Short Communications

Biosynthetic Relationship between Ubichromenol and Ubiquinone

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Ever since the discovery by Links (1960) that ubiquinone can be cyclized to its isomer, ubichromenol, by an alumina-catalysed reaction, the natural occurrence of ubichromenol has been one of doubtful status (Draper & Csallany, 1960). Morton (1961) has summarized the evidence in support of its natural occurrence. It was shown for the first time from this Laboratory (Joshi, Jayaraman & Ramasarma, 1963*a*) that [2-¹⁴C]mevalonate administered to the rat was incorporated into ubichromenol, as well as ubiquinone, thereby confirming its natural origin. In the present communication, data on the time-course of incorporation of [2-¹⁴C]mevalonate into ubiquinone and ubichromenol in the rat are presented and the possible biosynthetic relationship between these compounds is briefly reviewed.

[2-¹⁴C]Mevalonate was given orally to four groups of four rats each (5 μ C per rat). The rats in the different groups were killed at the end of 1, 2, 4 and 8 hr., and the livers, kidneys and intestines were removed. The tissues were saponified in the presence of pyrogallol and the unsaponifiable lipids were purified by column chromatography on alumina as described by Joshi, Jayaraman & Ramasarma (1963*b*). Ubiquinone and ubichromenol eluted in ether-light petroleum (1:19, v/v) and ether-light petroleum (1:9, v/v) respectively from the column were further purified by thin-layer chromatography on alumina with acetone-light petroleum, (1:24, v/v): the R_f values of ubiquinone and ubichromenol were 0.8 and 0.2 respectively. The specific radioactivities of ubichromenol and ubiquinone in liver, kidneys and intestine (Fig. 1) indicate a precursor-product relationship.

Several lines of evidence prompted us to suggest that ubichromenol may serve as a precursor of ubiquinone. Changes in the concentration of ubichromenol in the tissues of rats corresponded to changes of ubiquinone, as expected of a precursor, in vitamin A deficiency (Morton & Phillips, 1959), in vitamin E deficiency (Edwin, Diplock, Bunyan & Green, 1961) and on protein deprivation (Joshi, Jayaraman & Ramasarma, 1963*c*). Jayaraman & Ramasarma (1963) have shown that orally administered [¹⁴C]ubiquinone-50 was deposited in the rat

liver and was distributed in the cell fractions similar to the endogenous form. Though this exogenous ubiquinone equilibrated with the endogenous form (Joshi, Jayaraman, Ramakrishna Kurup & Ramasarma, 1964), it was not converted into ubichromenol (Joshi *et al.* 1963*b*). Further, under the experimental conditions used, the formation of ubichromenol during the isolation procedures, as an artifact, was negligible (Hemming, Laidman, Morton & Pennock, 1961; Joshi *et al.* 1963*b*). In all our experiments, with time-intervals of 1–8 hr., the specific radioactivity of ubichromenol was several-fold higher than that of ubiquinone in liver, kidneys and intestine. Green, Diplock, Bunyan & McHale (1963), though confirming the incorporation of radioactive mevalonate into ubichromenol of rat liver, reported a lower specific radioactivity of ubichromenol compared with that of ubiquinone. The time-interval used in their experiment was 24 hr. Interpreting the data at this single late time-interval, they claimed that ubichromenol cannot be a precursor of ubiquinone and therefore disagreed with our hypothesis. But, in fact, their results are not in contradiction to our hypothesis, since such a result is easily explained by the data in Fig. 1 where the specific radioactivity of ubichromenol decreases with time and that of ubiquinone increases. Thus it is concluded that ubichromenol could not have been formed from ubiquinone and, if at all, it can only serve as its precursor.

Direct experimental verification of this hypothesis by examining the conversion of ubichro-

Fig. 1. Specific radioactivities of ubiquinone (○) and ubichromenol (●) at various time-intervals in liver (a), kidneys (b) and intestine (c) of rats given [2-¹⁴C]mevalonate orally. The values in ordinate are given in logarithmic scale for convenience.

menol into ubiquinone was next attempted (Ramasarma & Joshi, 1964). Radioactive ubichromenol was administered to rats, both orally and intracardially in separate experiments, and ubiquinone and ubichromenol were purified from the total lipids of livers. Although large quantities of radioactive ubichromenol were present, ubiquinone was not labelled, showing thereby the absence of conversion of ubichromenol into ubiquinone under these conditions.

