medium during growth on maltose. This phenomenon is good presumptive evidence that malto-

phosphorylase is responsible for the initial stages of maltose metabolism by these lactobacilli. Presumably, the β -glucose 1-phosphate produced is metabolized by the cells, but this work gives little indication of its subsequent transformation. The infrared spectra of the preparations of bacterial β -glucose 1-phosphate indicate that they are free from the α -anomer, which might be expected as a contaminant if the next step in the transformation led to the production of α -glucose 1-phosphate.

The maltophosphorylase mechanism appears to be prodigal of carbohydrate, in that a molecule of glucose is rejected for every molecule of maltose utilized. However, it carries with it the biological advantage of sparing ATP, in that a molecule of phosphorylated glucose is produced without expenditure of ATP.

It is perhaps noteworthy that the natural habitat of these lactobacilli (beer and brewer's malt wort) contains maltose as the predominant sugar, so that the maltophosphorylase system may have been elaborated by them in response to prolonged culture in the presence of maltose.

SUMMARY

1. During the growth of six strains of beer lactobacilli on maltose, glucose accumulated in the medium.

2. Cell-free extracts of L1, a strain which grew

readily only on maltose, contained a maltophosphorylase, catalysing the reversible reaction:

3. The enzymically prepared sugar phosphate resembled authentic and synthetic samples of β -glucose 1-phosphate in physical properties, infrared spectrum and in its enzymic behaviour.

4. Maltophosphorylase was without phosphorolytic action on α -D-glucose 1-phosphate.

5. The preparation and some properties of β -glucose 1-phosphate (dipotassium salt) are described.

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The Cytidine Diphosphate Choline Content of Rat Brain

BY G. B. ANSELL AND BRENDA J. BAYLISS

Department of Experimental Psychiatry, The Medical School, Birmingham 15

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The importance of cytidine nucleotides in the biosynthesis of the di-ester linkage in phospholipid molecules has been increasingly recognized in recent years. Kennedy and his co-workers have unequivocally demonstrated the reactions by which phosphatidylcholine (Kennedy & Weiss, 1956) and sphingomyelin (Sribney & Kennedy, 1958) are synthesized by the reaction of cytidine 5'-diphosphate choline with a D- α -diglyceride and N-acylsphingosine respectively. Similarly, cytidine diphosphate ethanolamine reacts with a D- α -diglyceride to yield phosphatidylethanolamine (Kennedy & Weiss, 1956). The biosynthesis of

some other phospholipids is also dependent on cytidine derivatives [see, for example, Paulus & Kennedy (1959)]. No other nucleotide base can replace cytosine, although within the nucleotide D-ribose can be replaced by D-2-deoxyribose (Kennedy, Borkenhagen & Smith, 1959).

Studies on the cytidine nucleotide content of nervous tissues have shown that they account for only about 3% of the total nucleotides present (Mandel & Harth, 1957). Koransky (1958) in an elaborate study of rat-brain nucleotides concluded that cytidine nucleotides were absent from brain tissue; he could not isolate cytosine after total

degradation of the compounds present. In a personal communication, however, he has subsequently stated that small amounts of cytidine nucleotides are present; Miani (1956-57) has located both cytidine diphosphate choline and cytidine diphosphate ethanolamine on two-dimensional chromatograms of the total free nucleotides of brain tissue. It is apparent from the investigations of McMurray, Strickland, Berry & Rossiter (1957) that cytidine triphosphate is essential for the synthesis of cerebral lecithin via cytidine diphosphate choline, and recent work by the same group (Rossiter, Thompson & Strickland, 1960) indicates that this is also true for cerebral phosphatidylethanolamine.

In view of the pivotal position of cytidine diphosphate choline in lecithin and sphingomyelin biosynthesis it seemed of interest to determine its concentration in nervous tissue, with a method suitable for relatively small amounts of tissue. A preliminary communication has already been made (Ansell & Bayliss, 1960).

