of administration of the isotope, which was given intravenously in the rabbit and intraperitoneally in the cat.

SUMMARY

1. The degree of calcification (calcium/nitrogen ratio) of cortical bones in rabbits increases from about the fifth day of life and in the cat from about the ninth day of life. From the birth of kittens until they are 9 days old there is a continuous reduction in the degree of calcification.

2. The calcification of the cancellous bone in rabbits remains approximately constant during the whole of growth, whereas in cats this bone becomes less calcified during growth.

3. The amount of ^{32}P incorporated during the 4 hr. following administration declines throughout the growth of rabbits in both cancellous and cortical bone. In cats there is a maximum incorporation in both types of bone at an age of about 14 days. This maximum is more marked in the cancellous bone.

4. In both cats and rabbits there is a very marked change during growth in the incorporation of ^{32}P in the subperiosteal and endosteal layers of bone. The incorporation of ^{32}P in the former layers declines throughout growth, whereas that in the latter layers reaches its maximum in the adult.

We wish to thank Mr R. W. Lofthouse and Miss E. B. Morley for excellent technical assistance, and one of us (S.M.W.), would like to thank the Medical Research Council for a research grant received during the earlier part of this work.

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Enzyme Systems in Marine Algae

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS**

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Duncan, Manners & Ross (1956) reported that unfractionated extracts of four species of marine algae (*Cladophora rupestris*, *Laminaria digitata*, *Rhodymenia palmata* and *Ulva lactuca*) showed hydrolytic activity towards a number of carbohydrates, including maltose. In dilute aqueous solution (0·04 %) this sugar was completely hydrolysed to glucose, whereas in concentrated solution (7–17 %) the synthesis of oligosaccharides was observed. By contrast, incubation of an algal extract with a concentrated solution of glucose did not result in oligosaccharide synthesis. The enzymic reaction therefore involves the transfer of α-glucosyl residues to glucosaccharides and not the enzymic polymerization of glucose; in this respect, the algal enzyme systems differ from that in *Aspergillus niger* (strain NRRL 330), which can synthesize disaccharides from glucose (Peat, Whelan & Hinson, 1955).

In the present paper the characterization of the oligosaccharides synthesized from maltose by an extract of *Cladophora rupestris* is described, and the acceptor specificity of the trans-α-glucosylase system is discussed.

* The paper by Duncan, Manners & Ross (1956) is regarded as Part 1.

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METHODS AND MATERIALS

Analytical methods

Chromatography. (*a*) *Qualitative.* Paper chromatography of free sugars was carried out as previously described with either *n*-butanol–pyridine–water–benzene (5:3:3:1, by vol.) (*A*) or ethyl acetate–pyridine–water (10:4:3, by vol.) (*B*) as solvent. The rate of movement of sugars is expressed relative to D-glucose (R_G values) (Duncan *et al.* 1956). The corresponding *N*-benzylglucosylamines were chromatographed by the method of Bayly & Bourne (1953).

(*b*) *Preparative.* Columns containing a 1:1 (w/w) mixture of activated charcoal (British Drug Houses Ltd.) and Celite no. 545 (Johns Manville Co., New York, U.S.A.) were prepared by the general methods of Whistler & Durso (1950), Whelan, Bailey & Roberts (1953) and Bacon (1954). Oligosaccharides were eluted with an increasing concentration of aqueous ethanol; fractions of 700 ml. were collected from the larger columns (70 or 79 cm. × 4·5 cm.), and fractions of 7 ml. from smaller columns.

Electrophoresis. Paper electrophoresis of free sugars was carried out in a water-cooled apparatus (Foster, 1952) with Whatman no. 1 paper in a 0·1N-borate buffer (pH 10·0), with a 1 hr. period for equilibration. The current (500 v; 12·5 mA) was passed for 3·5 hr., the paper was air-dried and the sugars were located with an aniline oxalate–acetic acid spray reagent.

Periodate oxidation (with Mr F. B. Anderson). The oligosaccharides were oxidized with sodium metaperiodate in the presence of 0·05M-phosphate buffer (pH 8·0) as described by Hough & Perry (1956). The production of formaldehyde was determined with a phenylhydrazine–ferricyanide reagent (Hough, Powell & Woods, 1956). Under these conditions 1→4-linked oligosaccharides yield 1 mole of formaldehyde/glucose residue, whereas formaldehyde is not liberated from glucose residues which are joined by 1→6 linkages.

