Studies on the Oxidation of Gluconate by Animal Tissues

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The direct oxidation of glucose in animal tissue was first studied by Harrison (1931), who discovered an enzyme present in liver which was able to oxidize glucose to gluconic acid without previous phosphorylation. This dehydrogenase was later shown to be active with either diphosphopyridine nucleotide (DPN) (Andersson, 1934), or triphosphopyridine nucleotide (TPN) (Euler, Adler, Schlenk & Günther, 1935), and this activity with both coenzymes has recently been confirmed using a purified preparation of the enzyme (Strecker & Korkes, 1952). The primary oxidation product of glucose has now been identified as gluconolactone, the hydrolysis of the latter to gluconic acid being non-enzymic (Strecker & Korkes, 1952).

The further metabolism of gluconic acid has been studied mainly in intact animals. Stetten & Stetten (1950) showed that when rats were injected with ^{14}C-labelled sodium gluconate, approximately 57% of the administered ^{14}C was excreted in the urine and 14% appeared in the expired CO_2. This, as well as the presence of some isotope both in the liver glycogen and the liver nucleic acids, shows that gluconate can be fairly readily oxidized *in vivo*. It therefore seemed of interest to determine which tissues of the body break down gluconate, and to study the products of its metabolism.

MATERIALS AND METHODS

Calcium D-*gluconate*, D-*ribose*, D-*arabinose*, D-*arabo-ascorbic acid*, D-*gluconolactone*, D-*glucuronolactone*. These were commercial products.

Calcium 2-oxo-D-*gluconate*. This was a gift to Professor Dickens from Dr P. P. Regna of Chas. Pfizer, New York.

Sodium 5-oxo-D-*gluconate*. This was kindly supplied by Dr J. De Ley.

Potassium D-*arabonate* (m.p. 215°) *and potassium* D-*ribonate* (m.p. 187°). These were prepared from D-arabinose and D-ribose respectively by hypoiodite oxidation according to Moore & Link (1940).

Ribulose. An aqueous solution of ribulose was prepared by the method of Hassid, Doudoroff, Barker & Dore (1946).

Glycolaldehyde (m.p. 95–97°). This was prepared from dihydroxymaleic acid according to Fischer & Taube (1927).

Adenosine triphosphate (*ATP*). A solution of the sodium salt was made by dissolving the barium salt (Boots Pure Drug Co., Ltd.) in the minimum amount of N-HCl, precipitating the Ba^{2+} with Na_2SO_4 and neutralizing.

Diphosphopyridine nucleotide. This was purchased from Nutritional Biochemicals Inc. Enzymic analysis (see below) showed that it contained 35·8% DPN and 0·7% TPN.

Triphosphopyridine nucleotide. This was prepared from acetone-dried ox liver (Boots Pure Drug Co., Ltd.) by the method kindly communicated to us by Dr C. Liébecq in 1949, and described by Dickens & Glock (1951). (Found: 23·4% TPN, 1·1% DPN.)

N-*Methylphenazinium methyl sulphate* (m.p. 166–168°). This was prepared from phenazine by the method of Hillemann (1938).

Zwischenferment (*Glucose 6-phosphate dehydrogenase*). This was prepared from Löwenbräu (Munich) bottom lager yeast according to LePage & Mueller (1949).

Lactic dehydrogenase (*semi-purified*). This was prepared from pig heart by the method of Straub (1940) as modified by Slater (1950).

[^{14}C]*Glucose*. 2 μC of uniformly labelled [^{14}C]glucose obtained from the Radiochemical Centre, Amersham, Bucks, were diluted to 2 g. with inactive glucose.

Potassium [^{14}C]*gluconate* (m.p. 180°). This was prepared by hypoiodite oxidation of [^{14}C]glucose according to the method of Moore & Link (1940). The total carbon content of a sample of the potassium gluconate was determined by the Van Slyke & Folch (1940) wet-oxidation method. (Found: C, 30·7%; calc. for $C_6H_{11}O_7K$: C, 30·77%.)

Analysis of DPN and TPN. The reduced forms of DPN and TPN were determined spectrophotometrically at 340 mμ. in the Hilger Uvispek (Hilger and Watts, London), using the extinction coefficients of Horecker & Kornberg (1948). DPN was determined by the method of Slater (1950) and TPN according to LePage & Mueller (1949).

