phenomenon is not restricted to the adenylic deaminase of brain.

The observation that the enzyme is associated with insoluble particles is interesting in view of the adenylic deaminase activity of brain mitochondria (Weil-Malherbe & Green, 1955). Abood & Gerard (1954) found that in peripheral nerve of the rat adenylic deaminase is mainly associated with small microsomes of less than 100 m μ . in diameter.

SUMMARY

1. Adenylic deaminase of ox brain was purified 10- to 20-fold by fractionating the aqueous extract of acetone powder with ammonium sulphate and alumina gel C γ .

2. The activity is associated with insoluble particles which can be separated on the high-speed centrifuge. Treatment with sodium cholate, though inhibitory, produces some solubilization, as shown by the appearance of activity in the supernatant solution.

3. Under standard conditions the preparation is practically free of adenosinetriphosphatase, 5-nucleotidase, myokinase and adenosine deaminase activities.

4. The activity of the enzyme is greatly increased by adenosine triphosphate. The effect is specific; adenosine diphosphate, inosine tri- and diphosphates are inactive. Some activity persists even in complete absence of adenosine triphosphate. This activity cannot be accounted for by adenosine deaminase action.

5. The effect of varying adenosine triphosphate concentrations on the activity of the enzyme is independent of the concentration of adenylic acid.

6. The pH optimum is 6.2-6.8.

7. The enzyme is inhibited by some multivalent cations and by phosphate and fluoride. Other inhibitors were tested.

8. Adenosine triphosphate is recovered unchanged at the end of the reaction, while adenylic acid is converted into inosinic acid and ammonia.

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The Use of Paper Chromatography for *in vitro* Studies of Lactose Metabolism in Mammary-gland Preparations

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A major problem in the investigation of lactose synthesis by mammary-gland preparations is the estimation of lactose in the presence of other sugars, chiefly glucose. Previous workers (Grant, 1935, 1936; Knodt & Petersen, 1945; Malpress & Morrison, 1950; Hills & Stadie, 1952; Reithel, Horowitz, Davidson & Kittinger, 1952) have all used selective fermentation, combined with

chemical methods more or less specific for the carbohydrates concerned. The development of paper partition chromatography of sugars (Partidge, 1948) has given the opportunity of carrying out a preliminary physical separation of lactose.

In the work described here quantitative paper chromatography was so adapted that lactose could be estimated in the contents of Warburg vessels in

which the gas exchange of various mammary-gland preparations had been measured. A synthesis of lactose obtained by Grant (1935) in guinea-pig mammary gland was confirmed, and some factors influencing it were further investigated. In addition, unsuccessful attempts were made to obtain lactose synthesis in cell-free preparations of the glands.

MATERIALS

Sugar phosphates. Glucose 1-phosphate was prepared by the method of Hawk, Oser & Summerson (1947) and galactose 1-phosphate by the method of Colowick (1938). The method of preparation and the rotation of galactose 1-phosphate suggest that it is the α -isomer (Kosterlitz, 1939). The barium salt of glucose 6-phosphate and the calcium salt of fructose 1:6-diphosphate, both products of Schwarz Laboratories Inc., were converted into sodium salts in solution by the addition of Na_2SO_4 and sodium oxalate respectively.

Purines. Adenosine triphosphate (ATP) was prepared as the barium salt (LePage, 1949) and converted into the sodium salt (Eggleston & Hems, 1952). Inosine monophosphate (IMP) was a sample prepared by the method of Ostern (1932). Inosine was prepared by the method of Levene & Jacobs (1910).

Adenosine monophosphate (AMP) and hypoxanthine were the preparations of Roche Products, and adenosine the preparation of L. Light and Co. Ltd.

METHODS

Lactating Norway rats (Wistar strain) or guinea pigs were killed and the mammary glands immediately removed and transferred to a dish of ice and water.

Tissue slices. These were cut with the tissue-slicer of Stadie & Riggs (1944) and about 100 mg. fresh weight of slices weighed and transferred to 4.0 ml. chilled medium in each of a series of Warburg flasks; the first slice was always discarded. In each experiment additional slices were dried at 110° for 16 hr. for measurement of dry weight.

The following salines were used. (1) The bicarbonate medium I, without substrates, of Krebs (1950); atmosphere, $\text{O}_2 + \text{CO}_2$ (95:5), except in anaerobic experiments. (2) The calcium-free phosphate saline of Krebs & Eggleston (1940); 100% O_2 . (3) The medium III without substrates, of Krebs (1950); 100% O_2 . This medium contains low concentrations of phosphate and bicarbonate but the physiological concentration of calcium.

