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Biochem. J. (1961) **78**, 204

The Maltophosphorylase of Beer Lactobacilli

BY B. J. B. WOOD AND C. RAINBOW
Department of Applied Biochemistry, University of Birmingham*

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The observation that certain beer lactobacilli grow better on maltose than on glucose (Moore & Rainbow, 1955) indicates that these bacteria metabolize maltose by a process with initial steps that do not involve the intervention of maltase. Since then, two other beer lactobacilli 'preferring' maltose for growth have been found by us. One of these (L 1), which grew rapidly and well only on maltose, of many carbohydrate substrates tested, was selected for further study as being likely to possess a restricted system of carbohydrate-metabolizing enzymes, and therefore to be uncomplicated with respect to the means by which the initial steps of maltose breakdown take place.

EXPERIMENTAL

Materials

β-Maltose. This was the monohydrate (T. Kerfoot and Co. Ltd., Vale of Bardsley, Lancs.): it was chromatographically almost free from other reducing sugars.

α-D-*Glucose* 1-*phosphate* (*dipotassium salt*). This was synthesized by a modification of Hanes' (1940) enzymic method: it was crystalline material containing less than 0·05% of inorganic P.

β-D-*Glucose* 1-*phosphate.* This was synthesized chemically as the barium salt from α-acetobromoglucose by the method of Reithel (1945) and converted into the dipotassium salt by ion exchange as described below. We are also indebted to Dr W. Z. Hassid, University of California, for a gift of a derivative of authentic β-glucose 1-phosphate, which was converted into the dipotassium salt for infrared examination.

Enzymes. Glucose oxidase and catalase (ex liver, 20 mg./ml.) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Yeast autolysate. This was prepared by incubating a suspension of pressed yeast in an equal weight of water overnight at 45°. After separating the supernatant by centrifuging, it was autoclaved at 15 lb./in.^{2} for 15 min. with 1% of filter-aid and then filtered.

Organisms. These were the strains of lactobacilli designated L3, L4, L5 and L6 by Moore & Rainbow (1955) and also strains L1 and L2. L3 and L4 are strains of *Lactobacillus brevis*, and we are grateful to Dr M. Elizabeth Sharpe for carrying out a confirmatory identification of L4. L5 and L6 are also probably strains of this species, but L1 and L2 are unidentified lactobacilli isolated from brewer's top fermentation pitching yeast by Dr D. Kulka of this Department. Stock cultures of all strains were carried as stabs in malt agar.

Media. All strains were grown on the following medium (composition/l.): yeast autolysate, 100 ml.; casein acid hydrolysate, 10 g.; β-maltose monohydrate, 20 g.; KH_2PO_4 (A.R.), 0·5 g.; K_2HPO_4 (A.R.), 0·5 g.; $MgSO_4,7H_2O$ (A.R.), 0·1 g.; $MnSO_4,4H_2O$ (A.R.), 5 mg.; $FeCl_3$ (A.R.), 1 mg.; KOH soln. to pH 5·0. After dispensing in suitable vessels, the medium was sterilized by autoclaving at 15 lb./in.^{2}, momentarily (for volumes less than 50 ml.) or for 15 min. (for volumes exceeding 50 ml.).

Cultural methods

Batches of cells were usually grown in two portions, each of 750 ml., contained in 1 l. conical flasks. Each portion was inoculated with 5 ml. of a culture obtained by transferring from a stock stab to a 10 ml. portion of medium in

* Now Department of Biochemistry.

a test tube, which was then incubated for 2 days at 28°; the flasks were incubated at 28° for 5–7 days and shaken by hand once daily. The cells were harvested by centrifuging, combined and washed three times with sterile 0·85% NaCl soln.