Total reducing sugar. This was determined by the method of Hagedorn & Jensen (1923), and expressed as glucose. The reducing power of the gluconate and 2-oxogluconate employed corresponded to less than 0·1% of that of glucose.

Estimation of seven-minute hydrolysable phosphate. The two terminal phosphate groups of ATP were estimated by hydrolysis for 7 min. in N-HCl at 100° and subsequent determinations of P were made by the method of Fiske & Subbarow (1925).

Ribulose. This was estimated spectrophotometrically at 540 mμ. by the cysteine-carbazole method of Dische & Borenfreund (1951).

Pentose. This was determined by the orcinol method of Mejbaum (1939) with a 45 min. heating period (Albaum & Umbreit, 1947).

2-*Oxogluconic acid*. This was estimated by the *o*-phenylenediamine method of Lanning & Cohen (1951).

Manometric measurements. Oxygen uptake and CO_2 evolution were measured by means of Warburg manometers at 37° with O_2 as the gas phase. Tissue slices of thickness below 0·5 mm. were suspended in Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1949*a*) in the main part of the Warburg vessel, and the substrate was placed in the side arm. When oxygen uptake was measured, the centre well contained 0·2 ml. 20% (w/v) KOH as CO_2-absorbent. The direct method of Warburg was used for the

estimation of CO_2 and O_2 (Umbreit, Burris & Stauffer, 1949*b*) and approximately equal weights of tissue were placed in each of the two manometer cups. As it was desired to measure the pH of the solution at the end of the experiment, the customary acidification to determine the bound CO_2 was omitted. This was thought justifiable, since separate experiments showed that the bound CO_2 amounted to only 6% of the total CO_2 produced when kidney slices were used, and the final pH was 7·2. The effect of this small correction does not appreciably affect the conclusions drawn from these experiments.

Preparation of homogenates. The media used were 0·01 M sodium phosphate buffer, pH 7·4, and 0·9% (w/v) NaCl or KCl, all cooled to 0° for the homogenizing. The kidneys were quickly dissected, and homogenized for 3 min. in 5 vol. of one of the above media in a Potter–Elvehjem glass homogenizer cooled with ice. When NaCl or KCl was used, the pH was maintained at neutrality by the addition of dilute NaOH solution. For some experiments the homogenate was dialysed overnight against large volumes of distilled water at 4°.

Paper chromatography. This was carried out by the descending method; the solvent was prepared by shaking equal volumes of butanol and water with formic acid (400 ml. butanol, 400 ml. water, 37 ml. formic acid) to give a 2 N solution of the acid in the aqueous phase (Lugg & Overell, 1948). Whatman no. 1 filter paper was used. After being equilibrated with the aqueous stationary phase for 8 hr., the chromatogram was developed for 15 hr. The paper was dried at room temperature and then sprayed with a solution of aniline hydrogen phthalate in butanol (Partridge, 1949), which gives a pink colour with pentoses and 2-oxogluconate and brown with glucose. After being sprayed, the paper was partly dried in air and then in an oven at 100° for 5–10 min. 2 μg. of sugar could be detected in this way. As an alternative solvent, a mixture of butanol–water–ethanol (400:190:110, v/v/v) was used (Hough, Jones & Wadman, 1950).

Measurement of $^{14}CO_2$. The KOH in the centre well of the Warburg flasks, together with washings (4 × 0·2 ml. CO_2-free water) was transferred quantitatively by means of a syringe to a small gas generator attached to a high-vacuum apparatus. The total CO_2 present was liberated by the addition of 2–3 ml. acid consisting of 80% (v/v) concentrated H_2SO_4 and 20% (v/v) fuming H_2SO_4 (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949), freed from water by a solid CO_2–ethanol trap, and condensed for volume measurement in a McLeod gauge with liquid nitrogen. The KOH solution used in these experiments gave a zero blank for CO_2. The activity of the CO_2 samples was determined in standard gas counters with graphite cathodes (1 in. diameter, 100 ml. capacity) supplied by Twentieth Century Electronics, Dunbar Works, S.E. 27. The counting mixture of Brown & Miller (1947) was used (pressure in cm. Hg: 20 cm. CO_2 and 2 cm. CS_2) together with an operating voltage of 3200–3500 v. The quench unit was a modified Neher Harper circuit.