Blends. The mammary tissue of lactating guinea pigs was chopped into 40 ml. 0.9% (w/v) KCl at 0° and blended in the Waring Blendor for 90 sec. The pH was always found to be 7. Samples were added to chilled Warburg flasks containing the rest of the incubation mixture and other samples were dried. The dry weights were corrected for KCl. The atmosphere used was 100% O_2 or 100% N_2 .

Mitochondrial suspensions. Method 1. The lactating guinea-pig mammary tissue was chopped into 40 ml. 0.25M sucrose in the Waring Blendor at 0° and blended for 90 sec. Mitochondrial suspensions in 0.9% KCl were then prepared by differential centrifugation, according to Bartley & Davies (1954).

Method 2. Blends made with 40 ml. 0.9% KCl (above) were similarly centrifuged. The supernatant of extra-mitochondrial cytoplasm in KCl from the first centrifuging was added to 5 ml. boiling 0.9% KCl and boiled for a further 10 min. The cooled solution was used as a suspending medium for the mitochondria, as a source of possible cofactors. The atmosphere was 100% O_2 . The dry weights were corrected for KCl.

Incubation. The flasks were equilibrated in the Warburg bath at 40° for 10 min. before readings were taken. When 100% O_2 was used 0.2 ml. 2N-NaOH was placed in the centre well to absorb CO_2 ; in anaerobic experiments phosphorus was placed in the centre well.

Inactivation. Preliminary experiments showed that if rat-tissue slices were added to boiling water, dried at 110° for 16 hr. and then extracted with water, only 10% of the lactose was recovered. The procedure adopted was therefore to withdraw the control and incubated slices from the medium and to grind them with about 30 mg. HgCl_2 and a little sand. The incubation medium was added gradually during the grinding and the whole mixture centrifuged.

Blends and mitochondrial suspensions were deproteinized with 40% (w/v) trichloroacetic acid (TCA) (0.3 ml. for each 4.0 ml. of incubation mixture) and the mixtures centrifuged.

Paper partition chromatography. The apparatus and methods were essentially those of Bealing & Bacon (1953). The solvents were butanol-acetic acid-water (Partridge, 1948), isobutyric acid-ammonia (Krebs & Hems, 1953), and butanol-pyridine-water (6:4:3, by vol.). The first solvent, which was used for most purposes, does not separate AMP from IMP, or adenosine from hypoxanthine, and the second solvent was used for these separations. The third solvent was used to separate lactose from monosaccharide phosphates.

Sugars were detected with the benzidine-trichloroacetic acid spray of Bacon & Edelman (1951). Phosphates were detected according to Hanes & Isherwood (1949) and Bandurski & Axelrod (1951). Purines and their derivatives were detected as dark areas under u.v. light (Chromatolite Lamp, Hanovia Ltd., Slough, Bucks).

Quantitative estimation of lactose. Each supernatant was applied to the base line of a 24×24 in. sheet of Whatman no. 1 paper with an Agla micrometer syringe (Burroughs Wellcome Ltd.) in four groups of four 5 μl . spots, with five 5 μl . guide spots. The spots were dried with a hair drier (cold air in the case of TCA solutions) and the application was repeated twice more. Mixtures of 2% lactose, 0.5% galactose and 0.5% glucose (two 5 μl . spots) were used as guide spots on each chromatogram. The chromatograms were developed for 15 hr. or more in butanol-acetic acid-water. After drying and spraying the guide strips, the four corresponding lactose areas and similar blank areas in front of the monosaccharide position were cut from the chromatograms. The sugars were eluted by shaking with 8.0 ml. water in 6 in. glass-stoppered tubes and heating at 80° for 30 min. The paper was squeezed to the bottom of the tube and the solution filtered.

