The Actual Nitrogen Sources for Growth of Heterotrophic Bacteria in Non-limiting Media

By A. C. I. WARNER
*The Rowett Research Institute, Bucksburn, Aberdeenshire**

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There is a large amount of information available about the minimal nutritional needs of different bacterial species and about the ability of certain bacteria to use one compound in place of another for growth; however, very little information is available about the compounds that are actually utilized for growth when the bacteria are growing in a complex medium offering alternatives for the essential nutrients.

Animals are fairly stringently restricted in their ability to utilize various nitrogenous compounds for growth. Nevertheless, when rats were fed on ^{15}N-labelled ammonia together with their normal diet, the ^{15}N was found in many of the amino acids of their tissue proteins, including some of the amino acids known to be essential in the diet; no ^{15}N was, however, found in lysine (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg, Schoenheimer & Keston, 1939; Sprinson & Rittenberg, 1949). Different species of bacteria have widely differing nitrogenous needs, and it was thought of interest to determine what was the actual nitrogen source for growth of heterotrophic bacteria, either able or unable to use ammonia as sole nitrogen source, when growing in a medium containing peptides, free amino acids and ammonia. The only directly relevant evidence in the literature appears to be that *Escherichia coli* was found to utilize preformed amino acids in preference to synthesizing them from either carbon dioxide (Abelson, Bolton & Aldous, 1952) or glucose (Bolton & Abelson, 1952; Abelson, 1954), and that *Streptococcus faecalis* R utilized lysine peptides in addition to free lysine when growing in a partial protein hydrolysate (Pader, Melnick & Oser, 1948).

In this work, two strains of bacteria of widely differing nutritional needs were studied. In Expts. I and II the uptake of ammonia N from a complex medium was examined; in Expts. II and III the uptake of free lysine from the same medium was shown to be accompanied by an uptake of combined lysine; and in Expt. IV the effect of adding a single amino acid, lysine, to a medium containing ammonia as sole nitrogen source, was examined.

* Present address: Sheep Biology Laboratory, Prospect, N.S.W., Australia.

METHODS

Selection of bacterial species. Two species of bacteria were needed, one capable of growing well with ammonia as sole source of nitrogen but not inhibited in any way in a rich medium, the other capable of growing only in a medium supplying practically all the amino acids as such. For the latter organism, *Leuconostoc mesenteroides* strain P. 60 was chosen, as Dunn, Shankman, Camien, Frankl & Rockland (1944) had shown that it needed some 18 amino acids for anything other than very scanty growth; a strain freshly obtained from the National Collection of Industrial Bacteria was used. The selection of an organism capable of good growth both on ammonia and on a rich medium proved more difficult, and, after several unsuccessful trials had been made with various stock cultures, a suitable organism was isolated from the rumen of a sheep by enrichment culture in, successively, an ammonium–glucose–lactate–salt broth and a peptone–yeast extract–meat extract–glucose–salt broth. The organism that grew best in both these media proved to be a hitherto undescribed coliform organism, which on routine bacteriological testing had the following properties: Gram-negative rod, usually about 1·8 μ. × 0·4 μ., though the length varied from 0·9 to 7·0 μ.; motile. Fermentations: in 24 hr., arabinose, dulcitol, galactose, glucose, maltose, mannitol, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose were fermented, with the production of acid and gas. After 3 days, acid and gas were formed from lactose and a slight acid reaction was obtained with aesculin and glycerol. No fermentation of dextrin, glycogen, inulin, inositol, raffinose or starch was found after 7 days. Growth was obtained in Koser's citrate medium. Nitrates were reduced to nitrites. Litmus milk showed no change in 24 hr., but acid, clotting and reduction of litmus occurred after 5 days. The indole test was negative on the third day; the Voges–Proskauer test was positive; the methyl-red test weakly positive. Gelatin was not liquefied. Urease was not formed. H_2S was produced after 48 hr.

A subculture of this organism has been deposited with the National Collection of Type Cultures (Colindale Avenue, London, N.W. 9) where it has been given the number 9711; it is referred to by that number below.