Cell extracts

These were prepared from the washed cells in suspension in 8 ml. of sterile water by mechanical disintegration with Ballotini beads (0·2–0·3 mm. diam.) in a tissue disintegrator (H. Mickle, Gomshall, Surrey). Before shaking, the suspension and beads were cooled in ice, and then submitted to alternate cycles of shaking (5 min.) and cooling (5 min.) until a total time of shaking of 30–45 min. had elapsed. Cell residues and extract were then washed from the beads by successive portions of ice-cold water (50 ml. in all) and centrifuged for 20 min. at 2°. The residue, which had no relevant enzyme activity, was discarded and the supernatant frozen overnight. On thawing, a precipitate having no relevant enzyme activity was centrifuged off and the clear supernatant cell-free extract was used for enzyme experiments, usually within 2 days, although the extract retained much of its activity for months when stored at −20°. Extracts thus prepared contained about 160 μg. of total nitrogen/ml., as determined by micro-Kjeldahl.

Analytical methods

Paper chromatography. This was carried out as conventional unidimensional, descending, double runs (Jeanes, 1951) with butan-1-ol–ethanol–water (4:1:5) on Whatman no. 4 paper. Sugars were located by the aniline hydrogen phthalate reagent of Partridge (1949) and sugar phosphates by that of Hanes & Isherwood (1949).

Ion exchange. This was applied to convert barium hexose phosphate into the corresponding dipotassium salt. Columns (20 cm. × 1·2 cm.) were charged with Amberlite IR-120 resin in the H^+ form. The resin was converted into the K^+ form by treatment with aqueous 5% (w/v) KCl soln. (about 1·5 l.) until a neutral effluent was obtained. This treatment was followed by washing with water (about 2 l.) until a chloride-free effluent was obtained. An aqueous solution of barium hexose phosphate (approximately 0·5%, adjusted according to content of acid-labile phosphate) was then passed through the column at the rate of about 100 ml./hr., followed by 30 ml. of water, and the effluents were collected. The K^+ form of the resin was regenerated for further use by treatment with 5% KCl soln. until the effluent no longer gave a white precipitate with dil. H_2SO_4.

Reducing sugar. This was determined colorimetrically by the method of Schales & Schales (1945).

Esterified phosphate. This was determined as the difference between the inorganic P content of untreated samples and that of samples which had been heated for 7 min. in a boiling-water bath in the presence of N-HCl. Inorganic P was determined by Allen's (1940) method, the colour developed being measured against a reagent blank as reference solution in the EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with an OR-1 filter. Instrument readings were converted into quantity of inorganic P by reference to a standard curve previously constructed by submitting known concentrations of KH_2PO_4 (A.R.) to the Allen procedure.

Infrared spectra. These were carried out in a KCl disk with the Perkin–Elmer model 21 infrared spectrophotometer by Dr D. H. Whiffen and Dr K. J. Morgan of the Chemistry Department, University of Birmingham, to whom we are greatly indebted.

RESULTS

Growth of all strains on maltose. During growth on maltose medium, paper chromatography showed that, within 7 days, the maltose was almost completely removed by all strains. Simultaneously, a hexose (probably glucose), which was demonstrably absent from the inoculated culture medium, accumulated. Maltose metabolism by all strains thus appeared to involve a cleavage of the molecule to a metabolizable fragment and to glucose, which was rejected, wholly or in part, by the cells.

Further experiments to elucidate the nature of this cleavage were carried out with L 1 for reasons stated below.

Properties of strain L 1. L 1, the strain mostly used in this work, possessed unusual properties. Thus in the semi-defined CR medium of Chamberlain & Rainbow (1954) it grew well only on maltose and failed to grow on other disaccharides, hexoses, pentoses and pentose–hexose mixtures, except glucose–ribose, on which it grew relatively poorly after prolonged incubation. Best growth on maltose was obtained in CR medium adjusted initially to pH 4·8–5·0; growth took place also at more acid pH values, but not at pH values more alkaline than 5·2.

Because cells of L 1 failed to grow on glucose, it seemed unlikely that their utilization of maltose would depend on its hydrolysis to glucose by maltase. This was confirmed by incubating a cell-free extract of L 1 at 28° for 16 hr. at pH 6·8 with maltose: no glucose production was detected by paper chromatography. L 1 was therefore selected as the strain most suitable for studying the initial stages of maltose metabolism by our lactobacilli, since the situation in this case was unlikely to be complicated by the presence of maltase.