Estimation of degree of polymerization (*DP*). The sugars (about 5 mg.) were dissolved in water (10 ml.) and the reducing power of samples (2 ml.) was estimated with the iodometric Shaffer & Somogyi (1933) reagent as modified by Hanes & Cattle (1938). With the exception of M_0, which required 20 min. heating, a heating time of 60 min. was necessary for the full development of reducing power. The reducing powers were calculated as equivalents of maltose. Samples (2 ml.) were hydrolysed with sulphuric acid (36N; 0·12 ml.) at 100° for 2 hr., neutralized with sodium hydroxide (phenolphthalein), diluted to 5 ml. and the glucose content was determined.

Partial acid hydrolysis. The sugar (1%, w/v solution; 1 ml.) and sulphuric acid (0·5N; 0·6 ml.) were heated at 100° for 1 hr., neutralized, concentrated and analysed by paper chromatography. Alternatively, the sugar (1% solution; 5 ml.), iodine (30 mg.) and potassium hydroxide (1·0N; 0·2 ml.), mixed in this order, were shaken together, and after the solution had been kept for 30 min. a further portion of alkali (0·1 ml.) was added. After 15 hr. incubation at room temperature, a sample of the aldonic acid solution (1 ml.) was partly hydrolysed as described above.

Enzyme preparations

Extract of C. rupestris. Soluble protein was extracted from fresh minced algae as previously described (Duncan *et al.* 1956). The freeze-dried material contained 8·8% of nitrogen.

β-Amylase. A commercial preparation from barley was used (Liddle & Manners, 1957).

D-Enzyme. This enzyme was prepared from potato juice by the method of Peat, Whelan & Rees (1956).

EXPERIMENTAL AND RESULTS

Preparation and isolation of oligosaccharides

A digest was prepared containing recrystallized maltose (32·5 g.), 0·1M-acetate buffer (pH 5·0; 250 ml.), freeze-dried extract (1·25 g.) and toluene. After incubation at 35° for 16 days, the digest was heated to inactivate the enzyme and concentrated. Paper-chromatographic analysis showed the presence of glucose, maltose, and six additional oligosaccharides with R_G values (in solvent *A*) ranging from 0·38 to 0·03 (see Table 1). (R_G values are relative to D-glucose.)

To facilitate separation of the oligosaccharides, the concentrate was chromatographed on a charcoal–Celite column (79 cm. × 4·5 cm.) and the glucose and maltose were eluted with water and 4% (v/v) aqueous ethanol respectively. The oligosaccharides were then eluted with 30% ethanol and concentrated.

The resulting syrup was applied to a second column (70 cm. × 4·5 cm.) and the oligosaccharides were eluted with increasing concentrations of ethanol. Fractions (700 ml.) were collected, concentrated and examined by paper chromatography. None of the fractions became acid on concentration.

The oligosaccharides will be referred to as M_0, M_1, M_2, etc., in order of elution from the column; in a preliminary comment on the present work (Duncan *et al.* 1956) the two major sugars were named in order of paper-chromatographic mobility, so that M_1 and M_2 are now redesignated M_2 and M_1 respectively.

The following combined fractions were collected: *A*, glucose; *B*, maltose and M_0 (faint trace); *C*, maltose; *D*, maltose and M_1; *E*, M_1 (1·38 g.); *F*, M_1, M_2, and maltose (trace); *G*, M_2 and maltose (faint trace); *H*, mixed higher oligosaccharides.

Fraction *G* was chromatographed on a third column (65 cm. × 3 cm.), yielding pure M_2 (1·50 g.). Fraction *H* was applied to a fourth column (67 cm. × 3 cm.). Elution with 0–2 and 5% aqueous ethanol gave chromatographically pure glucose and M_0 (25 mg.) respectively. Elution with increasing concentrations of ethanol (6% → 18%) was continued, 7 ml. fractions being collected. The yields of the pure oligosaccharides, after freeze-drying or crystallization from ethanol, are given in Table 1. Mixed fractions were not analysed further, except that chromatography of a mixture

of M_2 and M_3 on Whatman no. 3 MM paper yielded pure M_3 (10 mg.).