Culture media. Medium *A* contained Oxoid bacteriological peptone, 50 g.; Oxoid Lab-Lemco, 10 g.; Oxoid yeast extract, 10 g.; glucose, 50 g.; KH_2PO_4, 25 g.; sodium acetate (hydrated), 50 g.; salt solution ($MgSO_4,7H_2O$, 10 g.; $MnSO_4,4H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·5 g.; HCl, 3 drops; water to 250 ml.), 25 ml.; tap water to 5 l., pH was adjusted to 7·0 and the whole autoclaved at 15 lb./in.^{2} for 20 min. Medium *B* contained L-lysine monohydrochloride, 0·5 g.; KH_2PO_4, 50 g.; Na_2HPO_4, 50 g.; salt solution (as above), 50 ml.; $(NH_4)_2SO_4$, 10·4 g.; tap water to 9 l., pH

was adjusted to 7·0 and the whole autoclaved at 15 lb./in.² for 20 min. and mixed with 1 l. of a 20% (w/v) glucose solution separately sterilized by filtration through a porcelain candle.

Isotopes. ^{15}N was obtained as $^{15}NH_4NO_3$ through the Isotope Division, Ministry of Supply Atomic Energy Research Establishment, Harwell. It was converted into $(^{15}NH_4)_2SO_4$ for use. Uniformly labelled [^{14}C]L-lysine was obtained from the Radiochemical Centre, Amersham.

Analyses. Ammonia was estimated according to Conway & O'Malley (1942), the NH_3 being liberated with K_2CO_3 and absorbed in the boric acid indicator solution. Total nitrogen was estimated by the micro-Kjeldahl procedure of Chibnall, Rees & Williams (1943), the NH_3 being distilled off in the apparatus of Markham (1942) and trapped in the boric acid indicator solution of Conway & O'Malley (1942). Carboxyl nitrogen was estimated by the technique of Van Slyke, Dillon, MacFadyen & Hamilton (1941), with the citrate buffer, pH 2·5, and the apparatus of Synge (1951).

Lysine was estimated by the manometric method of Gale (Gale & Epps, 1944; Gale, 1945), with a modified lysine-decarboxylase preparation (private communication from Dr G. Ellinger). In this, the medium employed for the growth of *Bacterium cadaveris* contained 50 g. of Oxoid Lab-Lemco, 50 g. of Oxoid bacteriological peptone, and 25 g. of NaCl in 5 l. of tap water; it was filtered after steaming for 30 min. at pH 8·4, the pH was adjusted to 7·8–8·0, 7·5 g. of glucose was added and the whole autoclaved at 15 lb./in.² for 20 min. After growth for 24 hr. at 26° the bacteria were separated in a Sharples Supercentrifuge, washed, made into a thick suspension and poured into 8 vol. of ice-cold acetone. After preliminary centrifuging, the deposit was washed on a Büchner funnel with acetone, acetone–ether and ether, dried at room temp. and stored in the refrigerator for 3–4 days while arginase activity disappeared. For use, the Warburg flasks held 1·0 ml. of solution containing 0·5–1·0 mg. of L-lysine at pH 6·0, 1·5 ml. of 0·2M sodium phosphate buffer (pH 6·0), and 0·5 ml. of a suspension containing 20 mg. of acetone-dried powder/ml. of buffer; when the reaction was complete, combined CO_2 was liberated with 0·25 ml. of 8N-H_2SO_4 and the results were calculated with reference to a standard curve prepared from control experiments with known amounts of L-lysine carried out at the same time.

Radioactivity was estimated by counting samples at infinite thickness with an end-window Geiger–Müller counter and 1 cm.² planchets, at least 2000 counts being made. ^{15}N was estimated by first converting the material into $(NH_4)_2SO_4$ and then into N_2, the ^{15}N abundance being determined in the mass spectrometer at the Rowett Research Institute by Dr E. I. McDougall or in the A.R.C. mass spectrometer at Reading University by Dr D. H. Tomlin.