The filtrates were estimated by the reducing-sugar method of Nelson (1944) and Somogyi (1945). Two samples (2.0 ml.) were transferred to 6 in. glass-stoppered tubes (internal diam. 1 cm.), graduated at 8.0 ml. Two standard lactose solutions (20 μg . in 2.0 ml.) and a reagent blank (2.0 ml. water) were also taken. Throughout the present work the copper reagent of Somogyi (1945) was prepared

with half the concentration (i.e. 0.4%, w/v) of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in an attempt to reduce the blank. The resultant blank was paler, but so were the colours of the lactose determinations, so that the use of this modified reagent eventually proved to be of no advantage. The copper reagent (2.0 ml.) was added, the solutions were mixed and the tubes heated in a boiling-water bath for 15 min.; this time was found to be sufficient for complete reduction. After cooling in a water bath (5 min.), 1.0 ml. of arsenomolybdate reagent (Nelson, 1944) was added and the solutions were mixed. The blue-green solutions were allowed to stand (5 min.) to allow the colour to develop and were then made up to the 8.0 ml. mark.

The absorption spectrum obtained by reading the colour from 50 μg . lactose against a reagent blank in the Unicam spectrophotometer showed a single peak with a maximum of 760 $\text{m}\mu$. Fig. 1 shows a lactose concentration curve made by using the Spekker absorptiometer with a Chance OR2 (red) glass filter (50% transmission at 610 $\text{m}\mu$; 94% transmission at 650 $\text{m}\mu$. and over) this procedure was used throughout.

Fig. 1. Concentration curve of lactose estimation. Details of procedure given under 'Methods'. Each point represents the mean of four readings and twice the S.E.M.

The average of six paper blank readings was subtracted from the average reading for each lactose 'unknown', before comparison with the average reading for the lactose standard. The recovery of lactose from paper chromatograms was $98 \pm 1\%$.

Statistical treatment. Results are given in the form:

mean $\pm$ S.E.M. (number of observations)
range of observations.

The test of significance applied to differences between means corresponds to a probability of 0.05.

RESULTS

Slices from guinea-pig mammary gland

Thirty-nine experiments were carried out with slices from lactating guinea-pig mammary glands; in twenty-six of these there was a significant

synthesis of lactose. The results given below illustrate some aspects of the process.

Time curve of lactose synthesis. The results of an experiment in which slices were incubated for periods up to 3 hr. are plotted in Fig. 2. There was a significant synthesis of lactose in 2 hr. The mean $-Q_{O_2}$ values were 9.3 at 10–60 min., 8.1 at 60–120 min. and 7.5 at 120–170 min.

Choice of saline. The three experiments in which the syntheses in the different salines were compared are summarized in Table 1. No significant synthesis was obtained in the calcium-free, phosphate saline (2). There were significant syntheses in the bicarbonate saline (1) and in saline (3). No further

Fig. 2. Time curve of lactose synthesis in slices of lactating guinea-pig mammary gland (2 days). Saline (3) with the following substrates added: 15 mM glucose, 5 mM glutamate, 5 mM pyruvate and 6 mM fumarate. The vertical lines represent twice the S.E.M. of three observations.

investigation of calcium-free saline was made. In most subsequent experiments saline (3) was preferred, because O_2 uptake could be measured with it.

By applying the $-Q_{O_2}$ values in saline (3) to the gas exchange in saline (1) the R.Q. values were calculated to be 1.3 (Expt. 2) and 1.4 (Expt. 3). These agree with other observations on tissue slices of lactating non-ruminant mammary gland, e.g. 1.6 for rat (Folley & French, 1949).

Distribution of lactose between tissue slice and medium. In five early experiments the slices and medium were analysed separately. For example, Table 2 shows a typical experiment in which the

Table 1. *Effect of different salines on lactose synthesis in tissue slices*

Details of experiments as described under 'Methods'; in each case the saline contained 17 mM glucose. Each experiment was carried out with tissue from a single guinea pig; the time in parenthesis indicates the stage of lactation.

Expt.	Time (hr.)	Saline	$-Q_{O_2}$	Lactose content (mg./g. dry wt.)	Synthesis
1 (3 days)	0	—	—	17 ± 3.0 (3) 14-23	—
	2	1	—	37 ± 4.5 (3) 28-42	Significant
	2	2	9.7	21 ± 3.9 (3) 14-27	—
2 (2 days)	0	—	—	12 ± 1.4 (3) 10-15	—
	2	1	—	28 ± 3.0 (3) 24-34	Significant
	2	2	7.1	15 ± 4.4 (3) 10-24	—
	2	3	7.2	21 ± 4.9 (3) 12-29	—
3 (2 days)	0	—	—	50 ± 12.0 (3) 38-74	—
	3	1	—	96 ± 6.6 (3) 83-105	Significant
	3	2	9.4	84 ± 6.8 (3) 77-98	—
	3	3	9.1	121 ± 23.6 (3) 84-165	Significant

Table 2. *Distribution of lactose between tissue slices and medium*

The incubation medium (saline 1) was applied directly to chromatograms for separation and estimation of lactose. The tissue slices were ground with sand and 1.0 ml. 10 mM-HgCl₂; the mixture was then centrifuged and the supernatants were applied to chromatograms for lactose estimation.