RESULTS

Oxygen uptake of tissue slices in presence of sodium gluconate and sodium 2-oxogluconate

The oxygen uptake of rat tissue slices was measured manometrically by the Warburg technique. Table 1 shows that kidney is the only tissue among those studied which gives an increase in O_2 consumption when incubated with either gluconate or 2-oxogluconate, 3·8 and 2·6 μmoles of extra O_2/μmole added substrate respectively being taken up in 6 hr. The effect of varying substrate concentrations on the O_2 uptake was investigated, but no increase could be demonstrated with increasing amounts of substrate. With liver slices, 2 μmoles O_2/μmole gluconate initially present were taken up, but there was no increased oxygen consumption in the presence of 2-oxogluconate. Neither brain nor mouse sarcoma 37 gave an appreciable increase in O_2 consumption with gluconate.

Fig. 1 illustrates the oxygen uptake of kidney slices in the presence of gluconate and 2-oxogluconate, compared with that of the control without substrate. It is unlikely that appreciable bacterial contamination occurred, as the values of O_2 uptake are proportional to the times of incubation (Fig. 1). The increases varied greatly from one sample of kidney to another, but the differences could not be accounted for by the season of the year or state of nutrition of the animals.

Table 1. *Oxygen uptake with sodium gluconate and sodium 2-oxogluconate as substrate*

Tissue slices, 70 mg. moist wt.; 1·8 ml. Krebs–Ringer phosphate, pH 7·4; 0·2 ml. 0·02 M substrate; 0·2 ml. 20% (w/v) KOH in centre well; gas phase O_2, temp. 37°.

Tissue	Substrate (0·002 M)	μl. O_2/6 hr.	Increase (μl. O_2)	$\frac{\mu\text{moles extra } O_2}{\mu\text{mole substrate}}$
Rat kidney cortex	Na gluconate	1218	342	3·8
	Control	876		
	Na 2-oxogluconate	905	238	2·6
	Control	667		
Rat liver	Na gluconate	306	155	2·0
	Control	151		
	Na 2-oxogluconate	166	15	0·17
	Control	151		
Rat brain cortex	Na gluconate	99	9	0·1
	Control	90		
	Na 2-oxogluconate	211	19	0·21
	Control	192		

The following experiments were performed in order to determine whether 2-oxogluconate, which has been shown to be an intermediate product in the oxidation of gluconate by both *Pseudomonas fluorescens* (Entner & Stanier, 1951) and *Aerobacter* sp. (De Ley & Cornut, 1951), is also formed from gluconate by kidney slices. When kidney slices were incubated with arsenite, which is known to inhibit the oxidation of several α-keto acids (Krebs, 1933), the O_2 uptake in the presence of gluconate was no greater than that of the control without substrate. After incubation at 37° for 1 hr., the contents of the vessels were deproteinized with perchloric acid, but no 2-oxogluconate (as determined spectrophotometrically with *o*-phenylenediamine by the method of Lanning & Cohen, 1951) could be detected in the filtrate. Sodium bisulphite and hydroxylamine were also tried as trapping agents for 2-oxogluconate. The oxygen uptake of kidney slices, both in the presence and absence of substrate, was greatly diminished by both these reagents. At the end of the experiments, the contents of the flasks were deproteinized with perchloric acid, and the protein-free filtrates were treated with *o*-phenylenediamine as above. The filtrates from the flasks to which bisulphite had been added were first treated with alkali to decompose any bisulphite addition compound which might have been formed. Again no 2-oxogluconate could be detected.

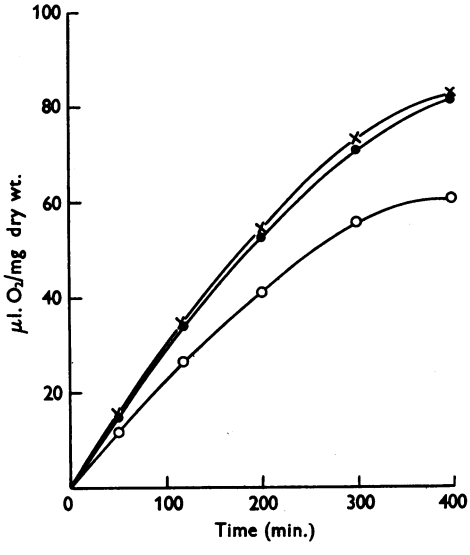

Fig. 1. Oxygen uptake of rat kidney cortex slices in presence of sodium gluconate and sodium 2-oxogluconate. ×—×, sodium gluconate; ●—●, sodium 2-oxogluconate; ○—○, control without substrate. Tissue slices, 70 mg. moist wt.; 1·8 ml. Krebs–Ringer phosphate, pH 7·4; 0·2 ml. 0·02M substrate; 0·2 ml. 20% (w/v) KOH in centre well; gas phase O_2, temp. 37°.