Preparation of samples for analysis. Cultures were centrifuged in a Sharples supercentrifuge, and the deposits washed three times with water at 2°, in an M.S.E. refrigerated centrifuge, at 4500 rev./min. for 90 min. The deposits were then left to stand overnight in 5% (w/v) trichloroacetic acid (TCA) at 2°. They were then washed once with more cold TCA and treated three times with 200 ml. of 5% TCA at 90° for 10 min., followed by cooling and centrifuging, and finally washed with cold 5% (v/v) acetic acid, then with cold 1% acetic acid, after the technique of Gale & Folkes (1953). The extracted cells were hydrolysed with 500 ml. of 6N-HCl under reflux for 24 hr., and the HCl was removed by repeated evaporation *in vacuo*. The hydrolysate was then filtered and a portion removed for preparation of amide and ninhydrin nitrogen, as described below.

The remaining hydrolysate was then treated with prepared charcoal, 3 g./100 ml. of hydrolysate, according to Partridge's (1949) method. The adsorbed tyrosine and phenylalanine were eluted from the charcoal with 5% (w/v) phenol in 20% (v/v) acetic acid, and the solution, after extracting the phenol with ether, was evaporated to dryness. After extracting the phenylalanine with cold water, the tyrosine was recrystallized from hot water, and nitrogen samples were prepared after measuring the radioactivity.

The cleared hydrolysate was then put on to the ion-exchange resin Zeo-Karb 215 (Permutit Co. Ltd., London), column sizes VI and VII of Partridge & Brimley (1952); the amino acids were displaced with 0·15N-NH_4OH. As the amount of material used nearly saturated the columns, the first amino acids came off quite soon. Four or five large fractions were collected before the NH_3 front left the columns, a small fraction of about 10–15 ml. just as the NH_3 front passed out, and a further 250 ml. This last fraction was found to contain lysine free of other basic amino acids and nearly free of neutral amino acids. The fractions were tested on one-dimensional paper chromatograms, with either phenol–ammonia–water or collidine–water as solvents, after Synge (1951). The first and second fractions usually contained virtually all the glutamic and aspartic acids with some contamination of neutral amino acids: the last fraction before the NH_3 front contained neutral amino acids, mainly valine, the leucines, proline, alanine and glycine, with little contamination of either acidic or basic amino acids. These three main fractions, lysine, aspartic and glutamic acids and neutral amino acids were further purified by ionophoretic fractionation in the four-compartment cell described by Synge (1951). Short exposure times (2–3 hr.) were used for the basic and acidic amino acids, and long exposure times (6–8 hr.) for the neutral amino acids, after which the contents of the cathode compartment, the acetic acid compartment, and the specimen compartment respectively were collected. Where paper chromatograms still showed impurities, this procedure was repeated. In the last two experiments reported here, the lysine was further purified by making the picrate, recrystallizing, and regenerating lysine as the hydrochloride after Rice (1949). After measuring the radioactivity of these three fractions, nitrogen samples were prepared.

Ammonia, derived mainly from amide groups, was recovered from the original filtered hydrolysate by making alkaline with 0·5 ml. of 40% (w/v) NaOH and steam-distilling into a slight excess of H_2SO_4. The residue after this distillation was concentrated *in vacuo*, adjusted to about pH 3 with H_2SO_4 and made up to 5 ml.: 2 ml. of this solution was put in a Thunberg tube with 100 mg. of ninhydrin and 100 mg. of citrate buffer (pH 2·5) of Van Slyke *et al.* (1941). After evacuation, the tube was placed for 14 min. in a boiling-water bath, cooled and gassed three times with H_2S, shaking well each time (MacFadyen, 1944). The contents were then filtered and the precipitate was washed with buffer solution prepared by dissolving 1 g. of the above citrate buffer in 20 ml. of water. The combined filtrate and washings were made alkaline and steam-distilled into a slight excess of H_2SO_4. About 65% of the non-

amide nitrogen of the filtered hydrolysate was recovered as $(NH_4)_2SO_4$ in this way, and is referred to below as ninhydrin nitrogen; it is presumed to be representative of the amino acid nitrogen.