Substrate	Incubation time (hr.)	Fresh tissue* (g.)	Lactose in tissue (mg./g.)	Vol. of medium* (ml.)	Lactose in medium (mg./ml.)	Total lactose* (mg./g. fresh wt.)
—	0	0.216	6.0	—	—	6.0
17 mM Glucose	2	0.189	2.0	12	0.27	19.3
17 mM Glucose + 50 mM glutamate	2	0.224	2.1	12	0.30	18.3

* Values for material from three Warburg vessels.

Table 3. *Effect of anaerobiosis on lactose synthesis*

Slices of a lactating guinea-pig mammary gland (2 days) were incubated in saline (1) under the conditions described.

Substrate (17 mM)	Atmosphere	Incubation time (hr.)	Lactose content (mg./g. dry wt.)
Glucose	—	0	39 ± 3.0 (3) 35-45
Glucose	CO ₂ + N ₂ (5:95)	2	31 ± 2.7 (3) 28-36
None	CO ₂ + O ₂ (5:95)	2	32 ± 0.9 (3) 31-34
Glucose	CO ₂ + O ₂ (5:95)	2	72 ± 2.9 (3) 66-75

final concentration of lactose in the tissue slice (mg./g. fresh wt.) was about 7 times that in the medium (mg./ml.). This procedure was discarded in favour of the procedure described under 'Methods'. The latter has the advantage that control slices and incubated tissue have identical treatment, and it also reduces the time spent on each experiment.

Essential conditions for synthesis. There was a significant synthesis when slices were incubated in the presence of O₂ and added glucose, but no synthesis in the absence of O₂ or of glucose (Table 3). There is no significant difference between the lactose contents of the first three groups in this table; the possible disappearance of lactose during incubation without glucose was investigated further (see below). Fig. 3 shows the relation of lactose synthesis and of O₂ uptake to glucose concentration;

the $-Q_{O_2}$ and the synthesis of lactose increased with glucose concentration over the range 2.5-17 mM.

In each of the remaining experiments reported in this section, a control incubation with glucose showed significant lactose synthesis; unless otherwise stated, the concentration of glucose was 2.5 mM.

Effect of 2:4-dinitrophenol (DNP). The effects of this inhibitor of oxidative phosphorylation (Lehninger, 1949) are given in Table 4. In Expt. 1, 0.02 mM DNP gave a greater $-Q_{O_2}$ and a greater synthesis; in Expt. 2, 0.02 mM DNP gave a greater $-Q_{O_2}$ but the same synthesis. In Expt. 1, 0.2 mM DNP did not change the $-Q_{O_2}$ further, but there was no synthesis; in Expt. 2, 0.2 mM DNP increased the $-Q_{O_2}$ further and again there was no

synthesis. Thus both concentrations increased $-Q_{O_2}$ and 0.2 mM DNP inhibited synthesis; all the differences referred to were significant.

Effect of substrates other than glucose. (a) No effect was found when glutamate (see Table 2), fumarate, or pyruvate was added to 17 mM glucose. In one experiment 2.5 mM glucose + 5 mM pyruvate + 2 mM fumarate gave the same synthesis as 5 mM glucose + 2 mM fumarate.

(b) No change occurred in the lactose concentration when tissue slices were incubated with 9 mM lactose as substrate. In this experiment the control, which showed synthesis, contained 17 mM glucose.

(c) Table 5 shows the effect on lactose synthesis of galactose as a substrate in addition to glucose. The $-Q_{O_2}$ was as great in 3 mM glucose as in 6 mM glucose, so that the amount of substrate available for respiration was not limiting the lactose synthesis. There was a significant synthesis with 3 mM glucose, and a significant increase on this synthesis with 6 mM glucose, so that the rate with 3 mM glucose was not already maximal. The synthesis in 3 mM glucose + 3 mM galactose was significantly less than the synthesis in 6 mM glucose.