Disappearance of 2-oxogluconate

Rat kidney cortex slices were suspended in Krebs–Ringer phosphate at pH 7·4 in presence of sodium 2-oxogluconate, as before. Oxygen uptake was measured for 6 hr. The contents of the vessels were then deproteinized by addition of 2 ml. 10% (w/v) perchloric acid, 2 ml. of the filtrate were neutralized with N-NaOH, and oxogluconate was estimated according to Lanning & Cohen (1951). Although some 2-oxogluconate seemed to have disappeared in this time, the amount was too small for accurate measurement (Table 2).

Substrates other than gluconate and 2-oxogluconate

The only other experiments on the metabolism of gluconate by animal tissues which have been reported were carried out with the intact animal, and nothing is known of the intermediate products. The following compounds were therefore incubated with kidney slices: sodium 5-oxogluconate, glucuronolactone, potassium saccharate, D-araboascorbic acid, D-ribose, D-arabinose, sodium D-ribonate, sodium D-arabonate, glycolaldehyde and D-ribulose. In all cases the increase in oxygen consumption above that of the blanks was negligible, providing no evidence that any of them is an intermediate in the metabolism of gluconate by kidney slices.

Production of carbon dioxide and test for other possible metabolic products

Carbon dioxide output and O_2 uptake were measured manometrically by the direct method (Umbreit *et al.* 1949*b*). The figures in Table 3 indicate an appreciable increase of CO_2 output in presence of both substrates, 4 μmoles of extra CO_2 being found/μmole of gluconate and 2·5 μmoles CO_2/μmole 2-oxogluconate added.

Table 2. *Disappearance of 2-oxogluconate*

Rat kidney cortex slices; 1·8 ml. Krebs–Ringer phosphate, pH 7·4; 0·2 ml. 0·02M sodium 2-oxogluconate; 0·2 ml. 20% (w/v) KOH in centre well; gas phase O_2, temp. 37°. After 6 hr., 2 ml. 10% (w/v) perchloric acid were added and the 2-oxogluconate (2-OG) content of the filtrate was estimated.

μmoles 2-OG originally present	μmoles 2-OG 0 hr.	μmoles 2-OG 6 hr.	μmoles 2-OG disappeared in 6 hr.	μl. O_2/mg. dry wt./6 hr.
4·0	3·96	3·78	0·18	78·0
0·0	0	0	—	60·0

Paper chromatography was employed in an attempt to identify any metabolites formed from the substrates. 0·5 ml. 0·02M sodium gluconate or sodium 2-oxogluconate was incubated with kidney slices in Krebs–Ringer phosphate solution in the usual way, with O_2 as the gas phase. After shaking in Warburg vessels for various periods 1 ml. 20% (w/v) perchloric acid was added to precipitate the protein and 0·2–0·5 ml. of the filtrate was applied to the paper. After developing as described under Methods, using either butanol–water–formic acid or butanol–water–ethanol as solvents and spraying with aniline hydrogen phthalate, and comparing the chromatogram with one from a control experiment without substrate, no metabolite could be recognized. Standard sugars applied to the same chromatogram for comparison of R_F values gave satisfactory spots.

No pentose formation could be detected in the deproteinized filtrate by the orcinol method of Mejbaum (1939), nor could any other metabolite be found by the cysteine–carbazole reaction (Dische & Borenfreund, 1951).

Table 3. *Oxgen uptake and carbon dioxide output of kidney slices in presence of gluconate and 2-oxogluconate*

Rat kidney cortex slices approximately 70 mg. moist wt.; 1·8 ml. Krebs–Ringer phosphate, pH 7·4; 0·2 ml. 0·02M substrate; ±0·2 ml. 20% (w/v) KOH in centre well; gas phase O_2, temp. 37°.