RESULTS

Uptake of ammonia nitrogen from rich medium A

In two experiments, Expts. I and II, $(^{15}NH_4)_2SO_4$ was added to medium *A* before sterilization at a concentration indicated in Table 1. Each 5 l. flask of medium was then inoculated with about 7–8 % of its volume of an 18 hr. culture of either organism 9711 or *Ln. mesenteroides*. In Expt. I, the two flasks were then incubated for 24 hr. without agitation, so that conditions would be relatively anaerobic. In Expt. II, a slow current of $O_2 + CO_2$ (95:5, v/v) (British Oxygen Co. Ltd.) was bubbled through the medium and then absorbed in saturated baryta solution; a solution of [^{14}C]lysine (10 μc in 0·25 mg.) was added in six portions at intervals over the 24 hr. incubation period. Analyses at the end of the incubation period are given in Table 1, and show that both organisms, though 9711 more than *Ln. mesenteroides*, produced ammonia from the culture medium. Mass spectrometer readings are given in Table 2; these show that about 6–7 % of the total nitrogen of the cells (Expt. II) and about 4 % of the nitrogen of the cell protein (Expt. I) of both organisms was derived from ammonia, to a large extent confirming with respect to the nitrogen moiety the finding of Abelson and co-workers with respect to the carbon moiety that bacteria do not synthesize amino acids when these are supplied preformed in the medium. The pattern of ^{15}N distribution in the various amino acids was essentially similar in both bacteria to that found by Foster *et al.* (1939) in the tissues of rats fed [^{15}N]-ammonia. As had been expected, *Ln. mesenteroides* took up less ^{15}N into its amino acids than did 9711; however, it took up considerably more into amide groups. No ^{15}N was incorporated in the lysine of either organism.

Uptake of free lysine from medium A

The results of the radioactivity measurements in Expt. II are given in Table 3. There was no evidence for anything more than minimal metabolism of the lysine. The activity of the lysine of the medium,

Table 1. *Analysis of medium A and cells after incubation*

Each 5 l. flask of medium contained initially 8600 mg. of total N, 485 mg. of total NH_3 N and 83 mg. of excess ^{15}N as NH_3 (Expt. I), or 8600 mg. of total N, 540 mg. of total NH_3 N and 76 mg. of excess ^{15}N as NH_3 (Expt. II); in Expt. II the total lysine content of the medium, measured after hydrolysis with 6 N-HCl for 24 hr. in a sealed tube at 105°, was 3100 mg. (590 mg. of N). The results shown are the total amounts found from the respective 5 l. flask.

	Expt. I		Expt. II	
	Organism 9711	*Ln. mesenteroides*	Organism 9711	*Ln. mesenteroides*
Ammonia N content of medium (mg.)	620	540	670	600
Total N content of washed cells (mg.)	—	—	213	200
Total N content of TCA-extracted cells (mg.)	152	155	153	143

Table 2. *Uptake of* NH_3 N *from medium A*

Incorporation of ^{15}N into the medium, cells and fractions after incubation of the two bacterial strains in medium *A* containing $(^{15}NH_4)_2SO_4$ is expressed as atom % excess ^{15}N. The normal ^{15}N abundance, measured with the Rowett Research Institute mass spectrometer, was 0·36 %; the figures have an error (95 % confidence limits) of about 5 % of the reading, except for the figures for tyrosine N and aspartic and glutamic acid N in Expt. II, where the error was probably 15–20 %; the figures for lysine N in Expt. II were determined with the mass spectrometer at Reading University, where the normal ^{15}N abundance was measured as 0·365 % with a 95 % confidence error of about 1 %.

	Incorporation of ^{15}N			
	Expt. I		Expt. II	
	Organism 9711	*Ln. mesenteroides*	Organism 9711	*Ln. mesenteroides*
Ammonia N of medium	10·8	10·7	9·0	9·8
N of washed bacterial cells	—	—	0·58	0·69
N of TCA-extracted cells	0·46	0·45	—	—
Amide N	—	—	3·4	5·8
Ninhydrin N	—	—	0·14	0·02
Lysine N	0·01	0·02	0·00	0·00
Tyrosine N	0·12	0·04	0·4	0·1
Aspartic + glutamic acid N	0·54	0·04	0·5	0·1
Neutral amino acid N	—	—	0·05	0·05

Table 3. *Metabolism and uptake of* [^{14}C]*lysine*

Radioactivity of medium, evolved CO_2 and amino acid fractions from bacterial cells, after incubation of bacteria in medium *A* containing [^{14}C]lysine (Expt. II) is given as counts/min. at infinite thickness, less background.

