in the presence of sugar, as much of the protein molecule as it needs for its nitrogen metabolism; in the presence of sugar, although more of the protein molecule has been used up, as manifested by the smaller amount of amino-nitrogen present, less ammonia has accumulated in the culture than in the sugar-free medium, simply because in this case the nitrogen has been built up into the fungus protein, the mycelium being found to contain about 4 % of nitrogen.

In order to study the influence of different quantities of sugar upon the production of ammonia, the following experiment was undertaken: Five liters of medium were made up according to the formula given above. One liter was left without any sugar; to the other 4 liters cane sugar was added in quantities amounting to 1, 3, 5 and 20%. The media were distributed, sterilized, inoculated, and incubated as outlined above. At the end of 5 and 15 days the flasks were taken out of the incubator and filtered. The mycelium was washed,

dried, and weighed, as stated above. The filtrate was used for the determination of ammonia, reducing sugar and amino nitrogen as before. All determinations are calculated back to the total amount of filtrate in each flask, originally containing 100 cc. of 2% peptone solution.

TABLE II.—THE INFLUENCE OF DIFFERENT QUANTITIES OF SUGAR UPON AMMONIA ACCUMULATION BY MICROÖRGANISMS FROM 2% PEPTONE SOLUTION.

Period of incubation in days.	Sugar content. in %.	Organism used.	Mg.NH_3-N. per 100 cc. of medium.	Mg.NH_2-N. per 100 cc. of medium.	Mg. invert sugar per 100 cc. of medium.	Wt. of mycelium per 100 cc. of medium in g.
0	..	*Aspergillus niger*	0	40.60	0	0
5	0	*Aspergillus niger*	44.80	33.06	0	0.200
5	1	*Aspergillus niger*	40.74	30.21	305	0.280
5	3	*Aspergillus niger*	14.14	23.94	387	1.304
5	5	*Aspergillus niger*	1.26	19.38	470	1.500
5	20	*Aspergillus niger*	0	19.38	750	1.620
15	0	*Aspergillus niger*	73.08	32.49	0	0.360
15	1	*Aspergillus niger*	50.68	30.78	0	0.930
15	3	*Aspergillus niger*	36.54	22.80	0	3.270
15	5	*Aspergillus niger*	33.04	16.67	120	5.220
15	20	*Aspergillus niger*	0	10.83	491	11.210
15	0	*Citromyces glaber*	13.30	66.98	0	..
15	1	*Citromyces glaber*	6.44	75.53	240	..
15	3	*Citromyces glaber*	1.82	80.94	370	..
15	5	*Citromyces glaber*	0.56	67.12	430	..

The data brought out in Table II confirm the observations made in the first experiment. The larger the amount of sugar added to the medium, the less is the amount of ammonia accumulated by *A. niger*, till finally where a large excess of sugar was present, no ammonia was found in the medium even after fifteen days of incubation, although the mycelium produced on 100 cc. of medium weighed 11.210 g.; it looks as if in the presence of a large excess of sugar all the ammonia was used up for the growth of the fungus. It will be observed that, where the ammonia accumulated was least, the amount of amino-nitrogen was also small; this is probably due to the fact that, in the presence of an excess of sugar, the organism was feeding so heavily that the greatest part of the nitrogen originally added was used by the organism and built up into fungus mycelium. The amount of invert sugar present can be taken as a measure of the total sugar present in the medium, since the organism is a strong invertase producer, and most of the sugar would be inverted. We can find in Table II a direct correlation between the sugar present and the amount of ammonia accumulated in the medium, for the ammonia accumulation becomes pronounced when the sugar is exhausted.

As to the *Citromyces glaber*, which ordinarily produces small quantities of ammonia and which was selected for that purpose, the same thing holds true. The excess of sugar corresponds to a decrease in the amount of ammonia present in the medium. In the production of amino nitro-

gen, *Citromyces glaber* behaves in an entirely different manner from *A. niger;* it was found[1] that many organisms which are not able to reduce the proteins to ammonia, whether in the presence or absence of available carbohydrates, may split the proteins to amino acids which accumulate in the medium.

This experiment shows again that, when available carbohydrates are present, the organism will utilize all the nitrogen split off from the protein for its own metabolism; while in the absence of available carbohydrates, or where these have been used up, the protein molecule will be attacked not only for its nitrogen content, but also for its carbon content.

Discussion.

The results brought out in this paper will explain the peculiar effect of available carbohydrates upon the different processes taking place in the soil. The majority of soil bacteria and fungi attack the protein molecule in the soil to derive from it the nitrogen needed for their structural purposes, if available carbohydrates are present to supply the energy requirements of the microörganisms; only small quantities of ammonia are then liberated as a waste product. But when carbohydrates are absent in the soil or are present in an unavailable form, the microörganisms will attack the protein molecule not only for their nitrogen requirement, but for the carbon part of it; since the amount of carbon compounds necessary for the energy of the organism is much greater than that of nitrogen, only a small part of the protein nitrogen will be used by the organisms and the larger part of it will be liberated in the form of ammonia. The nitrifying organisms will then oxidize the ammonia to nitrates and make the nitrogen available for the higher plants. The check exerted by carbohydrates upon nitrification will be a secondary effect, the ammonia being primarily affected, and its presence or absence will determine the amount of nitrates formed. This also expains the fact observed by Lipman and Brown[2] and Hutchinson and Marr[2] that the addition of carbohydrates results in an increase in bacterial numbers, because the presence of available carbohydrates will stimulate the development of bacteria and fungi, which may also result in a greater attack upon the protein of the soil, but not in an accumulation of ammonia, since the nitrogen is built up again into bacterial or fungus protein. This may lead us to think that an addition of carbohydrates to the soil, though producing at first a checking effect upon crop production, will result in an increase in the successive crops, since the large numbers of bacteria will be able to decompose more organic matter and liberate more ammonia for utilization of succeeding crops. In the same way we may interpret the fact observed by Lipman and Brown[2]