These results provide evidence for the independent biosynthesis of ubiquinone and ubichromenol in the rat. From the results with *Torula* yeast, Stevenson, Hayward, Hemming & Morton (1962) suggested the need for a common precursor and that ubichromenol might have been formed from this precursor by a non-enzymic process. In *Aspergillus niger*, a mould containing ubiquinone but not ubichromenol, the incorporation of mevalonate into ubiquinone and sterol, as in rat liver, was demonstrated (Jayaraman & Raman, 1964). If the process of conversion of the common precursor into ubichromenol was non-enzymic, ubichromenol should have been detected in *Aspergillus niger*, since the methods employed were the same as for the rat liver. Therefore the presence of ubichromenol will probably depend on its enzymic formation, which appears to be characteristic of few species.

The biosynthetic relationship between ubichromenol and ubiquinone can be summarized as follows. The possibility of the formation of ubichromenol by isomerization of ubiquinone can be ruled out without any ambiguity. Ubichromenol may not serve as a precursor of ubiquinone. It is possible that ubichromenol is formed enzymically from a precursor of ubiquinone and thus the pattern of its specific radioactivity reflects that of an apparent precursor.

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Some Kinetic Properties of Pig-Heart Oxoglutarate Dehydrogenase that Provide a Basis for Metabolic Control of the Enzyme Activity and also a Stoichiometric Assay for Coenzyme A in Tissue Extracts

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The importance in metabolic control of CoA and some of its acyl derivatives has recently been stressed (Tubbs & Garland, 1963, 1964; Bortz &

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Lynen, 1963; Weiland & Weiss, 1963; Krebs, 1964). Enzymic assay of CoASH (thiol form) in tissue extracts requires rapid completion at pH 7 or below to avoid interfering effects that increase with increasing pH, such as the oxidation of CoASH, the

liberation of CoASH disulphides through reactions with thiols, and the hydrolysis of acyl derivatives of CoA.

The present report describes the inhibition of oxoglutarate dehydrogenase (EC 1.2.4.2) by its products NADH and succinyl-CoA, and conditions under which the same enzyme may be used to assay CoASH stoichiometrically with minimal interference from other CoA derivatives.

Oxoglutarate dehydrogenase was prepared according to the method of Sanadi, Littlefield & Bock (1952). NAD⁺, NADH and CoASH were purchased from Boehringer und Soehne G.m.b.H., Mannheim, Germany. 3-Acetylpyridine-adenine dinucleotide (APyAD⁺) was kindly provided by Dr C. J. R. Thorne of this Department, and reduced to APyADH as described for NAD by Rafter & Colowick (1957). Succinyl-CoA was synthesized as described by Simon (1957). Standard solutions of CoASH were assayed and also the acid-soluble fraction of rat-liver CoA as described by Tubbs & Garland (1964). Solutions of NAD⁺ and NADH were assayed as described by Klingenberg (1963), and APyAD⁺ and APyADH in a similar manner. Spectrophotometric assays were performed at 25° in a Beckman DK 2 recording spectrophotometer by following the extinction change at 340 mμ for NADH production, at 365 mμ for APyADH production, and at 344 mμ when production of NADH and APyADH were to be compared directly (Siegel, Montgomery & Bock, 1959). Assays were also performed in an Eppendorf photometer, modified to record, with a 4.0 cm. light-path cell at 25° and at 366 mμ. Phosphate buffers were prepared by adjusting KH₂PO₄ solutions with KOH.

To assay CoASH in perchloric acid extracts of rat liver, the extracts were carefully adjusted with *N*-KOH to pH 5 (glass electrode) while being continuously stirred. Extracted CoASH was then assayed by measuring the change in extinction at 366 mμ on adding oxoglutarate dehydrogenase (60 μg.) to a 4.0 cm. light-path cell containing: phosphate buffer, pH 6.8 (45 mM); MgCl₂ (0.9 mM); EDTA (0.45 mM); GSH (0.25 mM); APyAD⁺ (0.2 mM); 2-oxoglutarate (1.0 mM); CoASH (0–10 μM) from tissue extract; the final volume was 2.2 ml.