	Radioactivity (counts/min.)	
	Organism 9711	*Ln. mesenteroides*
Cell-free medium	143	133
$BaCO_3$	0·7	0·8
Neutral amino acids	6·8	3·2
Aspartic + glutamic acid	3·8	0·8
Lysine	1908	1621
Lysine of medium (calc.)	7700	7300

calculated from the known total lysine concentration (Table 1) and the total activity of the culture, was considerably higher than that of the lysine isolated from the cells, suggesting that unlabelled, and hence presumably peptide, lysine had been utilized as well as free lysine; synthesis of lysine from unlabelled precursors seems unlikely since no ammonia nitrogen was incorporated. Since, however, the method of calculation of the activity of the lysine of the medium was subject to considerable error, and since there was some evidence for the presence of a small amount of a non-nitrogenous impurity in the isolated cell lysine, this experiment was repeated with organism 9711 only. In Expt. III, medium *A* was used, without added [^{15}N]-ammonia and with a small (0·1 %) inoculum, but otherwise as in Expt. II. At the conclusion of the experiment, 500 ml. of cell-free medium was hydrolysed with 6N-HCl for 18 hr. under reflux, evaporated to dryness and extracted with 0·5 % (v/v) HCl in absolute ethanol (Baliga, Krishnamurthy, Ragagopolan & Giri, 1955). The extract was then evaporated to dryness, taken up in 200 ml. of water, filtered and put on to a Zeo-Karb column; lysine was isolated in the usual way. In this experiment, and in Expt. IV mentioned below, the lysine was further purified through the picrate as already described. The radioactivity of the lysine of the cells was 2600 counts/min.; the radioactivity of the lysine of the medium was 1150 counts/min. However, the ratio of free lysine to total lysine (measured after hydrolysis with 6N-HCl in a sealed tube for 24 hr. at 105°) was 1:4 at the conclusion of the experiment. It proved impossible to measure with any accuracy the initial small amount of free lysine, but the ratio of free to total amino acids, measured as the ratio of carboxyl nitrogen before and after hydrolysis as above, was 1:27 at the start of the experiment, compared with 1:5 at the conclusion, and there seems no reason to suspect that lysine would behave markedly differently from the other amino acids in this respect. Hence at all times throughout the incubation free lysine in the medium constituted less, probably considerably less, than one-quarter of the total lysine, so that the radioactivity of the free lysine would have been at least four times that of the lysine measured, probably more than twice that of the lysine isolated from the cells. Hence in the synthesis of protein, at least one peptide lysine molecule was used for every free lysine molecule. In view of the similarity of the results for the two organisms shown in Table 3, it is probable that a similar ratio would hold for *Ln. mesenteroides*.

Uptake of ammonia and of lysine from synthetic medium B

In Expts. I–III ammonia nitrogen constituted about 6 % of the total nitrogen available for growth; the effect of growing organism 9711 in a medium in which ammonia nitrogen constituted 96 % of the total nitrogen, the remainder being supplied as lysine, was investigated in Expt. IV. A volume (10 l.) of medium *B* was prepared, with $(^{15}NH_4)_2SO_4$ recovered from Expts. I and II. The inoculum was 1 ml. of a 24 hr. culture in a similar, but unlabelled, medium and incubation was continued for 40 hr. with $O_2 + CO_2$ bubbling through the medium. About 17 % of the total nitrogen of the medium was incorporated into the bacteria, about three-quarters of this remaining after TCA extraction, hydrolysis and filtration. By necessity, the largest part of the cell protein must have been synthesized from ammonia. Table 4 shows the ^{15}N abundance in the medium and the amino acid fractions of the cells after growth, and the calculated proportion of each

Table 4. *Uptake of ammonia* N *from medium B by organism* 9711

A very small inoculum of organism 9711 was grown in 10 l. of medium *B* containing initially 2200 mg. of total ammonia N (labelled with ^{15}N) and, by calculation, 77 mg. of lysine N. After growth, the washed bacterial cells contained 380 mg. of N, of which 280 mg. remained after TCA extraction, hydrolysis and filtration (Expt. IV). The readings were made with the mass spectrometer at Reading University, and have a 95 % confidence error of about 1 %, except for the figures for amide N and ninhydrin N, which were determined with the Rowett Research Institute mass spectrometer, which had an error of about 5 %. All the readings were made after incubation.