A control digest containing maltose (8·1 g.) and 0·1 M-acetate buffer (pH 5·0; 63 ml.) was incubated at 35° for a similar period. After heating, it was concentrated, and applied to a charcoal–Celite column (21 cm. × 4·5 cm.) and developed with increasing concentrations of aqueous ethanol. No sugar other than maltose could be detected. The observed synthesis of oligosaccharides in the main digest is not therefore due to microbial contamination of the buffer and sugar solution. Further, the purity of the maltose used as substrate is confirmed.

Preliminary characterization of the oligosaccharides

The purified oligosaccharides, which were homogeneous by paper chromatography and electrophoresis, were reducing sugars which contained glucose and no other sugar.

The results in Table 1 indicate that M_2 and M_3 are trisaccharides and that M_4, M_5 and M_6 are tetrasaccharides. Further, since the degree of polymerization (DP) values of M_2 and M_5 are very close to whole numbers, these sugars are probably maltosaccharides (cf. Whelan *et al.* 1953).

N-Benzylglycosylamine derivatives of M_1, M_2, maltose and maltotriose were prepared; the R_G values were 0·29, 0·31, 0·57 and 0·31 respectively.

The four main oligosaccharides and the corresponding aldonic acids were partly hydrolysed with acid, and the products were tentatively identified by paper chromatography (Table 2).

Further evidence of the structure of M_1, M_2, M_4 and M_5 was obtained by determining the formaldehyde liberated during 'over-oxidation' with periodate (see Table 1). In a control experiment, maltose gave 1·8 moles/mole (theoretical, 2·0).

In addition to the above observations, molecular rotation and paper-chromatographic mobility [expressed as $\log R_G/(1-R_G)$] data support the suggestion that M_2 and M_5 are maltotriose and maltotetraose respectively. A comparison of the figures with those of maltose shows that a linear relationship exists with the DP of the sugar. This indicates that maltose, M_2 and M_5 belong to the same polymeric series of sugars (cf. Whelan *et al.* 1953). Further, the paper-chromatographic mobilities of M_1 and M_4 are intermediate between those of the maltosaccharides and the isomaltosaccharide series, suggesting that M_1 and M_4 may contain both α-1 → 4- and α-1 → 6-glucosidic linkages (cf. French & Wild, 1953). M_0, M_3 and M_6 have not been examined further.

Characterization of M_1 *as panose*

M_1 had $[\alpha]_D^{15} + 148°$ in water (*c*, 3·7); cf. $[\alpha]_D + 150°$, reported by Pazur & French (1952) for panose. M_1 had the same R_G in three solvents as an authentic sample of panose, and was not attacked by barley β-amylase or maltase-free saliva. The infrared spectrum of M_1 (panose), kindly determined by Dr D. M. W. Anderson, showed that it was present in the form 2 described recently by Wolfrom & Thompson (1957). M_1 (150 mg.) in water (3 ml.)

Table 1. *Properties of oligosaccharides synthesized from maltose by an extract of* Cladophora rupestris

The periodate-oxidation values are given in moles of formaldehyde/mole of oligosaccharide; theoretical values for the assigned structures are given in parentheses.

Oligosaccharide	Yield (g.)	R_G values *A*	R_G values *B*	DP	Periodate-oxidation value
M_0	0·03	0·38*	0·50*	1·94	—
M_1	2·16	0·18	0·28	—	1·0 (1)
M_2	2·11	0·35†	0·41†	3·04	2·7 (3)
M_3	0·01	0·07	0·14	2·63	—
M_4	0·31	0·11	0·17	3·72	1·8 (2)
M_5	0·47	0·19	0·26	3·95	4·1 (4)
M_6	0·02	0·03	0·09	3·69	—

* Authentic isomaltose had R_G values *A*, 0·38; *B*, 0·48.
† Authentic maltotriose had R_G values *A*, 0·33; *B*, 0·41.

Table 2. *Products of partial acid hydrolysis of the oligosaccharides and of the corresponding aldonic acids*

	Oligosaccharide hydrolysate*	Aldonic acid hydrolysate*	Tentative structure
M_1	M_0, maltose, glucose	M_0, glucose	Panose
M_2	Maltose, glucose	Maltose, glucose	Maltotriose
M_4	M_1, M_2, M_0, maltose, glucose	M_1, M_0, maltose, glucose	6^3-α-Glucosylmaltotriose†
M_5	M_2, maltose, glucose	M_2, maltose, glucose	Maltotetraose

* Unchanged oligosaccharide is not recorded.
† For full systematic name see text.