Sanadi & Littlefield (1953) reported that in a NAD-linked assay system for oxoglutarate dehydrogenase, limiting CoASH was not completely succinylated in the reaction:

In a similar assay system Sanadi, Langley & White (1959) observed non-linear progress curves for the production of NADH, despite the fact that

all of the substrates were present in considerable excess of their *K_m* values (oxoglutarate 13 μM, CoA < 0.1 μM, NAD⁺ 4.5 μM; Sanadi, 1963). A similar effect is seen in Fig. 1 (curve A). However, at a higher initial NAD⁺ concentration, the reaction proceeded to the theoretical CoASH-limited completion (curve B). The same completion is obtained at a lower concentration of APyAD⁺, and the reaction course is more nearly linear (curve C). The slower initial rate with APyAD⁺ is due to the higher *K_m* (80 μM). Curve D demonstrates the inhibitory effect of added NADH.

The pH-dependence of the inhibition by NADH was studied over the pH range 5.5–7.5. At each pH taken, the initial velocity in the absence of added NADH was assigned a value of 100 %, and the percentage inhibition in the presence of various concentrations of added NADH was determined. It was found that, for any given percentage inhibition of the enzyme at a set pH, the value of $[\text{NAD}^+]/[\text{NADH}][\text{H}^+]$ was constant and could be expressed as the oxidation-reduction potential, *E_h*, of the NAD system. With increasing *E_h* values (i.e. towards oxidation) the percentage inhibition decreased, and conversely so. The *E_h* required for a given percentage inhibition was unchanged when NAD was replaced by APyAD. These findings indicate that increasing reduction of part of the enzyme complex is associated with inhibition of the overall oxoglutarate-dehydrogenase activity.

Fig. 1. Product inhibition of oxoglutarate dehydrogenase. The system (2.2 ml.) contained: phosphate buffer, pH 6.5 (45 mM); MgCl₂ (0.9 mM); EDTA (0.45 mM); GSH (0.25 mM); 2-oxoglutarate (1.0 mM); oxoglutarate dehydrogenase (20 μg.). Other components were: curve A: NAD⁺ (90 μM); curve B: NAD⁺ (900 μM); curve C: APyAD⁺ (90 μM); curve D: NAD⁺ (90 μM) and NADH (20 μM); curve E: NAD⁺ (90 μM), NADH (20 μM) and succinyl-CoA (11 μM); curve F: NAD⁺ (90 μM) and succinyl-CoA (11 μM). In all cases the reaction was started with CoASH (20 μM) added at the points marked with an arrow. The vertical bar to the right of the Figure represents the theoretically predicted extinction change for the complete assay of 20 μM-CoASH. Graphs were taken from recordings made with a Beckman DK 2 spectrophotometer.

Curves *E* and *F* (Fig. 1) show that added succinyl-CoA inhibited in the presence but not in the absence of added NADH. Inhibition by succinyl-CoA is abolished at infinite [CoASH], and also by increasing the E_h of the NAD system or by replacing NAD with a comparable concentration of APyAD. The requirement for a partially reduced enzyme complex and the reversal by infinite [CoASH] suggest that the inhibitory effects of succinyl-CoA are associated with succinylation of lipoyl residues in the enzyme.

A possible common mechanism for the inhibitory effects of NADH and succinyl-CoA is that accumulation of reduced and succinylated lipoyl residues in the enzyme complex results in a decrease of oxidized lipoyl residues, which then behave as a rate-limiting cofactor in the overall oxoglutarate dehydrogenation. Product inhibition of oxoglutarate dehydrogenase provides a mechanism whereby the enzyme activity could be related to the state of mitochondrial reduction and, via succinyl-CoA synthetase (EC 6.2.1.4), to the phosphorylation state of mitochondrial nucleotides. The behaviour of oxoglutarate dehydrogenase resembles that of pig-heart pyruvate dehydrogenase, which is inhibited by its products acetyl-CoA and NADH (Garland & Randle, 1964, and unpublished work).