	^{15}N content (atom % excess)	% N derived from NH_3 of medium
Ammonia of medium	8·97	(100)
Amide N	7·84	88
Ninhydrin N	8·24	92
Neutral amino acid N	8·67	97
Tyrosine N	8·64	96
Aspartic + glutamic acid N	7·20	81
Lysine N	0·164	1·8

nitrogen fraction derived from the ammonia of the medium, assuming that no lysine was converted into ammonia during growth. It can be calculated that at least one-third of the lysine of the medium was incorporated into cell protein, either as such or as other amino acids, etc. Since there had obviously been metabolism of the lysine of the medium, it is probable that its final concentration was very low, though it was not found possible to detect lysine at such a low concentration in a high-glucose, high-ammonia medium; it is hence possible that the small amount of ammonia nitrogen incorporated into the cell lysine was due to the virtual exhaustion of the lysine in the medium. The pattern of distribution of ^{15}N in these cells is almost the reverse of that found in Expts. I and II, that is, the ^{15}N abundance is lowest in aspartic and glutamic acid nitrogen and in amide nitrogen, and high in tyrosine nitrogen and neutral amino acid nitrogen; this is consistent with lysine nitrogen entering the nitrogen pool of the cells by a route involving aspartic or glutamic acid in an early stage, possibly followed by amide nitrogen.

DISCUSSION

The close similarity in actual behaviour towards the components of the culture medium of two micro-organisms of such widely differing minimal nutritional requirements is striking. The only major difference appears to be in the amide groups, which in *Ln. mesenteroides* were nearly in equilibrium with the external ammonia, whereas in 9711 much unlabelled nitrogen had been incorporated; possibly *Ln. mesenteroides* was more restricted in the sources of amide nitrogen available to it. The utilization of preformed amino acid in preference to synthesis was, with lysine and organism 9711, very nearly absolute; in a medium containing 96% of its nitrogen as ammonia, about one-sixth of the total nitrogen was used for growth, yet not only was the preformed lysine used almost exclusively in the formation of cell lysine, but it was also used to some extent in the synthesis of other amino acids; despite this, the addition of this amount of lysine to the culture medium made no apparent difference to either the rate of growth of the organism or to the total amount of cell material finally synthesized.

Previous work on the utilization of peptides by bacteria deals mainly with the response of nutritionally exacting organisms to peptides containing an amino acid essential to their growth [see, for example, Fruton & Simmonds (1949), Waelsch (1952) and Kihara & Snell (1955)], and to this the finding mentioned above that *Ln. mesenteroides*, known to be exacting towards lysine, utilizes lysine peptides, is completely analogous; indeed, it is probably little more than chance that led Pader *et al.* (1948) to use *Streptococcus faecalis* rather than *Ln. mesenteroides* as test organism for the estimation of lysine in partial protein hydrolysates. The finding that a coliform organism, capable of synthesizing all its amino acids from ammonia, also utilizes lysine peptides as well as free lysine is a little more unexpected, and suggests that the phenomenon is a much more general one than had been supposed. Perhaps the hypothesis, recently re-stated by Fisher (1954), that peptides rather than amino acids are the 'normal currency' of protein metabolism, that much of the absorption and metabolism of amino acids occurs when they are conjugated as peptides, applies to micro-organisms in appropriate circumstances.

SUMMARY

1. *Leuconostoc mesenteroides* P. 60, needing 18 amino acids for growth, and a coliform organism, N.C.T.C. 9711, shown to grow well with either ammonia or peptone, etc., as nitrogen source, were grown in a medium containing salts, yeast and meat extracts, bacteriological peptone and added L-[^{14}C]lysine and [^{15}N]ammonia, and the synthesized proteins were isolated, hydrolysed and analysed.