Action of extracts of L 1. Having rejected maltase activity as a factor in maltose cleavage by L 1, three other possibilities were considered: (*a*) a reaction of the type n maltose $\rightleftharpoons n$ glucose + $(\text{glucose})_n$ as described by Doudoroff, Hassid, Putman & Potter (1949) for *Escherichia coli*; (*b*) the formation of a maltose phosphate under the action of an appropriate kinase and adenosine triphosphate (ATP), followed by cleavage of this phosphate; (*c*) a reaction of the type reported by Fitting & Doudoroff (1952) for *Neisseria meningitidis*, catalysed by a maltophosphorylase:

$$\text{maltose} + (PO_4)^{3-} \rightleftharpoons \text{glucose} + \text{glucose 1-phosphate}.$$

There was no visible indication that L 1 metabolized maltose by alternative (*a*), which implies

the synthesis of polysaccharide material; this should show itself as an increased viscosity of cultures as growth proceeds. Again, in testing for (*b*), no chromatographic evidence of the formation of esterified phosphate was obtained when extracts of L 1 were incubated at pH 6·3 with 0·02 M-maltose and 0·01 M-ATP. However, esterified phosphate was demonstrated, both chromatographically and by formation of acid-labile phosphate, when mixtures containing maltose and KH_2PO_4 were incubated at pH 6·8 with extracts of L 1 (Table 1). Esterification was not increased by added Mg^{2+} ions or L-cysteine and it was not inhibited by 0·01 M-fluoride. However, it was dependent on the presence of inorganic P and maltose and it did not take place when the reaction mixture was boiled, nor when glucose was substituted for maltose in the reaction mixture. A maltophosphorylase reaction of type (*c*) was therefore indicated.

Factors affecting the activity of L 1 *enzyme.* In mixtures containing only glycylglycine (0·01 M), maltose (0·02 M), KH_2PO_4 (0·01 M) and enzyme, there was little difference in the amount of esterification at equilibrium (24 hr.) at 32° and 37°, but the amount diminished with fall in temperature down to 21°. In similar mixtures incubated at 32° for 24 hr., most esterification took place at pH values between 6 and 7. Incubation at 32° and pH 6·8 were therefore selected as suitable conditions for further enzyme studies.

When mixtures at pH 6·8 containing L 1 enzyme, 0·01 M-KH_2PO_4 and 0·01 M-maltose were incubated for 24 hr. at 32°, about 25 % of the initial inorganic P appeared as ester P, compared with about 40 % when a molar excess of maltose (0·05 M) was present (Table 1). The equilibrium of the reaction thus appeared markedly to favour maltose synthesis (see also Table 4). For the preparation of the phosphate ester (see below) it was preferred to drive the reaction in favour of the ester by using a molar excess of KH_2PO_4.

In a mixture containing KH_2PO_4 (0·01 M) and maltose (0·01 M) at pH 6·8, in which about 20 % of phosphate was esterified after incubation for 5 hr. at 32°, the amount of esterification was decreased to less than 10 % by the presence of 0·01 M-glucose (Table 1). This might be anticipated for a reversible reaction in which maltose synthesis was favoured at equilibrium.

Nature of the end products. Examination of mixtures after incubation at pH 6·8 with maltose, KH_2PO_4 and cell-free extracts of L 1 by chromatography and by electrophoresis in 0·05 M-borate buffer (pH 10) indicated that glucose and a phosphate having the mobility of α-glucose 1-phosphate were produced.