EXPERIMENTAL

Materials. Cytidine diphosphate choline, cytidine diphosphate ethanolamine, cytidine 5'-monophosphate, 2-deoxycytidine 5'-monophosphate and cytosine were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Phosphorylcholine was prepared as the calcium salt by the method of Plimmer & Burch (1937) and freed from calcium by passing through a column of Amberlite IRC-50 resin (H^+ form). Celite 535 was purchased from Johns Manville and Co. Ltd., 20 Albert Embankment, London, S.E. 11. A generous gift of Norit-SX-Plus charcoal was made by Haller and Phillips Ltd., 14 Wool Exchange, Basinghall St., London, E.C. 2; this was boiled with $N-HCl$, washed first with water and then with 10% (v/v) *sec.*-octanol in ethanol (Threlfall, 1957).

[^{32}P]Orthophosphate in sterile buffered solution, containing carrier P, was purchased from The Radiochemical Centre, Amersham, Bucks.

Miscellaneous methods. Phosphorus was estimated by either the method of Fiske & Subbarow (1925) for amounts of 10-50 $\mu g.$, or that of Berenblum & Chain (1938) for amounts of 1-10 $\mu g.$ Phosphorus compounds on chromatograms were detected by spraying with the reagent of Hanes & Isherwood (1949), drying at room temperature and exposing to unfiltered light from a Hanovia mercury-vapour lamp. Nucleotides were located on chromatograms by reflex photography on Kodak Duostat paper; the light source was conveniently provided by a Phillips OZ4 w lamp (unfiltered). After elution from chromatograms with dilute HCl , nucleotides could be estimated by reading the extinction at 260 and 280 $m\mu$, and correcting for the extinction given by an eluate of a blank area.

Separation of cytidine diphosphate choline from brain tissue. Young rats (body weight 50 g.) were frozen whole in liquid N_2 and the frozen brain (5 g.) was extracted by one of the following procedures.

(a) The frozen tissue was homogenized in a mechanical blender with 10 vol. of ice-cold 10% trichloroacetic acid

solution. After centrifuging, the extract, together with a single washing, was shaken with successive equal volumes of ether until the pH was about 4. After adjustment of the pH to 8.5-9.0 with aq. 3*N*- NH_3 soln., the solution was filtered on to a column of Dowex-1 (Cl^- form) (see below). If necessary the solution could be stored at 0° at pH 7.0.

(b) Frozen tissue was homogenized with 10 vol. of ice-cold ethanol-water (2:1, v/v) (Kennedy & Weiss, 1956), the ethanol extract was centrifuged and then, together with washings, was reduced to about one-third of the original volume under reduced pressure at a temperature < 40°. After centrifuging, the supernatant was extracted with ether, the pH adjusted to 8.5-9.0, and the supernatant filtered as in (a).

The extract was slowly run through a short column of Dowex-1 (Cl^-) ion-exchange resin (50-100 mesh), followed by 30 ml. of water. Measurement of the absorption of the effluent at 260 $m\mu$ at pH 1 (the maximum for adenine nucleotides which predominate in brain) showed that at least 95% of the nucleotide material in the original extract had been retained by the resin. Elution of the resin with 100 ml. of 0.1*M*- NH_4Cl -aq. 0.1*M*- NH_3 soln. brought off a small amount of material absorbing at 280 $m\mu$ after acidification, and the acidified eluate was passed immediately through a short column of Norit-SX-Plus charcoal (0.3 g.) mixed with Celite 535 (1.5 g.) (cf. Threlfall, 1957) to remove nucleotides. The charcoal column was washed with water until the effluent was neutral, and the nucleotides were then eluted with 30 ml. of ethanol-water (1:1, v/v) containing aq. 0.3% NH_3 soln. After removal of solvent under reduced pressure at a temperature < 40°, the nucleotides were subjected to two-dimensional chromatography on Whatman no. 1 paper (unwashed) in ethanol-acetic acid (Kennedy, 1956), and ethanol-ammonium acetate-ethylenediaminetetra-acetic acid (EDTA) (Berger & Gimenez, 1956). The position of cytidine diphosphate choline was located with an appropriate marker and was clearly separated from a number of other nucleotides (see Results section).