As shown in Fig. 1, CoASH can be assayed by measuring the reduction of APyAD (curve *C*). Under the conditions described for assay of CoASH in tissue extracts, the extinction change is proportional to the CoASH added and is completed within 1 min. of adding the enzyme. Any continued slow increase in extinction due to hydrolysis of succinyl-CoA and recycling of CoA can be corrected graphically (Bergmeyer, 1963). Added acetyl-CoA, butyryl-CoA or 3-hydroxybutyryl-CoA (20 μ m-moles) does not interfere.

The content of CoASH in liver from fed rats was 47 ± 3.3 (5) μ m-moles of CoASH/g. wet wt. of

tissue (mean $\pm$ S.E.M.; number of observations in parentheses). In liver from rats starved for 36 hr. the content was 76 ± 6.0 (5), and from alloxan-diabetic rats 77 ± 8.9 (6). The percentage of total acid-soluble CoA (see Tubbs & Garland, 1964) accounted for as CoASH was 39 ± 2.7 (5), 50 ± 1.9 (5) and 45 ± 2.1 (6) in liver from normal, starved and alloxan-diabetic rats respectively.

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The Structure of Cardiolipin

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From the time of Thudichum the pathways of identification of natural phospholipids have been enlivened by controversy and strewn with 'red herrings'. To this, cardiolipin is no exception. It is common ground that it is a polyglycerophosphatide (Pangborn, 1942) with a molar ratio of fatty acids to

phosphorus of 2:1, which on mild alkaline saponification gives a single water-soluble polyglycerophosphate without liberation of phosphoric acid. In the last few years structure (I) (Table 1), proposed by Pangborn (1947), has generally lost ground in favour of structure (II); but recently

Rose (1964) suggested structure (III), and de Haas & van Deenen (1963) consider that the choice between structures (I) and (II) is still open. It is perhaps timely to consider various structures for the molecule (Table 1) by the null hypothesis, i.e. a postulated structure is 'in' until it is 'out' by experimental results. The nature of the fatty acids is immaterial for this.

Structure (I) is a hexa-acyl $\alpha\gamma$ -polyglycerophosphatide with no free hydroxyl group. Macfarlane & Gray (1957, 1958) proposed structure (II), a diphosphatidylglycerol in which the phosphatidyl groups were arbitrarily assigned to the $\alpha\gamma$ -positions, leaving a median β -hydroxyl group free. Rose (1964) proposed structure (III), triphosphatidylglycerol, postulating that the β -phosphatidyl group would be very labile to acid hydrolysis, giving compound (II) and phosphatidic acid. Structure (IV), now set down for consideration, is the $\alpha\beta$ -isomer of structure (II), and structure (V) is a variant of structure (II) in which there is a primary or secondary hydroxyl group free in a terminal glycerol.

Evidence on chain structure of deacylated glycerol.

(i) Oxidation of deacylated cardiolipin with periodate yielded 1 mol. of formaldehyde/mol. of phosphorus (Macfarlane, 1958). This excludes structure (I) and any chain longer than in structures (II) or (III), in which the number of vicinal hydroxyl groups gives a ratio less than one.

(ii) Benson & Strickland (1960) synthesized $\alpha\gamma$ -bis(glycerylphosphoryl)glycerol (GPGPG) by treatment of synthetic glycerodiphosphate (PGP, formed by phosphorylation of 1,3-dichloropropan-2-ol) with glycidol. This compound co-chromatographed with the deacylation product from *Chlorella* polyglycerophosphatide, which in turn co-chromatographed with the corresponding product from sheep-heart mitochondria (Strickland & Benson, 1960). The synthetic GPGPG also co-electrochromatographed with the *Chlorella* ester, and the electrophoretic mobility was consistent with the presence of two phosphoric diester groups in the molecule. Brief hydrolysis of the synthetic and the *Chlorella* ester at 100° in 0.1N-hydrochloric acid gave the same initial products, α -

glycerophosphate and glycerylphosphorylglycerol (GPG), the latter decreasing in time with increase in glycerophosphate. These facts virtually exclude structure (III) (as well as structure I), whose deacylation product contains 3 phosphoric diester groups/mol. and is therefore unlikely to co-chromatograph with GPGPG, and which should give different initial products on hydrolysis. As Benson & Strickland's (1960) data do not specifically distinguish between the deacylation products of compounds (II) and (IV), which are presumably very similar in behaviour, both these structures remain 'in'.