(d) A significant synthesis of lactose occurred when tissue slices were incubated with 2.5 mM glucose 1-phosphate as substrate. Paper chromatography showed that about 90% of glucose 1-phosphate disappeared and glucose and inorganic phosphate appeared.

(e) A significant synthesis of lactose occurred in 2.5 mM glucose (Table 6), but the addition of

Fig. 3. The effect of glucose concentration on O_2 uptake and on lactose synthesis in tissue slices of lactating guinea-pig mammary gland. The curves are based on seven experiments in which there was a significant synthesis with saline (3) and with glucose as sole substrate. $-Q_G$ (lactose) and $-Q_G$ (O_2) are μ l. glucose used/mg. dry wt. of tissue/hr. calculated from the lactose synthesized and from O_2 uptake, respectively, on the assumptions that glucose is the sole substrate for synthesis and for oxidation. The vertical lines indicate the S.E.M. of the mean values from the separate experiments. The squares include the S.E.M. for $-Q_G$ (O_2).

Table 4. *Effect of 2:4-dinitrophenol (DNP)*

In each experiment mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3) plus 5 mM glucose, with or without DNP. The zero-hour samples included one from each DNP concentration.

Expt.	DNP (mM)	Incubation time (hr.)	$-Q_{O_2}$	Lactose content (mg./g. dry wt.)
1	—	0	—	33 ± 3.2 (3) 27–38
	0	2	5.0 ± 0.1	47 ± 1.2 (3) 45–49
	0.02	2	7.5 ± 0.3	56 ± 2.8 (3) 53–62
	0.20	2	6.6 ± 0.5	38 ± 1.7 (3) 35–41
2	—	0	—	26 ± 0.9 (3) 25–28
	0	2	7.3 ± 0.4	52 ± 2.3 (3) 48–56
	0.02	2	9.5 ± 0.1	49 ± 4.4 (3) 42–57
	0.20	2	10.2 ± 0.1	34 ± 3.4 (3) 28–39

Table 5. *Effect of galactose as an additional substrate*

Mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3) plus mM glutamate, 6 mM fumarate and 5 mM pyruvate, with the additions of monosaccharide specified.

Further substrates (3 mM)	Incubation time (hr.)	Average $-Q_{O_2}$	Lactose content (mg./g. dry wt.)
Glucose + galactose	0	—	18 ± 2.6 (3) 13–22
Glucose	2	7.3	29 ± 1.2 (3) 27–31
Glucose + galactose	2	7.3	35 ± 1.6 (3) 31–37
Glucose (6 mM)	2	7.4	47 ± 1.7 (3) 44–50

Table 6. *Glucose plus α -galactose 1-phosphate as substrates for lactose synthesis*

Mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3). The zero-hour samples included one of each substrate type.

Substrates (2.5 mm)	Incubation time (hr.)	$-Q_{O_2}$	Lactose content (mg./g. dry wt.)
—	0	—	39 $\pm$ 3.3 (3) 32-42
Glucose	2	4.6	51 $\pm$ 1.2 (3) 49-53
Glucose + galactose 1-phosphate	2	4.7	55 $\pm$ 3.6 (3) 44-57
Glucose (5 mm)	2	4.7	66 $\pm$ 6.3 (3) 53-73

2.5 mm galactose 1-phosphate gave no greater synthesis. Chromatography showed the formation of free galactose though much galactose 1-phosphate remained.

Dry/fresh weight quotient. In each experiment there was close agreement between duplicates and the result from all experiments was: 0.24 ± 0.004 (72) 0.19-0.35.

Slices of rat mammary gland

There was much variation in the dry/fresh weight quotients, even between duplicate determinations in individual experiments, and the result from all experiments was 0.34 ± 0.019 (18) 0.21-0.47.

No synthesis of lactose was gained when slices of glands from lactating rats (7-17 days) were incubated under the same conditions as guinea-pig slices, e.g. in saline (1) with 17 mm glucose as substrate. There was always a net output of gas, indicating an R.Q. greater than 1. Incubation periods up to 6 hr.; the addition of 5 mm glutamate + 5 mm fumarate; the addition of 0.1% (w/v) Pronutrin (Atomised Food Products and Herts Pharmaceuticals Ltd.) + 0.005% (w/v) tyrosine, as a source of amino acids; and the addition of 0.25% (w/v) 'Anterior lobe of pituitary gland substance' (Armour Laboratories, Chicago) did not produce a synthesis of lactose. In a typical experiment, in which 0.1% (w/v) Pronutrin, 0.005% (w/v) tyrosine and 5% (fresh w/v) of a rat-liver extract were added, the lactose content (mg./g. dry wt.) was 13.2 at 10 min. and remained at 13.5 after 20, 40 and 60 min. incubation.