Preparation of the phosphate ester. The phosphate ester produced by the action of L 1 enzyme on maltose and inorganic phosphate was prepared as follows. The reaction mixture, consisting of KH_2PO_4 (102 g.), KOH (24·5 g.) and β-maltose monohydrate (30 g.) in 1 l. of water, was adjusted to pH 6·8 and incubated at 32° for 24 hr. with the cell-free extract derived from the cells grown in a 1 l. culture of L 1. Inorganic phosphate was then removed from the reaction mixture by precipitation as magnesium ammonium phosphate and the filtrate was concentrated *in vacuo* at 40° to about 50 ml. To this concentrate, ethanol was added, with shaking, until a slight turbidity developed. After refrigeration of the mixture overnight, the

Table 1. *Esterification of phosphate catalysed by extracts of* L 1

In Expt. A the complete reaction mixture contained (in a volume of 1 ml.): extract of cells, 0·1 ml. (16 μg. of nitrogen); KH_2PO_4, 10 μmoles; $MgCl_2$, 10 μmoles; maltose, 10 μmoles. Cysteine–HCl, NaF or glucose (each 10 μmoles) were added as shown. In Expt. B, $MgCl_2$ was omitted. Adjustment of reaction mixtures to pH 6·8 was made by addition of 10 % KOH immediately before the addition of enzyme.

		P_i (μg.) after incubation				
Incubation time (hr.)		0	3	5	22	P esterified (μg./ml./22 hr.)
Expt.	Reaction mixture					
A	Complete	62	51·5	49	47	15
	Complete, boiled	62	62	62	62	0
	No KH_2PO_4	0	0	0	0	0
	No maltose	62	62	62	62	0
	Plus glucose, no maltose	62	62	61·5	61	1
	No $MgCl_2$	62	51	49	46	16
	Plus cysteine	62	52	50	49	13
	Plus NaF	62	52·5	49	48	14
Incubation time (hr.)		0	2	5	24	(μg./ml./24 hr.)
B	Complete	62	55	49	48	14
	No maltose	62	62	62	61	1
	Plus glucose	62	61	57	56·5	5·5
	Plus extra maltose to 0·05 M	62	53	39	38	24

Table 2. *Analysis of bacterial and synthetic β-glucose 1-phosphate*

Bacterial ester	Quantity of sample analysed		Reducing sugar: Found		Reducing sugar: Purity of sample*	Inorganic P: Found		Inorganic P: Purity of sample*	$[\alpha]_D^{21}$*
	μg.	μmoles*	μg.	μmoles	(%)	μg.	μg.atoms	(%)	
(*a*) After hydrolysis	641	1·91	340	—	—	54	—	—	
(*b*) Before hydrolysis	641	1·91	15	—	—	2	—	—	+8·2° (*c* 11·2)
(*a*) − (*b*)	—	—	325	1·81	95	52	1·68	88	
Synthetic ester									
(*c*) After hydrolysis	622	1·85	285	—	—	48	—	—	
(*d*) Before hydrolysis	622	1·85	15	—	—	2	—	—	+8·7° (*c* 7·5)†
(*c*) − (*d*)	—	—	270	1·50	81	46	1·48	80	

* Calculated assuming the molecular formula of the ester as $C_6H_{11}O_9PK_2$; specific rotations are calculated assuming all impurity to be optically inactive.
† Barium salt.

Table 3. *Manometric demonstration of the presence of glucose in acid hydrolysates of bacterial ester*

Each Warburg flask contained 1 ml. of KH_2PO_4 (0·2 M, pH 5·6), 0·2 ml. of glucose oxidase (750 units/ml.), 0·1 ml. (2 mg.) of catalase and 1 ml. of substrate [glucose (1 mg./ml.), phosphate ester (2 mg./ml.) or hydrolysed phosphate ester (equivalent to 2 mg. of ester/ml.)]. Incubation was at 30°.

	O_2 absorbed (μl.)				
Time (min.)	5	10	15	20	30
Glucose	34	46	52	57	62
Phosphate ester					
(*a*) Before hydrolysis	3	3	3	3	3
(*b*) After hydrolysis	40	50	57	57	55

supernatant was decanted from the precipitated thin syrup and discarded. The syrup was dissolved in a little water and reprecipitated several times as before to remove inorganic P. This crude syrupy dipotassium salt was purified by treating its aqueous solution with a small excess of barium acetate solution and precipitating the barium hexose phosphate by careful addition of an equal volume of ethanol. The barium compound was centrifuged off, dissolved in water and the dipotassium salt was regenerated from it by ion exchange. The neutral effluent and washings were freeze-dried, the residue was taken up in water, the solution was decolorized with charcoal and filtered and freeze-dried again to yield about 50 mg. of an almost colourless solid.