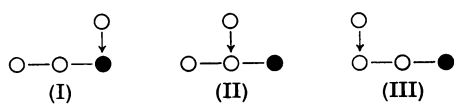
was reduced with potassium borohydride solution (4·5%, 1 ml.). After 2 hr. at room temperature, the solution was acidified with acetic acid (to decompose excess of borohydride), neutralized and evaporated to dryness. The product (120 mg.) was acetylated with sodium acetate (162 mg.) and acetic anhydride (3 ml.) at 135° for 20 min. The sugar acetate was isolated from ice–water and recrystallized from ethanol [m.p. and mixed m.p. with an authentic sample of panitol dodecaacetate, 148–149°; $[\alpha]_D^{15}+119°$ in chloroform (*c*, 1·0) (cf. +119° and +120° reported by Peat, Whelan & Edwards, 1955)]. M_1 is therefore identified as panose (6^2-α-glucosylmaltose).

Characterization of M_2 as maltotriose

M_2 had $[\alpha]_D^{15}+164°$ in water (*c*, 2·8); cf. $[\alpha]_D+160°$, reported by Whelan *et al.* (1953) for maltotriose. M_2 was slowly hydrolysed by maltase-free saliva and by barley β-amylase, giving glucose and maltose, as shown by paper-chromatography and reducing-power measurements. On incubation with D-enzyme, glucose and higher oligosaccharides were formed. The data reported in Tables 1 and 2 and the enzymic studies identify M_2 as maltotriose.

Characterization of M_4 as 6^3-α-glucosylmaltotriose

M_4 had $[\alpha]_D^{15}+177°$ in water (*c*, 0·5) and differed in R_G and M_G (electrophoretic mobility relative to that of D-glucose) values from maltotriose, maltotetraose, isomaltotriose and isomaltotetraose. It was not attacked by α- or β-amylase. On the assumption that M_4 is formed by trans-α-glucosylation from maltotriose, three possible structures have been considered:



where ○ and ● represent respectively non-reducing and reducing glucose residues, and the symbols — and ↓ represent 1 → 4- and 1 → 6-linkages.

On periodate oxidation at pH 8, the structures would yield 0, 1 and 2 moles of formaldehyde repectively. M_4 gave 1·8 moles. Structure (I) and the aldonic acid of compound (II) on partial acid hydrolysis could not yield panose. These results and those reported in Tables 1 and 2 therefore identify M_4 as 6^3-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose].

Characterization of M_5 as maltotetraose

M_5 had $[\alpha]_D^{15}+175°$ in water (*c*, 1·3); cf. $[\alpha]_D$ values of +176° and +177° reported by Whistler & Hickson (1955) and Whelan *et al.* (1953) for maltotetraose. M_5 was rapidly hydrolysed by maltase-free saliva to give maltose and glucose, and by barley β-amylase to give maltose. The enzymic studies and the data in Tables 1 and 2 characterize M_5 as maltotetraose.

Attempted transfer of α-glucosyl radicals to other sugars

Digests were prepared containing maltose (300 mg.), acceptor sugar (300 mg.), *C. rupestris* preparation (20 mg.) and 0·1 M-acetate buffer (pH 5·6, 2 ml.), and were examined at intervals, after incubation at 35°, by paper chromatography. The following acceptor sugars were tested: D-xylose, L-xylose, *N*-acetylglucosamine and L-sorbose. In all digests the same series of oligosaccharides were produced as were present in a control digest containing only maltose and extract.

DISCUSSION

The ability of many carbohydrases to catalyse both the hydrolysis and synthesis of oligosaccharides is now widely recognized (cf. Baumann & Pigman, 1957). The reactions may be formulated as follows:

$$\text{G–O–X} + \text{Enzyme-H} \rightarrow \text{Enzyme-G} + \text{H–O–X}, \quad (1)$$

$$\text{Enzyme-G} + \text{H–O–R} \rightarrow \text{Enzyme-H} + \text{G–O–R}, \quad (2)$$

where G–O–X represents a glucoside (the glucosyl donor) and H–O–R the acceptor molecule. When R is a hydrogen atom, hydrolysis takes place; if R is a univalent radical the synthesis of a new glucoside with the same configuration as the glucosyl donor occurs.