Blends

A blend (264 mg. dry wt./4.0 ml.), prepared from the glands of a lactating guinea pig (3 days), was incubated with 10 mm glucose, 5 mm ATP, 5 mm-MgCl₂ and 10 mm phosphate buffer (pH 7.4) in O₂ or N₂ for 20 or 60 min. The $-Q_{O_2}$ fell from 7.2 (10-20 min.) to 1.2 (50-60 min.). Some adenosine diphosphate (ADP) remained after 20 min. aerobic incubation, but in all other samples no ATP or ADP was detectable by paper chromatography. Little glucose disappeared; there was no synthesis of lactose.

Similarly, there was no change in the lactose present when blends were incubated for up to 1 hr. in O₂ with 12 mm α -oxoglutarate, mm ATP, mm-MgCl₂, 10 mm phosphate buffer (pH 7.4), and either 5 mm glucose or 5 mm lactose.

Paper chromatography showed that during the incubations with blends ATP was broken down and AMP, inosine, and hypoxanthine appeared. No adenosine was detected, but the incubation of blends with adenosine gave rise to inosine and hypoxanthine, so that it is not excluded that adenosine was an intermediate.

Mitochondrial suspensions

In the experiment described in Table 7 the $-Q_{O_2}$ decreased rapidly in the presence of succinate or α -oxoglutarate, but was greater and continued

Table 7. *Incubation of mitochondria with various substrates*

The mitochondrial suspension was prepared by method 1 from the mammary glands of a lactating guinea pig (4 days). Each incubation mixture (4.0 ml.) contained 113 mg. dry wt. mitochondria, 5 mm glucose, 2 mm ATP, mm-MgCl₂ and 10 mm phosphate buffer (pH 7.4).

Additional substrates (5 mm)	$-Q_{O_2}$	
	10-20 min.	20-30 min.
Succinate	10	1
α -Oxoglutarate	16	5
Pyruvate + fumarate (0.5 mm)	23	21

with only a small decrease in the presence of pyruvate. Fumarate was added in catalytic amounts to aid the oxidation of pyruvate by the tricarboxylic acid cycle, but it appeared from the incubations with succinate and α -oxoglutarate that oxidation was not proceeding beyond fumarate. Chromatograms showed that ATP concentration was highest in the presence of pyruvate and fumarate, but had nevertheless fallen from its original level. No lactose was formed.

In incubations of mitochondrial suspensions (method 2) in the presence of 10 mm pyruvate and mm fumarate, there was no change in the lactose

concentration or in the appearance of butanol-acetic acid or butanol-pyridine chromatograms with the following additional substrates: glucose + galactose, glucose + glucose 1-phosphate; glucose + glucose 6-phosphate; glucose + hexosediphosphate; glucose + glucose 1-phosphate + glucose 6-phosphate + galactose 1-phosphate (all at 2.5 mM); nor with glucose, galactose, or glucose 1-phosphate (all at 5 mM).

Mammary-gland lactase

Extracts of lactating guinea-pig or rat mammary gland were prepared by grinding with sand. When these extracts were incubated in stoppered tubes with 4% (w/v) lactose at 37° in the presence of CHCl_3 , for 24 hr. there was a formation of glucose and galactose and a slight formation of oligosaccharides. Extracts prepared in a stainless-steel homogenizer of the Potter & Elvehjem (1936) type, and acetone powder extracts, had similar activity, but preparations made in the Waring Blendor were more active. The lactase activity was strongest at pH 4-5 and was unaffected by dialysis of the extracts or by the addition of inorganic phosphate or fluoride. No phosphate corresponded with the sugar positions on butanol-acetic acid chromatograms and there was no correlation of the lactase activity with acid phosphatase; one preparation was obtained with no acid phosphatase activity. In a typical experiment, in which an extract made with sand was incubated with added lactose for 22 hr. at pH 5 with 0.2 M acetate buffer and 0.02 M KH_2PO_4 at 37°, there was a breakdown of lactose from 2.1 to 1.8%, and a corresponding increase in monosaccharide.