Evidence from acid hydrolysis of cardiolipin. On hydrolysis of cardiolipin in 90 % (v/v) acetic acid Macfarlane & Wheeldon (1959) obtained 76 % of the fatty acids as diglycerides, with only 5 % of free acids, and 80 % of the phosphorus as water-soluble esters of which not more than 10 % was glycerophosphate and the rest was glycerodiphosphate. This excludes structures (I) and (V) but, as Rose (1964) points out, does not exclude structure (III), as the residual ether-soluble phosphorus was not identified. However, Coulon-Morelec, Faure & Maréchal (1960) found that on hydrolysis of cardiolipin in acetic acid the first products were diglycerides and phosphatidylecloglycerophosphate. On hydrolysis of cardiolipin as the free acid in water at 18° (Coulon-Morelec, Faure & Maréchal, 1962) the first products were a triacyl-lysocardiolipin and phosphatidylglycerophosphate; phosphatidic acid and glycerophosphate were not detected. These facts exclude structure (III) (whose first product is postulated to be phosphatidic acid) and are consistent with either structure (II) or structure (IV).

Synthesis of an acetyl-cardiolipin. Coulon-Morelec (cited by Faure & Coulon-Morelec, 1963) synthesized an acetyl derivative of cardiolipin by the action of excess of acetic acid and dicyclohexylcarbodi-imide in pyridine; it contained 0.48 mol. of acetyl group/mol. of phosphorus. This is the first direct evidence that cardiolipin contains a free hydroxyl group, but it does not distinguish between structures (II) and (IV).

Table 1. *Structures for cardiolipin*

(GP), Glycerylphosphoryl; phosphatidyl and lysophosphatidyl, di- and mono-acylglycerylphosphoryl respectively.

Structure	Designation	Deacylation product	Lipid product of acid hydrolysis (moles/mole)
(I)	Polyglycerophospholipid	GPGPGPG	2 Diglyceride + 2 fatty acid
(II)	$\alpha\gamma$ -Diphosphatidylglycerol	$\alpha\gamma$ -Di(GP)G	2 Diglyceride
(III)	Triphosphatidylglycerol	Tri(GP)G	Phosphatidic acid + 2 diglyceride
(IV)	$\alpha\beta$ -Diphosphatidylglycerol	$\alpha\beta$ -Di(GP)G	2 Diglyceride
(V)	α -Phosphatidyl- β -acyl- γ -lysophosphatidylglycerol	$\alpha\gamma$ -Di(GP)G	1 Diglyceride + 1 mono-glyceride + 1 fatty acid

Synthesis of an acyl derivative of diphosphatidylglycerol. A derivative of compound (II), $\alpha\gamma$ -bis-(γ -oleoyl- β -palmitoyl-L- α -glycerylphosphoryl)- β -myristoylglycerol (VI), was synthesized by de Haas & van Deenen (1963); this is clearly not cardiolipin as the ratio of fatty acids to phosphorus is different. They compared the action of phospholipase A on cardiolipin and on compound (VI), examining the products chromatographically and by gas-liquid chromatography. Cardiolipin after 6 hr. incubation gave two products: after 18 hr. the original cardiolipin and the faster product (presumably an intermediate triacyl-lysocardiolipin) had disappeared and half the original fatty acids had been released. The enzyme did not release any of the ' β -located' myristic acid from compound (VI), and the rate of hydrolysis was much less. After 18 hr. much of the material was unhydrolysed and two products were present, apparently with the same R_f values as those from cardiolipin. This result is puzzling, for if no myristic acid was liberated the expected products of compound (VI) would be different from those of compound (II), i.e. a tetra-acyl intermediate and a triacyl final product.