[1] Data unpublished.

[2] *Loc. cit.*

that the accumulation of nitrates in the soil is at first checked by the addition of carbohydrates, but later the amount of nitrates formed increases; we would expect that after the small amount of carbohydrate added to the soil has been used up, the larger number of microörganisms now present would liberate more ammonia from the soil, and that this would be subsequently oxidized to nitrates.

The data obtained by Lipman and his associates[1] give ample proof in support of this theory. Where the amount of dextrose added was small, the accumulation of ammonia began to take place on the third day of incubation, with a daily increase up to the end of the experiment; where the largest amount of dextrose was added, an increase in the ammonia content was observed on the eighth day; had the period of incubation been continued long enough, no doubt that the ammonia content would have gradually increased even in the case in which the largest quantity of carbohydrate was added.

It was also mentioned in the discussion that took place after the reading of the paper of Hutchinson and Marr[1] that in Koch's experiments the second crop benefited enormously from the sugar applied. Golding also stated at that meeting that he found a very considerable increase over control in the growth of crops, where these were grown in pots of sand and soil manured with cane sugar, but the quantity of sugar was very small. He thought that the increase in plant growth was due to the direct feeding of the plants on cane sugar. The failure of Hutchinson and Marr to obtain increased crop production due to the application of carbohydrates is probably due to the fact that the low temperature prevailing, when the sugar or starch were applied, did not stimulat seed germination, while the growth of fungi, which were especially stimulated by the addition of available carbohydrates, in proximity to the seeds, might have destroyed the vitality of the seeds.

It is very possible that the addition of small quantities of available carbohydrates may stimulate plant production in the soil by increasing the numbers of microörganisms which will subsequently attack the organic matter in the soil liberating large quantities of amino acids and ammonia, which may be either directly utilized by the plants, or easily converted by other organisms, such as nitrifiers, into utilizable forms. This may hold particularly true under certain abnormal conditions, when the organic matter in the soil is in such a form that it cannot be attacked easily by the soil organisms. The addition of small quantities of available carbohydrates will stimulate bacterial growth; the bacteria will have to derive their nitrogen mainly from the organic matter of the soil, this will start the process of decomposition of the soil organic matter. When the available carbohydrates are exhausted, the bacteria will have to at-

[1] *Loc. cit.*

tack further the organic matter both for their nitrogen and, what is yet more important, for their carbon supply, thus liberating the nitrogen in the form of ammonia from the organic matter, which otherwise would be decomposed very slowly. The amounts of carbohydrate added should not be very large, otherwise the microörganisms will merely live on that source of energy, breaking up only as much of the organic matter in the soil as is needed for their nitrogen metabolism. The higher plants would in that case only lose from the addition of carbohydrates, since the microorganisms would compete with them for the available plant food in the soil and would become injurious instead of beneficial. It would appear that microörganisms will only attack the complex protein molecule, thus liberating nitrogen in a form utilizable by higher plants, when they are actually in a condition of starvation for lack of available carbohydrates.

The writer's thanks are due to Dr. T. B. Robertson and Dr. C. B. Lipman for reading the manuscript.

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NOTES.

On the Use of Large Glass-stoppered Containers in Autoclaving.—There are a large number of reactions usually carried out under pressure in sealed glass tubes. The small capacity of such tubes makes their employment for the production of larger quantities of materials costly and laborious, and one usually turns to the use of a glass enameled autoclave.

Any process in which it is necessary to heat under pressure liquids and solids in quantity may be carried out in an iron autoclave according to the following scheme: The material to be heated under pressure is placed in a glass bottle with a ground stopper. The clean, dry stopper is carefully twisted tightly into the neck of the bottle and fastened securely by a clamp of suitable design. The bottle is placed in the autoclave which is half filled with water and the apparatus then closed and heated to the desired temperature. Bottles used in this way have stood gage pressures of 5000 lbs. per sq. in., when heated in a specially designed autoclave. It is obvious that under proper conditions the internal and external pressures on the bottle are practically equal.

The autoclave constructed for us, has a maximum working pressure of 10,000 lbs. per sq. in. with a 50% factor of safety. The body of the autoclave was drop-forged from one piece of armor-plate steel and then machined.

The dimensions are as follows: Thickness of metal at all points is 2″. Inside it is 10″ in diameter and 18″ deep. Outside its diameter is 20.5″ and its height is 20″. The flange is 5″ wide. The lid is secured