2. The ^{15}N abundance was very high in the amide nitrogen, particularly of *Ln. mesenteroides*, and several amino acids had a low content of ^{15}N, but none was found in lysine.

3. The [^{14}C]lysine was incorporated at a level of radioactivity lower than that of free lysine in the medium, suggesting that both bacteria utilized peptide in addition to free lysine.

4. When the coliform organism was grown in a medium containing salts, glucose, lysine and [^{15}N]-ammonia only, a very small amount of ^{15}N was incorporated into the lysine of its cell protein.

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A Possible Role of Pantothenic Acid in Diabetic Cholesterologenesis

By S. MOOKERJEA* and D. P. SADHU
Department of Biochemistry, Nagpur University, Nagpur, India and Department of Physiology, Bengal Veterinary College, Calcutta, India

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The mechanism of cholesterol synthesis in diabetes has been studied by Hotta & Chaikoff (1952), who reported a tenfold increase in the conversion of labelled acetate into cholesterol in liver slices from alloxan-diabetic rats. The investigation of Brady & Gurin (1950) shows that in diabetes lack of insulin prevents conversion of the short-chain into long-chain fatty acids and therefore results in accumulation of the short-chain acids as the fat depots become depleted. Their investigation indicates that the accumulation of these substances accentuates the formation of cholesterol in diabetes as an alternative path of acetate metabolism. The relationship between the capacity of tissues to form acetoacetate and cholesterol synthesis has also been stressed (Gurin & Brady, 1951). Another observation (Van Bruggen, Yamada, Huchens & West, 1954), however, shows that although in the intact rat fatty acid synthesis from [1-^{14}C]acetate is impared in alloxan diabetes, the incorporation of labelled acetate into liver, gut, carcass and skin cholesterol is unchanged, suggesting unaltered cholesterologenesis.

Our previous findings indicate a relationship between decreased oxidation of acetate and acetoacetate and increased cholesterol synthesis in hypothyroidism (Mookerjea & Sadhu, 1954*a*, 1955*b*), nephrosis (Sadhu & Mookerjea, 1955) and chronic poisoning by malonate and arsenite (Mookerjea & Sadhu, 1955*c*), but similar studies in diabetes (Mookerjea & Sadhu, 1956) appear to show a less clear picture. There is a small rise of cholesterol only in a prolonged state of diabetes of 6–7 weeks. Liver slices, however, show decreased oxidation of acetate and a considerable increase in ketogenesis. This, together with ketonuria and ketonaemia, the characteristic features of diabetes, shows that the availability of acetate and acetoacetate, the potential precursors of cholesterol, is increased in diabetes, but the less obvious rise of cholesterol indicates that they are not completely used for cholesterol synthesis.

The role of pantothenic acid in cholesterol synthesis is well established. Pantothenic acid deficiency has been shown to cause a decrease in tissue cholesterol due to lack of coenzyme A (Klein & Lipmann, 1953; Mookerjea & Sadhu, 1954*b*). The interesting observations by Migicovsky & Greenberg (1954) show the importance of relative requirement of both acetate and coenzyme A for cholesterol synthesis. They have shown that even in small amounts coenzyme A behaves as an inhibitor of cholesterol synthesis in the presence of relatively low acetate concentrations. When the acetate concentration is raised, the same amount of coenzyme A increases cholesterol formation. It appears from this that both these factors, namely availability of coenzyme A and size of acetate pool, are necessary for the synthesis of cholesterol.

The observations of Ralli & Dumm (1953), showing a great rise of adrenal cholesterol when excess of pantothenate is fed, further prompted us to investigate the importance of adequate levels of pantothenic acid for diabetic cholesterologenesis.

EXPERIMENTAL

Forty-eight male albino rats weighing 100–136 g. were fed with stock laboratory diet containing 70% of whole-wheat flour, 17% of whole-milk powder, 6% of groundnut oil, 2·5% of dried brewer's yeast, 2·5% of crude casein and 2% of salt mixture. Two drops of cod-liver oil was fed to each animal twice weekly. The average food intake/day/animal

* Present address: Post-doctorate Fellow, Atlantic Regional Laboratory, National Research Council, Halifax, N.S., Canada.