DISCUSSION

Despite the observations of Malpress & Morrison (1949), paper chromatography has proved to be a convenient method of following lactose synthesis in tissue slices. It has the advantage that the nature of the sugar being estimated can be confirmed, and usually it is possible to decide by inspection of the chromatograms whether synthesis has taken place. Any unusual carbohydrate in the extract may also be discovered (cf. Heyworth & Bacon, 1954). Even when relatively large amounts of glucose are present a preliminary fermentation with yeast is not needed; this avoids some of the complications, e.g. production of dextrans, introduced by the use of yeast.

In the present work lactose has been estimated with smaller amounts of tissue and shorter times of incubation than those used by previous workers: 25 mg. dry wt. and 2 hr. respectively [cf. 80 mg., 6 hr. (Grant, 1935); 450 mg., 4 hr. (Malpress & Morrison, 1950)]. The rates of lactose synthesis measured with slices of guinea-pig gland

were somewhat higher than those recorded previously; an average of 18 mg./g. dry wt./hr. was found with 17 mM glucose as substrate. Grant (1935) found about 8 mg., and Malpress & Morrison (1950), 3 mg. The rate was dependent upon glucose concentration within the physiological range.

The failure to demonstrate synthesis with guinea-pig tissue in calcium-free phosphate saline may have some connexion with the calcium-secreting activity of the mammary gland. The possibility that lactose secretion in slices was linked to synthesis of other milk constituents was also considered when experiments with rat tissue failed to show signs of synthesis from glucose alone; however, the addition of an amino acid mixture had no effect. We can offer no explanation of the failure to demonstrate synthesis in rat tissue slices. It would seem unlikely that the lactose level in these experiments represented a balance of synthesis and breakdown, because it remained always remarkably constant, and no breakdown of lactose in the absence of added glucose was ever observed. Certain precautions were found to be necessary in measuring the total lactose content of an incubation system. A falsely low value of the lactose content of slices may be obtained if they are dried and afterwards extracted. As Table 2 shows, lactose may be lost from slices to medium, so that the lactose content of the slices may fall even when a net synthesis of lactose is taking place. Judged by these criteria, the experimental details given by Hills & Stadie (1952), who found a synthesis of about 5 mg. lactose/g. dry wt./hr. in rat tissue slices, are insufficient.

Attempts to elucidate the mechanism of lactose synthesis with tissue slices were for the greater part unsuccessful. The absence of synthesis under anaerobic conditions (cf. Malpress, 1950) and with 2:4-dinitrophenol indicates that it is dependent upon oxidative phosphorylation. [This conclusion must be qualified by a reference to the rates of synthesis which can be measured by our method. Reithel *et al.* (1952) and Kittinger & Reithel (1954), who obtained a synthesis under anaerobic conditions, have used radioactive substrates as a means of measuring rates of synthesis too small to be measured by our method.]

The possibility that free galactose is an intermediate in the synthesis of lactose from glucose was tested by incubations with mixtures of glucose and galactose. The fact that galactose was not as effective as glucose in supplementing a suboptimum concentration of glucose may be taken as evidence against this possibility, but only if it is assumed that both sugars are absorbed by slices at an adequate rate.

Although lactose is a β -galactoside, the discovery that maltose, an α -glucoside, may be phosphorylated to yield β -glucose 1-phosphate (Fitting & Doudoroff, 1952) makes it conceivable that

α -galactose 1-phosphate could be a precursor of lactose; McGeown & Malpress (1952) found evidence for its presence in cow's milk. This substance is formed when galactose is phosphorylated by galactokinase, and also by the action of galactowaldenase (see Rutter & Hansen, 1953). When tested as a supplement to suboptimum glucose, it gave no significant increase in lactose synthesis. This again might be the result of a failure to reach the site of synthesis, either because of the existence of physical barriers, or because the substance is hydrolysed rapidly after gaining entry.

Many of the uncertainties mentioned above would be eliminated if a cell-free preparation could be obtained showing comparable rates of lactose synthesis. All our attempts to obtain such a preparation were unsuccessful. Reithel *et al.* (1952) found a variable but definite synthesis in cell-free preparations by a colorimetric method, but later resorted to the use of isotopes to measure the very small rates of synthesis achieved with their partially purified extracts (about 0.1 mg./g. dry wt./hr.). It is possible that the preparations of Turner (1954), which respire more actively, might be capable of a higher rate of synthesis.