Substrate	µl. O_2/6 hr.	Increase (µl. O_2)	µmoles O_2/µmoles substrate	µl. CO_2/6 hr.*	Increase (µl. CO_2)	µmoles CO_2/µmoles substrate added
Na gluconate	1090	469	5	710	341	4
Control	621			369		
Na 2-oxogluconate	879	260	3	635	244	2·5
Control	619			391		

* The low R.Q. may be partly due to the bound CO_2 held by the phosphate buffer as described in the experimental section.

Table 4. $^{14}CO_2$ *output by various tissues in the presence of labelled glucose and gluconate*

Tissue slices, approximately 70 mg. wet wt.; 1·8 ml. Krebs–Ringer phosphate, pH 7·4; 0·2 ml. 0·02M substrate, i.e. 4 µmoles; 0·2 ml. 20% (w/v) KOH in centre well; gas phase O_2, temp. 37°. Incubated 4 hr. The glucose and gluconate (added as K salt) gave 10975 and 4663 counts/min. respectively, except in experiments marked (1) and (2), where the values were 8836 and 4457 counts/min. respectively.

Tissue	Substrate	Activity of CO_2 (counts/min.)	$^{14}CO_2$ from substrate (µl.)	Total CO_2 (µl.)	Proportion of total respiration due to substrate (%)
Rat kidney cortex	Glucose (1)	2370	144	869	16·6
	Gluconate (2)	194	23·4	949	2·5
	Control	—	—	718	—
Rat kidney medulla	Glucose	3141	154	744	20·7
	Gluconate	84	9·1	456	2·0
	Control	—	—	503	—
Rat brain cortex	Glucose	4320	212	477	44·5
	Gluconate	0	0	410	0
	Control	—	—	322	—
Rat spleen	Glucose	1261	62	374	16·7
	Gluconate	0	0	380	0
	Control	—	—	385	—
Rat adrenals	Glucose	118	5·8	94	6·2
	Gluconate	0	0	90	0
Rat liver	Glucose	51	3·6	400	1·0
	Gluconate	0	0	389	0
Rat diaphragm	Glucose	34	1·7	82	2·0
	Gluconate	0	0	49	0
Rat leg muscle	Glucose	26	1·2	114	1·0
	Gluconate	0	0	—	0
Rabbit kidney cortex	Gluconate (2)	132	15·9	722	2·2
Rabbit liver	Gluconate (2)	0	0	—	0
Mouse sarcoma 37	Glucose	167	82	537	15·3
	Gluconate	43	5·0	492	1·0
	Control	—	—	603	—

Homogenates

Dehydrogenase activity. Kidney homogenates, prepared as described under methods, were dialysed against large volumes of distilled water at 4° overnight; undialysed preparations were unsuitable owing to high blanks. The rates of O_2 uptake were determined by incubating the homogenate in the presence of phosphate buffer, coenzyme, substrate and hydrogen carrier in Warburg vessels. No O_2 uptake above the blank was obtained, however, on the addition of TPN, DPN or other hydrogen acceptors, such as cytochrome *c*, methylene blue and *N*-methylphenazinium methyl sulphate. The results were similar when homogenates in water, KCl or phosphate buffer were employed.

Gluconokinase activity. The method of Colowick & Kalckar (1943) was used in order to determine whether gluconate is phosphorylated, as was shown by Cohen & McNair Scott (1950) to take place in extracts of *Escherichia coli*. The method is based on the liberation of CO_2 from bicarbonate, due to the formation of an extra acidic group on transfer of phosphate from ATP. Although an active glucokinase was present in the kidney preparation, no phosphorylation of gluconate was detectable. This was confirmed by measuring the disappearance of easily hydrolysable phosphate when a homogenate was incubated with substrate and ATP in the presence of magnesium ions and fluoride, the latter inhibiting adenosine triphosphatase. The decrease in 7 min. hydrolysable phosphate was used to measure the extent of phosphorylation. No phosphorylation of gluconate had occurred. It can be concluded from these and the previous results that there was no gluconokinase activity in this tissue dispersion.

Isotopically labelled substrates

Uniformly labelled [^{14}C]glucose and gluconate were incubated with tissue slices in Warburg vessels, as usual. After deproteinizing, $^{14}CO_2$, which had been absorbed by the KOH in the centre well, was measured as described (see Methods). Table 4 shows that of the normal tissues tested, kidney is the only one which is reasonably active in metabolizing gluconate. There was, however, some slight decarboxylation of gluconate by mouse sarcoma 37, 1% of the total respiratory CO_2 being $^{14}CO_2$ derived from the labelled substrates.