The present study shows that extracts of *C. rupestris* catalyse the synthesis of oligosaccharides from maltose when the concentration of water is sufficiently low. The main products of enzyme action, which have been characterized by chemical and enzymic methods, are panose (6^2-α-glucosylmaltose), maltotriose, 6^3-α-glucosylmaltotriose and maltotetraose. In addition, very small amounts of other oligosaccharides are also formed.

Although the yields of the oligosaccharides recorded in Table 1 are not quantitative, they are a

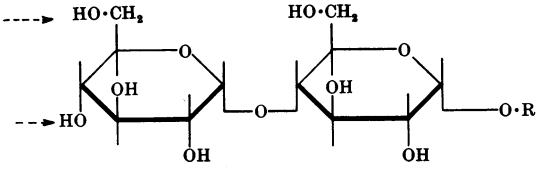


Fig. 1. Carbohydrate acceptors for α-glucosyl radicals transferred from maltose by extracts of *C. rupestris*. The arrows denote possible points of attachment of α-glucosyl radicals. R represents a hydrogen atom (maltose) or a glucose residue (maltotriose).

Table 3. *Trans-α-glucosylation: oligosaccharides synthesized from maltose by various enzyme preparations*

Biological source	Oligosaccharides	Reference
Penicillium chrysogenum Q. 176	Isomaltose, isomaltotriose, panose, 6^3-α-isomaltosylmaltose	Saroja, Venkataraman & Giri (1955)
Aspergillus niger (strain 152)	Isomaltose, isomaltotriose, panose	Barker & Carrington (1953)
Aspergillus oryzae	Isomaltose, isomaltotriose, panose, 6^3-α-isomaltosylmaltose	Pazur & French (1952)
Escherichia coli	Maltotriose and higher maltosaccharides	Barker & Bourne (1952)
Rat liver	Maltotriose, maltotetraose	Giri, Nagabhushanam, Nigam & Belavadi (1955)

measure of the relative proportion of the various sugars present in the equilibrium between synthesis and hydrolysis, under our particular experimental conditions (with 13% maltose solution). They show that the main transfer of α-glucosyl residues from maltose is to the hydroxyl group at $C_{(4)}$ or $C_{(6)}$ of the non-reducing end-group in maltose or maltotriose (Fig. 1). Glucose or panose do not appear to act readily as acceptor molecules.

These results may be compared with similar studies on trans-α-glucosylation by enzyme preparations from other biological sources, in which maltose was used as the α-glucosyl donor (Table 3).

It will be noted that the mould enzymes readily transfer α-glucosyl radicals to the primary alcoholic group in glucosaccharides, including glucose and panose, whereas the animal and bacterial enzyme systems can transfer only to the hydroxyl group at $C_{(4)}$ of the non-reducing end-group of the acceptor.

Experiments in this Laboratory (unpublished work) have also shown that the acceptor specificity for α-glucosyl radicals of the *C. rupestris* enzyme system differs from that found with cell-free extracts of the protozoan *Tetrahymena pyriformis* and with extracts of brewer's yeast.

In a further study of this acceptor specificity, the action of the algal extract on a mixture of maltose and a second sugar has been examined by paper chromatography. If the second sugar functions as an acceptor, new oligosaccharides will be formed in addition to those described here. As reported previously (Duncan *et al.* 1956), trans-α-glucosylation to D-galactose, D-fructose (and methanol) could not be detected. Similar experiments with D-xylose, L-xylose, L-sorbose and *N*-acetylglucosamine were unsuccessful. These six sugars do not therefore fulfil the acceptor-specificity requirements of the maltose trans-α-glucosylase. The results with the pentose sugars are in contrast with those obtained in an analogous study of trans-β-glucosylation in which cellobiose was used as β-glucosyl donor, since β-glucosylpentose disaccharides are formed in the presence of pentoses (D. J. Manners & J. L. Thompson, paper in preparation).

SUMMARY

1. An extract of *Cladophora rupestris* catalysed the synthesis of higher oligosaccharides from maltose.

2. The oligosaccharides were separated by charcoal–Celite chromatography and four sugars characterized by chemical and enzymic methods. They are panose, maltotriose, 6^3-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose] and maltotetraose.