Experiments with radioactive phosphorus. [^{32}P]Orthophosphate (usually 50 μc) was injected into the subarachnoid space of young rats, essentially as described by Lindberg & Ernster (1950), except that the rats were lightly anaesthetized with 2:2:2-tribromoethanol and 2-methylbutan-2-ol (Avertin, Winthrop Laboratories, New York) to facilitate the procedure. The rats recovered within 30 min. and a high incorporation of radioactive P was found in the brain nucleotide fraction after a few hours. Counting of ^{32}P was carried out in M6 liquid counting tubes (20th Century Electronics Ltd.) with conventional ancillary equipment. Samples were assayed for radioactivity either in dilute HCl or, if the samples had been wet-ashed with perchloric acid, in 0.7*N*-perchloric acid. Radioautographs of chromatograms were prepared with Kodak Blue Brand No-Screen X-ray film.

RESULTS

Identification of cytidine diphosphate choline in brain tissue

When the total nucleotides from rat brain were adsorbed on Dowex-1 (Cl^- form) and the column was eluted with 0.1*M*- NH_4Cl -aq. 0.1*M*- NH_3 soln.,

only a small amount of the total nucleotides were eluted. Calculation on the basis of an $\epsilon_{280\text{ m}\mu}$ of 14 200 showed that the material eluted by 70–80 ml. amounted to only 10% of the nucleotides present in the original extract. Comparison, between an extract equivalent to 1.859 g. of tissue and an identical extract containing added synthetic cytidine diphosphate choline (0.94 μ mole), indicated that this nucleotide was completely eluted with NH_4Cl -aq. NH_3 soln. The result of this experiment is shown in Fig. 1, in which the absorption of the fractions at 280 $\text{m}\mu$ (the maximum for cytidine nucleotides) is plotted against the volume of the eluate. In this particular experiment the recovery amounted to only 71% but subsequent experiments demonstrated recoveries of 80–100%. It was concluded that 70–80 ml. of 0.1M- NH_4Cl -aq. 0.1M- NH_3 soln. would elute all the cytidine diphosphate choline in a brain extract and separate it from 90% of the other nucleotides. Both cytidine diphosphate-ethanolamine and cytidine 5'-monophosphate were also eluted but could be separated at a later step.

After adsorption of this fraction on charcoal, elution with ammoniacal 50% ethanol and two-dimensional paper chromatography of the nucleotides in ethanol-acetic acid and ethanol-ammonium acetate-EDTA a number of substances were shown to be present both by ultraviolet photography and by radioautographs. The largest spot,

to judge from the $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$ ratio at pH 1, seemed to be an adenine nucleotide. The position of cytidine diphosphate choline was first determined by adding synthetic material as a marker. In ethanol-acetic acid its R_f value was 0.68 and in ethanol-ammonium acetate-EDTA, 0.43. Cytidine 5'-monophosphate ran to the same position as cytidine diphosphate choline in ethanol-acetic acid but had an R_f of 0.28 in the second solvent. Cytidine diphosphate ethanolamine had an R_f of 0.57 in ethanol-acetic acid and 0.33 in the second solvent. The identity of the synthetic material with that present in the brain was further established by the following evidence:

(1) Synthetic cytidine diphosphate choline ran to the position of that present in brain in a variety of chromatographic solvents.