An early attempt to determine the structure of cardiolipin by oxidation of the hydroxyl group and identification of the oxidation product was incon-

clusive (Macfarlane & Gray, 1957). At present it appears that cardiolipin can be either compound (II) or compound (IV). The route of biosynthesis is not yet known and perhaps the possibility that it proceeds through compound (IV) to compound (II) should be considered.

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Gluconic Acid in the Human Lens

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This paper reports the presence of free gluconic acid in the human lens. The formation of xylonic acid from xylose by the intact calf lens *in vitro* has already been shown (van Heyningen, 1958), and also the presence of an enzyme, in dialysed extracts of bovine and rat lens, which will reduce NAD^+ in the presence of a high concentration of glucose or xylose.

Lens extracts. Human cataractous lenses were removed at operation; a few normal lenses were obtained *post mortem*; both were stored frozen until required. Animal lenses were used soon after death. Trichloroacetic acid was used to precipitate lens proteins. It was removed from the protein-free extracts by shaking with methyldioctylamine in chloroform (Hughes & Williamson, 1951). The extracts (pH 7) were then concentrated *in vacuo*.

Distribution and preparation of acid. The lens extracts were found, by means of paper electro-

phoresis in pyridine (2.5 %, v/v)-acetic acid (0.25 %, v/v), pH 6 (Runeckles & Krotkov, 1959), to contain a negatively charged substance revealed by AgNO_3 -NaOH (Trevelyan, Procter & Harrison, 1950). The concentration of the acid was judged by the strength of the spot on an electrophoresis paper, compared with that given by known amounts of gluconic acid. In three clear (post-mortem) lenses from non-diabetics its concentration was 0.1 $\mu\text{mole/g.}$ of lens or less. In two clear (post-mortem) lenses from diabetics it was at concentrations of 2.5 $\mu\text{moles/g.}$ of lens and 0.4 $\mu\text{mole/g.}$ of lens. About 100 cataractous lenses were examined from non-diabetic and diabetic patients (Pirie & van Heyningen, 1964). The amount of the acid in these lenses was not assessed, but it was present in nearly all, and at a considerably higher concentration (probably up to 0.5 $\mu\text{mole/g.}$ of lens) in those from diabetics.

The acid was not detected in this way in the normal lens of the rat, rabbit or sheep; however, there is evidence of its presence in the lens of rabbits made diabetic with alloxan (A. Pirie & B. Bullard, personal communication). The normal cattle lens also contains a trace, less than $0.1 \mu\text{mole/g}$.

The acid was eluted from paper, after electrophoresis at pH 6 of pooled extracts of cataractous lenses. The eluate was brought to pH 7.5 with dilute ammonia.

Comparison of the acid in lens with gluconic acid, before and after lactonization, by using paper chromatography and electrophoresis. The eluted acid did not separate from authentic gluconic acid in paper electrophoresis in borate buffer at pH 9.2, and had the same R_f as gluconic acid in four chromatographic solvents. These were butan-1-ol-acetic acid-water (40:9:20, by vol.), ethyl acetate-pyridine-water (12:5:4, by vol.), pentanol-5M-formic acid (1:1, v/v) and ethanol-ammonia (sp.gr. 0.88)-water (16:1:3, by vol.). The acid did not react with the usual dips and sprays devised to reveal the presence of phosphorus, amino groups, aldehyde and keto groups, uronic acid, lactones or esters.

The eluted neutralized acid was treated with N-HCl for 15 min. at room temperature. Ammonium gluconate (an aqueous solution of glucono-1 $\rightarrow$ 5-lactone from British Drug Houses Ltd., brought to pH 7.5 with ammonia) was subjected to the same treatment. In both cases paper chromatography in the solvent system of Takahashi & Mitsumoto (1963) resulted in the separation of five AgNO_3 -NaOH-positive spots, R_f 0, 0.09, 0.15, 0.26 and 0.46. Three of these spots reacted with hydroxylamine-ferric chloride (Abdel-Akher & Smith, 1951), R_f 0, 0.26 (probably the 1 $\rightarrow$ 5-lactone) and 0.46 (probably the 1 $\rightarrow$ 4-lactone). Boiling in N-HCl for 15 min. did not alter this pattern.