A number of preparations made in this way were bulked and purified by conversion into the barium salt, which was repeatedly precipitated from aqueous solution by careful addition of ethanol (to 50 %, v/v). The dipotassium salt was then regenerated by ion exchange and the combined eluate and washings were evaporated *in vacuo* at 30–35° to about 5 ml. Absolute ethanol (5 ml.) was added dropwise to this concentrate and the supernatant was decanted from the precipitated colourless syrup. Attempts to induce crystallization of this syrup failed: it was dried to a white powder by teasing it into repeated changes of absolute ethanol, and finally dried *in vacuo* at room temperature.

Nature of the phosphate ester. The purified ester did not reduce Fehling's solution, but was readily hydrolysed by heating at 100° for 7 min. with N-HCl to inorganic P and a reducing sugar, chromatographically identical with glucose. Analysis of the preparation showed that, on hydrolysis, it yielded equimolar proportions of inorganic P and reducing sugar as glucose (Table 2). Assuming the preparation to be an anhydrous dipotassium glucose monophosphate, the analytical figures indicate it to be approx. 90 % pure.

The identity of the reducing sugar in the hydrolysate was confirmed as glucose by conventional Warburg manometric measurements of the oxygen absorbed in the presence of glucose oxidase and catalase (Bentley, 1955). Oxygen was taken up when hydrolysed, but not when unhydrolysed, ester was substrate (Table 3). With glucose, oxygen absorption was theoretical; with the hydrolysed ester, it was 85 % of theoretical, in good agreement with the results of chemical analysis.

These results, and the lability of the ester to acid, indicate that the bacterial phosphate ester is a glucose 1-monophosphate. However, two facts are indicative that the ester has the β- rather than the α-configuration: (*a*) the dipotassium salt failed to crystallize; (*b*) the previously discovered bacterial maltophosphorylase (Fitting & Doudoroff, 1952) yielded β-glucose 1-phosphate from maltose. Confirmation of the configuration was sought through its specific rotation and through its infra-red-absorption spectrum.

The specific rotations of the bacterial and synthetic esters (Table 2) are in good agreement, and the small positive rotation found is consistent

with the β-configuration ($[\alpha]_D$ of α-D-glucose 1-phosphate, dipotassium salt, is +78°).

Fig. 1 shows the close similarity between the infrared spectra of the dipotassium salts of Hassid's authentic β-glucose 1-phosphate and of bacterial β-glucose 1-phosphate. The spectra of these preparations, although lacking the strong bands at 835 and 868 cm.$^{-1}$ given by the dipotassium salt of the α-ester, have bands at 906 or 907 cm.$^{-1}$ which the α-ester lacks. According to Barker, Bourne, Stacey & Whiffen (1954), a strong band at 844 ± 8 cm.$^{-1}$ is characteristic of α-anomers of free pyranose sugars and α-glucosides, whereas the corresponding β-anomers and β-glycosides have bands at 891 ± 7 cm.$^{-1}$. Possibly the bands at 835 or 868 cm.$^{-1}$ of the α-phosphate, and that at 906 or 907 cm.$^{-1}$ of the β-phosphate, correspond respectively to those at 844 and 891 cm.$^{-1}$ reported by Barker *et al.*, the observed displacement resulting from the presence of the phosphate radical.

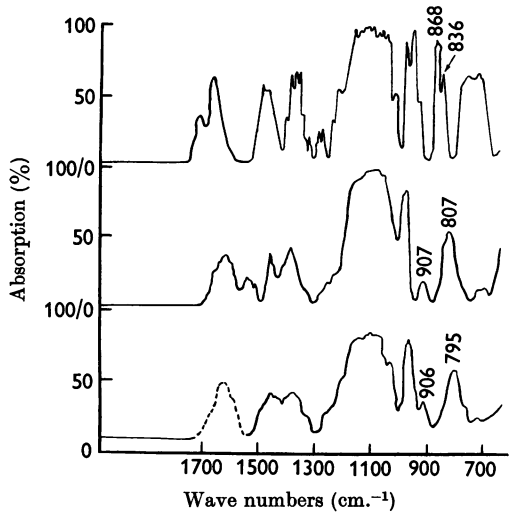

Fig. 1. Infrared spectrograms of: (*a*) α-D-glucose 1-phosphate, (*b*) β-D-glucose 1-phosphate (authentic) and (*c*) bacterial β-D-glucose 1-phosphate.