(2) An eluate of the spot located by means of a radioautograph was hydrolysed in N-HCl at 100° for 1 hr. (Kennedy, 1956); the hydrolysate was dried and run in one dimension in ethanol-ammonium acetate-EDTA (Berger & Gimenez, 1956). Two radioactive spots appeared in the position of cytidine 5'-monophosphate (R_f 0.26) and phosphorylcholine (R_f 0.5). The single ultraviolet-absorbing area on the chromatogram corresponded exactly with that of the radioactivity in the cytidine 5'-monophosphate position; this spot was eluted and heated with 12N-perchloric acid at 100° for 1 hr. (Marshak & Vogel, 1951), to free nucleotide bases. This hydrolysate was diluted and freed from perchloric acid by adding KOH soln. until the neutral point was reached, and, after removal of insoluble KClO_4 , the solution was passed over a column of Dowex-1 (Cl^-) and washed through with water. Elution of adsorbed material with 0.1N- HCl , evaporation to dryness *in vacuo* and subsequent chromatography in propan-2-ol- HCl (Wyatt, 1951) revealed the presence of only one nucleotide base, cytosine. In an experiment in which an amount of material was separated from 145 g. of ox brain, sufficient cytidine 5'-monophosphate and phosphorylcholine were obtained on hydrolysis to permit quantitative estimation of the molar ratio of phosphorus, which was 1:1. The number of μ moles of cytidine 5'-monophosphate calculated from the absorption at 280 $\text{m}\mu$ corresponded exactly to that calculated from a phosphorus estimation.

(3) The radioactive material obtained after fractionation of a brain extract, to which had been added 0.8 μ mole of synthetic cytidine diphosphate choline, was eluted from the final chromatogram, and the amounts of radioactivity and total material were determined. It was then adsorbed on to a column of Dowex-1 (Cl^-) when all the radioactivity was retained. Elution of the column with 0.1M- NH_3 soln.-0.1M- NH_4Cl and subsequent radioactive

Fig. 1. Elution of brain nucleotides from Dowex-1 (Cl^-) with 0.1M- NH_4Cl -aq. 0.1M- NH_3 soln. O, Brain extract ($\approx$ 1.86 g. of tissue); ●, brain extract ($\approx$ 1.85 g. of tissue) + 0.94 μ mole of cytidine diphosphate choline.

and spectrophotometric assay of 10 ml. fractions showed that the specific radioactivity was virtually unchanged (Fig. 2). Recovery of material added originally to the column both in terms of radioactivity and material absorbing at $280\text{ m}\mu$ amounted to 89%.

(4) It was considered that the cytidine diphosphate choline might be mixed with 2-deoxycytidine diphosphate choline, which has been found in animal tissues (Kennedy *et al.* 1959). To investigate this possibility $0.28\text{ }\mu\text{mole}$ of cytidine diphosphate choline obtained from ox brain were hydrolysed with N-HCl at 100° for 1 hr., and the hydrolysate, after being freed from HCl , was subjected to paper chromatography in ammonium acetate-sodium tetraborate-ethanol-EDTA (Reichard, 1958). Only one ultraviolet-absorbing spot was obtained corresponding in position to cytidine 5'-monophosphate (R_f 0.21). Synthetic 2-deoxycytidine 5'-monophosphate ran to a different position (R_f 0.41). A further sample ($0.99\text{ }\mu\text{mole}$) of cytidine 5'-monophosphate from the hydrolysate was tested for the presence of deoxyribose by the method of Brody (1953). Although deoxycytidylic acids react in this modified Dische reaction to give a lower colour yield than deoxyadenylic acids, the colour yield of synthetic deoxycytidine 5'-monophosphate ($\epsilon_{474\text{ m}\mu}$ 1300) was sufficient to show that the amount of deoxy sugar in the material from brain was less than 3%.

Fig. 2. Elution of radioactive brain cytidine diphosphate choline in the presence of synthetic cytidine diphosphate choline from Dowex-1 (Cl^-). Eluent, $0.1\text{ M-NH}_4\text{Cl-aq. } 0.1\text{ M-NH}_3$ soln. ○, Radioactivity in counts/min.; ●, $E_{280\text{ m}\mu}^{1\text{ cm.}}$.

Concentration of cytidine diphosphate choline in brain tissue

Recovery of synthetic material added to brain extracts and eluted from Dowex-1 (Cl^-) was 80–100%. Subsequent acidification, adsorption on charcoal and elution with ammoniacal 50% ethanol did not lead to any further loss, but there was a considerable loss during the subsequent transference and chromatography. The average final recovery was $60 \pm 2\%$ (S.E.M.), and the amount in young rat brain, calculated from recovery in seven experiments, was 0.029 ± 0.012 (S.D.) $\mu\text{mole/g.}$ of brain. Additional experiments were carried out in which radioactive extracts were divided into two equal parts and carrier cytidine diphosphate choline added to one part. Calculation of the amount present from the resultant specific radioactivities of the separated cytidine diphosphate choline agreed with amounts calculated from the recovery. There was no evidence that there was any rapid post-mortem loss of the compound when extracts from the brains of animals frozen whole in liquid nitrogen were compared with extracts prepared quickly from decapitated animals.