3. The above and other evidence shows that, with the *C. rupestris* extract, maltose and maltotriose can function as α-glucosyl acceptors, whereas D-glucose, D-fructose, D-galactose, D-xylose, L-xylose, L-sorbose, *N*-acetylglucosamine and panose cannot.

4. The metabolism of maltose by transglucosylases from various biological sources is discussed.

We wish to thank Professor E. L. Hirst, F.R.S., for his advice and encouragement, and Dr W. J. Whelan for the gift of samples of maltotriose, panose and panitol acetate. The work described in this paper forms part of a research programme sponsored by the Institute of Seaweed Research, to whom W.A.M.D. is indebted for a research grant.

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The Effects of Manganese on the Solute Content of Rat-liver Mitochondria

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Manganese is known to affect the metabolic activities of isolated mitochondria (see e.g. Lindberg & Ernster, 1954) and also to prevent the loss of ultraviolet-light-absorbing substances from the mitochondria (Siekevitz & Potter, 1955). In view of these observations it was thought desirable to study the effect of Mn^{2+} ions on isolated mitochondria under a variety of conditions.

MATERIALS AND METHODS

Special chemicals

Sodium 1-azo-2-hydroxy-3-(2:4-dimethylcarboxanilido)-naphthalene-1′-(2-hydroxybenzene-5-sulphonate), used in the estimation of Mg^{2+} ions by the method of Mann & Yoe (1956), was purchased from LaMotte Chemical Products Co., Baltimore, Maryland, U.S.A. Ethylenediaminetetra-acetic acid (EDTA), formaldoxime hydrochloride and 8-hydroxyquinoline (AnalaR) were obtained from British Drug Houses Ltd.

Mitochondrial fractionation

This followed the procedure of Werkheiser & Bartley (1957). The mitochondrial pellet was suspended in sufficient 0·25 M-sucrose to give about 350 mg. wet wt. of mitochondria/ml. Usually 1 ml. of this suspension was added to 4 ml. of 0·25 M-sucrose or 4 ml. of 0·125 M-KCl, either of which contained $MnCl_2$, usually m-molar in the final suspension. After incubation, a portion (2 ml.) of the mixture was centrifuged, usually for 10 min. at 24 000 *g*. The supernatant fluid was decanted into 15 ml. conical centrifuge tubes containing 1 ml. of 30% trichloroacetic acid. After clarification by centrifuging the supernatant was set aside for analysis. The treatment of the mitochondrial pellet for the determination of the acid-soluble solutes and the acid-insoluble dry matter was described by Werkheiser & Bartley (1957). All analytical data refer to the samples (2 ml.) which were taken for centrifuging.

Analytical methods

Potassium was measured with a lithium internal standard flame photometer (Amoore, Parsons & Werkheiser, 1958). Manganese was measured by the use of formaldoxime (Bartley, Notton & Werkheiser, 1957). Calcium was estimated flame-photometrically with the apparatus of Exley (to be published) and chloride by the method of Sanderson (1952) with the slight modifications introduced by Amoore & Bartley (1958). The ultraviolet absorption at 260 mμ of the $HClO_4$ extracts of the supernatant medium was measured in a quartz cell with a 1 cm. light path in a Beckman spectrophotometer. From these measurements the absorption due to these substances in the original medium was calculated. The changes in absorption at this wavelength were assumed to be due to changes in the nucleotide content of the solutions. The estimation of the water content, and of the dry matter non-extractable with trichloroacetic acid, of the mitochondrial pellet was according to Werkheiser & Bartley (1957). Total phosphate was estimated by the method of Berenblum & Chain (1938), as modified by Bartley (1953), on a sample of the extract after wet-ashing according to Hanes & Isherwood (1949).

Measurements of pH were made with the Beckman probe assembly, type 14400, and a Beckman pH meter, model G. Measurements were made on the suspensions remaining after the sample (2 ml.) had been abstracted for centrifuging. The temperature of measurement was that at which the mitochondria had been incubated.

Magnesium was estimated either by the modification of the colorimetric method of Mann & Yoe (1956) described by Amoore & Bartley (1958) or by the flame-photometric method of Exley (to be published). Mn^{2+} ion interferes in