Preparations of mitochondria, which broke down ATP much less rapidly than unfractionated blends, also showed no synthesis. Although some synthetic reactions, e.g. citrulline synthesis (Müller & Leuthardt, 1949) and *p*-aminohippuric acid synthesis (Kielley & Schneider, 1950), are carried out by mitochondrial preparations, it would seem less likely that lactose synthesis would be found in such a preparation than in crude homogenates. Like the cyclophorase preparations of Moore & Nelson (1952), our mitochondrial preparations from mammary gland did not apparently oxidize succinate and α -oxoglutarate beyond the stage of fumarate. However, unlike theirs, our preparations would oxidize pyruvate beyond citrate without the addition of nucleotides.

We have obtained no evidence for the existence of a lactose phosphorylase, but there was weak lactase activity, with an optimum at pH 5. There was no detectable breakdown of lactose by tissue slices under more physiological conditions. The appearance of oligosaccharide with an R_f value less than that of lactose suggests that the action of mammary-gland lactase, like that of the lactases of certain micro-organisms, involves transgalactosylation (Wallenfels & Bernt, 1952; Roberts & McFarren, 1953; Aronson, 1952; Pazur, 1953).

SUMMARY

1. Various preparations from lactating mammary glands of guinea pigs and rats were used to study the metabolism of lactose *in vitro*.

2. The formation of lactose and the fate of substrates added to the preparations were followed by paper chromatography. A chromatographic method was developed by which rates of synthesis of lactose could be measured after incubation of tissue slices in Warburg manometer vessels.

3. Lactose was not broken down by respiring tissue slices, blends or mitochondrial suspensions, but crude aqueous extracts showed some lactase activity, particularly when the pH was lowered.

4. The synthesis of lactose obtained by Grant (1935) in surviving mammary tissue slices from guinea pigs was confirmed, but no synthesis was obtained with rat tissue. The synthesis was supported by glucose and showed a greater proportional increase with glucose concentration than did glucose oxidation. It was not increased by the partial replacement of glucose by other substrates. The synthesis was dependent on aerobic conditions and was inhibited by 0.2 mM DNP.

5. No synthesis of lactose was gained with blends or with mitochondrial suspensions prepared from guinea-pig tissue.

6. Succinate and α -oxoglutarate were apparently not oxidized beyond fumarate by mitochondrial suspensions from guinea-pig mammary gland.

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The Substrate Specificity and Inhibition of Alkaline Phosphatases of Cow's Milk and Calf Intestinal Mucosa

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It is somewhat surprising, in view of the extensive literature relating to alkaline phosphatases, that the substrate specificity of these enzymes has not been clearly established. Although it has been shown that they hydrolyse a number of different phosphate esters (Folley & Kay, 1936; Roche & Thoai, 1950), this information is derived from studies with crude or partially purified preparations. The widely accepted belief that hydrolysis is limited to orthophosphate monoesters (Roche & Thoai, 1950; Roche, 1950) is not well-founded, especially since Schmidt & Thannhauser (1943) showed that highly purified preparations from calf intestinal mucosa hydrolysed phosphate diesters and pyrophosphates as well as phosphate monoesters.

The substrate specificity has now been determined using purified phosphatases from cow's milk (Morton, 1950, 1953*a*) and calf intestinal mucosa (Morton, 1950, 1954). It has been found that only orthophosphate monoesters and closely related compounds, but not diesters or pyrophosphates, are hydrolysed.

Since these alkaline phosphatases may therefore be regarded as monoesterases of a special type, it

seemed possible that they might be inhibited by compounds which inhibit other esterases. The failure to obtain appreciable inhibition by a range of organic pyrophosphates, phosphites and phosphonates shows the marked difference between these alkaline phosphatases and certain other esterases.

MATERIALS AND METHODS

Enzymes and buffers

Unless otherwise indicated, the experiments described here were carried out in 1950 and early 1951 using alkaline phosphatases purified from cow's milk (Morton, 1950, 1953*a*) and from calf intestinal mucosa (Morton, 1950, 1954). The method of handling these preparations and of purifying and preparing the buffers used in this work is described in another paper (Morton, 1955*a*).

Substrates

Except where otherwise indicated, commercial preparations were used. They were generally examined for the presence of contaminant phosphorylated compounds (Morton, 1955*a*), and, unless otherwise mentioned, were used without further purification.

Phosphocreatine (PC). The crystalline sodium salt was prepared by the method of Ennor & Stocken (1948).

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