When labelled glucose and gluconate were incubated with kidney homogenate, no decarboxylation of gluconate could be detected, and similar results were obtained with homogenates prepared as described under Methods and fortified with ATP, cytochrome *c*, sodium succinate and magnesium ions.

DISCUSSION

The most probable pathways of gluconate breakdown are: (1) phosphorylation to 6-phosphogluconate, and the further metabolism of the latter via the hexose monophosphate 'shunt', or (2) direct non-phosphorylative oxidation, possibly to 2- or 3-oxogluconate, the former compound having been shown to be formed in *Pseudomonas fluorescens* (Entner & Stanier, 1951), followed by decarboxylation of the keto acid to pentose. As no phosphorylation of gluconate could be demonstrated in kidney homogenates, it is unlikely that further oxidation occurs via the hexose monophosphate 'shunt', at least in the homogenized tissue. So far, no direct evidence has been obtained for the metabolism of gluconate in animal tissues by direct non-phosphorylative oxidation, as no intermediate products have been identified.

The experiments with labelled gluconate, described above, have shown that rat kidney is the only one of the normal tissues used which is able to metabolize added gluconate to a measurable extent, although the rate of oxidation is only about one-sixth of that of glucose. It can be concluded that the direct oxidative pathway of gluconate breakdown plays a very small role in the metabolism of the rat.

Stetten & Stetten (1950) and Stetten & Topper (1953) report a much more extensive carbon dioxide output from injected gluconate in the rat than can be accounted for by the above figures. They have obtained evidence supporting a fairly extensive conversion of gluconate into glucose in the whole animal, however, and it is possible that under their conditions this pathway may account for the large metabolism of gluconate which they observed.

SUMMARY

1. The oxygen uptake of various tissue slices in the presence of gluconate and 2-oxogluconate was measured. Kidney was found to be the only one of the tissues studied where an appreciable oxidation of both substrates was observed.

2. No 2-oxogluconate could be detected as an intermediate product of gluconate oxidation in kidney slices.

3. Decarboxylation of both gluconate and 2-oxogluconate by kidney slices was found to occur, with simultaneous increase of the oxygen uptake. The formation of carbon dioxide from gluconate in this tissue was confirmed with ^{14}C-labelled gluconate. The amount of gluconate oxidized was only about one-sixth of the glucose oxidized.

4. No oxidation of gluconate was observed with kidney homogenates in the presence of coenzymes and hydrogen carriers.

5. No phosphorylation of gluconate by kidney homogenate could be detected under conditions where glucose was readily phosphorylated.

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The Carotenoids of the Petals of Two Species of *Tecoma*

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Two species of the tropical *Tecoma* trees, belonging to the Bignoniaceae family, carry flowers of different colour. Those of *T. stans* are yellow, whilst those of *T. capensis* are deep red. Such variation in colour between two species of the same botanical family stimulated the study of the distribution of their carotenoid pigments, especially as no flowers of other members of the Bignoniaceae have been previously investigated, although Zechmeister & Sandoval (1945) demonstrated the presence of the colourless polyene phytofluene in species of *Bignonia*.

METHODS

Extraction of pigments. Freshly collected flowers (50 g.) were chopped with a sharp knife and heated with ethanolic KOH (300 ml., 10%, w/v) for 10 min. at 70° and then left overnight under N_2 at room temperature. The pigments were extracted according to the method of Goodwin & Taha (1950) and dissolved in light petroleum (except where otherwise stated light petroleum b.p. 40–60° was used). Sometimes a few drops of ethanol were added before the light petroleum to facilitate the dissolution of xanthophylls. After standing overnight under N_2 in the refrigerator, the extract was filtered from a white residue, and examined as described below.

Fractionation of pigments. The pigments were separated into epiphasic and hypophasic fractions by partition between light petroleum and 90% (v/v) aqueous methanol, and each fraction was chromatographed separately. The monohydroxy carotenoids were assumed to be in the epiphasic fraction.

Chromatography. The pigments were separated on columns of either activated alumina (British Drug Houses Ltd.) or alumina deactivated by the method of Goodwin & Taha (1950). Alumina was also weakened by making a paste of it with ethanol and spreading out the paste in layers about an