Formation of acid in vitro. After incubation of extracts of human lens with glucose and NAD^+ (van Heyningen, 1958), a strong spot with the same mobility as that of gluconic acid was separated by paper electrophoresis at pH 6. This spot was very faint in the control incubation without NAD^+ .

Enzymic identification of acid. Since the eluted acid was inevitably mixed with traces of carbohydrate from the paper, it was considered not possible to identify it by such methods as measurement of optical rotation or infrared-absorption spectrum. The acid was therefore identified by means of two successive enzyme reactions (Leder, 1963). Sodium [^{14}C]gluconate (The Radiochemical Centre, Amersham, Bucks.) ($0.01 \mu\text{mole}$) was added to a portion of the eluate which was calculated, from the size and intensity of a chromatographic spot, to contain about $0.2 \mu\text{mole}$ of

gluconic acid. This was then treated with gluconokinase (ATP-D-gluconate 6-phosphotransferase; EC 2.7.1.12) made from pig liver by the method of Leder (1963). The 6-phosphogluconic acid so formed was precipitated from the mixture as the barium salt (Umbreit, Burris & Stauffer, 1949). After removal of the Ba^{2+} ions by shaking with Amberlite resin IR-120 (H^+ form) the 6-phosphogluconic acid was separated by paper electrophoresis at pH 6, located by radioautography, eluted and assayed by its reduction of NADP^+ in the presence of 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate-NADP oxidoreductase (decarboxylating); EC 1.1.1.44] (from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). The reduction of NADP^+ was equivalent to $0.042 \mu\text{mole}$ of gluconate. Since 24 % of the added radioactivity was recovered in the eluted 6-phosphogluconic acid, $0.165 \mu\text{mole}$ of gluconic acid was present in the original eluate from the lens extract.

Discussion. The presence of free gluconic acid in a mammalian tissue does not appear to have been reported previously. Glucose dehydrogenase [β -D-glucose-NAD(P) oxidoreductase; EC 1.1.1.47] was detected in the liver by Harrison (1931) and has since been purified and examined (Strecker & Korkes, 1952; Brink, 1953). Brink suggests that the oxidation of glucose to gluconic acid does not occur in the liver, because of the low turnover number of the enzyme, its high Michaelis constant for glucose and the fact that it is inhibited by a low concentration of glucose 6-phosphate. Why gluconic acid is formed in the lens is not known, but its increased formation in diabetes is probably due to the raised concentration of glucose in the aqueous humour.

Free glucose is not only oxidized in the lens; it and other aldoses are also reduced to the corresponding sugar alcohols (van Heyningen, 1959) by an enzyme, probably the same as that described by Hers (1957) in the seminal vesicles and placenta of the sheep (polyol-NADP oxidoreductase; EC 1.1.1.21). In the normal human lens glucose, gluconic acid, sorbitol and fructose are all found, and in diabetes the concentrations of all these carbohydrates are elevated. No study has been made of the relative contributions at different glucose concentrations of the three enzymes that attack glucose (hexokinase, glucose dehydrogenase and aldose reductase).

In liver D-glucono-1 $\rightarrow$ 5-lactone is the primary product of glucose dehydrogenation (Strecker & Korkes, 1952); it is not known whether this is the case in the lens.

Gluconic acid is metabolized in the mammal to some extent (Stetten & Topper, 1953). The liver and kidney contain an enzyme that will phosphorylate gluconic acid to form 6-phosphogluconic acid, which can then be metabolized by the pentose

phosphate pathway (Leder, 1957). Experiments in progress have shown that gluconic acid is metabolized by extracts of human and rabbit lens, with the formation of lactic acid.

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The Molecular Weight of Avidin

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Measurements of the molecular weight of avidin in solutions of guanidine hydrochloride (Green, 1963*b*) showed that it was dissociated into sub-units whose molecular weight was close to the equivalent weight with respect to biotin. The number of sub-units was assumed to be three since Fraenkel-Conrat, Snell & Ducay (1952*b*) had based their calculations of molar amino acid composition on a molecular weight of 58 000 for avidin A. Other values quoted by Fraenkel-Conrat, Snell & Ducay (1952*a*) suggested a higher molecular weight, though hardly as high as 71 000, which would be required by a molecule of four sub-units.