The sample of synthetic β-glucose 1-phosphate reported in Table 2 also gave an infrared spectrum similar to that of the authentic and bacterial esters: it lacked the characteristic α-band, but that at 906 cm.$^{-1}$ was less well defined, perhaps because the preparation was less pure.

These results confirm the identity of the bacterial phosphate as β-glucose 1-phosphate, with the exception that Reithel (1945) reports the $[\alpha]_D^{30}$ of his synthetic barium salt as +20·5°. The discrepancy between this value and our own cannot be ascribed to the metal ion present in the preparations studied, since the barium salt of our synthetic preparation had $[\alpha]_D^{21}$ +8·7°, which compares well with $[\alpha]_D^{20}$ +10° found by Fitting & Doudoroff (1952) for their barium salt.

Synthesis of maltose by L 1 *enzyme.* Cell-free extracts of L 1 synthesized maltose from bacterial or from synthetic β-glucose 1-phosphate in a reaction for which glucose was an essential co-substrate; 64% of the total P of the system containing equimolar initial concentrations of glucose and β-glucose 1-phosphate was released as inorganic P in 22 hr. at pH 6·8 and 32° (Table 4). The equilibrium of the system thus favours maltose synthesis. Neither maltose nor inorganic P was produced when α-D-glucose 1-phosphate was substituted for the β-anomer in this system.

Table 4. *Enzymic synthesis of maltose*

Reaction mixtures (1 ml.) contained glycylglycine buffer (10 μmoles, pH 6·8), $MgCl_2$ (3 μmoles) and enzyme (0·1 ml.; 16 μg. of nitrogen). Other ingredients were added, as indicated, in the following concentrations: D-glucose (10 μmoles); α-D-glucose 1-phosphate (dipotassium salt, 10 μmoles); bacterial β-glucose 1-phosphate (dipotassium salt, 10 μmoles). After incubation at 32°, samples were withdrawn for analysis as follows: 10 μl. for maltose (by paper chromatography) and 0·25 ml. for inorganic P. + signifies maltose clearly detected; 0 signifies maltose not detected. The total content of acid-labile P in the reaction mixtures containing α- or β-glucose 1-phosphate was 286 μg./ml.

	Formation of		
	Maltose	Inorganic P (μg./ml.)	
Time (hr.)	22	3½	22
Additions to reaction mixture			
None	0	4	8
Glucose	0	0	12
β-Glucose 1-phosphate	0	12	12
β-Glucose 1-phosphate + glucose	+	120	184
α-Glucose 1-phosphate	0	4	8
α-Glucose 1-phosphate + glucose	0	4	8

DISCUSSION

This work shows that certain beer lactobacilli contain a maltophosphorylase, like that previously found in *Neisseria meningitidis* by Fitting & Doudoroff (1952), catalysing the reversible reaction:

maltose + $[PO_4]^{3-}$ ⇌ β-glucose 1-phosphate + glucose.

The carbohydrate requirement of these lactobacilli is remarkable in that maltose is 'preferred' to glucose, which is, in fact, excreted into the

medium during growth on maltose. This phenomenon is good presumptive evidence that maltophosphorylase 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 L 1, a strain which grew readily only on maltose, contained a maltophosphorylase, catalysing the reversible reaction:

maltose + inorganic orthophosphate ⇌ glucose + sugar phosphate.

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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Biochem. J. (1961) **78**, 209

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-αβ-diglyceride and *N*-acylsphingosine respectively. Similarly, cytidine diphosphate ethanolamine reacts with a D-αβ-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