DISCUSSION

From the investigation described in this paper it is apparent that cytidine diphosphate choline represents only a tiny fraction of the free nucleotides in rat brain and a very small proportion of the water-soluble choline derivatives. Phosphorylcholine is present at a concentration of $0.3\text{ }\mu\text{mole/g.}$ (Dawson, 1955a; Porcellati, 1958; Ansell & Spanner, 1959), and in addition there are measurable amounts of glycerylphosphorylcholine (Dawson, 1955b) and acetylcholine.

In the liver the cytidine diphosphate choline concentration is $0.1\text{ }\mu\text{mole/g.}$ (Kennedy & Weiss, 1956) [$0.05\text{ }\mu\text{mole/g.}$ according to a more recent publication by Wilgram, Holoway & Kennedy (1960)]. This contrasts with $2\text{--}4\text{ }\mu\text{moles/g.}$ for phosphorylcholine (Dawson, 1955a) and $0.08\text{--}0.8\text{ }\mu\text{mole/g.}$ for glycerylphosphorylcholine (Dawson, 1955b).

According to S. Harth & P. Mandel (unpublished work) cytidine 5'-monophosphate is the predominant cytidine compound in brain tissue. Although this material would be eluted from Dowex-1 (Cl^-) by the eluent described here, there was no evidence of any considerable concentration of the material on chromatograms. Small amounts of cytidine diphosphate ethanolamine could be detected though there was an 80% loss of synthetic material in this procedure.

In view of the fact that sphingomyelin formation is most intense in the 2- to 4-week-old rat it might

be expected that the concentration of cytidine diphosphate choline would be higher in young than in adult animals, but no evidence for this was obtained. The phosphorylcholine concentrations in young rat brain (Ansell & Spanner, 1959) did not differ significantly from those in adult animals (Dawson, 1955a; Porcellati, 1958).

In view of the rapid incorporation of [^{32}P]orthophosphate into cerebral phosphorylcholine *in vivo* (Ansell & Spanner, 1959) and the steady formation of phosphatidylcholine (Ansell & Dohmen, 1957; Ansell & Spanner, 1959), there should be a very high turnover of the cytidine diphosphate choline to account for the transfer of phosphate to the D- $\alpha\beta$ -diglyceride. It also seems likely that either the enzymic reaction leading to the formation of cytidine diphosphate choline or the reaction leading to the formation of the complete phosphatidylcholine molecule is affected in insulin hypoglycaemia (Ansell & Spanner, 1959) and thiopentone anaesthesia (Ansell, 1960). In any event the role of those cytidine molecules concerned with phospholipid synthesis in cerebral tissues would appear to be a central one, although turnover studies *in vivo* are difficult in view of the small amount present.

SUMMARY

1. A method for the separation of cytidine diphosphate choline from brain tissue is described.

2. Recovery of added cytidine diphosphate choline amounted to about 60% and the amount present in rat brain was calculated to be about 0.03 $\mu\text{mole/g.}$ of fresh tissue.

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Techniques in Tissue Metabolism

5. CHOPPING AND SLICING TISSUE SAMPLES*

By H. McILWAIN

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

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Chopping and slicing tissue samples for metabolic work have as their objective the preparation of portions small enough to receive metabolites by diffusion, but with minimal damage to cell struc-

ture. They are therefore distinct from the blending, grinding and homogenizing which typically yield cell-free systems. Description of apparatus and procedures for chopping and slicing are relatively rare, in comparison with those for blending and homogenizing (Allfrey, 1959, includes over 40 of

* Part 4: Heald & McIlwain (1956).