The molecular weight of avidin has now been determined by equilibrium sedimentation as well as from the sedimentation and diffusion coefficients and the results favour a molecule of four sub-units.

Results. In addition to the results summarized in Table 1 the following observations are significant: (1) the avidin sedimented as a single symmetrical boundary; (2) there was no systematic dependence of the calculated diffusion coefficient on the fringe position or on the time; (3) the graphs of $\ln c$ against x^2 from the equilibrium sedimentation were linear throughout the measurable concentration range.

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By these criteria the avidin was homogeneous with respect to size and shape. The calculated molecular weights indicate a slight dependence of S on concentration, but neither S nor D was appreciably affected when the avidin combined with biotin.

The sedimentation coefficients agree with those of A. Rothen (quoted by Woolley & Longworth, 1962) and of W. H. Ward (quoted by Fraenkel-Conrat *et al.* 1952*a*, and personal communication) obtained on less pure avidin preparations. The mean molecular weight from the equilibrium-sedimentation data was 68 300 and the observed variations were within the limits of error of the method (Yphantis, 1964). The chief source of uncertainty in this value is the calculated value of $\bar{v}$. If the avidin contains no inactive impurities a molecule of four sub-units would require a molecular weight of 71 000. If such impurities were present the true value would be lower.

If the molecular weight is taken to be 70 000 it is necessary to make some corrections to the previously published data.

The molar amino acid composition (Melamed & Green, 1963) should now be calculated relative to four rather than three moles of biotin. The data do not fit a molecule containing four identical peptide chains any better than they fit a three-chain model. However, a molecule containing two pairs of similar

Table 1. *Molecular weight and the coefficients of sedimentation and diffusion of avidin*

The avidin preparation D. 31 of Melamed & Green (1963) was used. The solvent was 0.1 M-NaCl-0.01 M-sodium acetate buffer, pH 5.5, unless otherwise indicated. All measurements were performed in a Spinco model E analytical ultracentrifuge at 15°. The diffusion coefficients were determined from the spreading of a synthetic boundary sedimenting at low speed (4010 rev./min.). They were calculated from the Rayleigh fringes by the method of Longworth (1952). The value given is the mean of six measurements at times between 14 and 150 min. The molecular weight was determined directly by the high-speed equilibrium-sedimentation method of Yphantis (1964) in the six-compartment cell described by him. The results were computed from the Rayleigh fringes after equilibration at 20400 rev./min. The partial specific volume, $\bar{v}$, was calculated from the amino acid composition by using the data given by Schachman (1957) and the reciprocal of the dry density for the contribution of the carbohydrate components.

	Concn. of protein (mg./ml.)	$S_{20,w}$ (s)	$10^7 \times D_{20,w}$ (cm. ² sec. ⁻¹)	f/f_0	Mol.wt.
Avidin	8.3	4.55	5.98 ± 0.04	1.33	66000
	0.5	Equilibrium sedimentation			67700
	0.25				70000
	0.12				67000
Avidin-biotin complex	7.4	4.58 (in 0.1 N-HCl)	6.08 ± 0.02		

but not identical chains would be consistent with the results.

The end points of the spectrophotometric titrations (Green, 1963*a, b*) should be changed from three to four moles of ligand bound/mole of avidin.

The results of the fluorescence-polarization experiments require re-evaluation since ρ_0 , the theoretical relaxation time of an anhydrous spherical molecule of molecular weight 70000, is 55 μ sec. The values of ρ_h/ρ_0 are consequently decreased to 1.0 for avidin and 0.9 for the avidin-biotin complex. These low values suggest some freedom of internal rotation within the avidin molecule. However, recent measurements of polarization of fluorescence as a function of sucrose concentration at constant temperature, by methods described by Green (1963*b*), have shown that the molecule is probably rigid. The ρ_h of avidin was 100 μ sec. and that of the avidin-biotin complex was 105 μ sec. The lower value of ρ_h obtained in the earlier experiments, where the temperature was varied, was probably due to temperature-dependent rotation of the dimethylaminonaphthalene-sulphonyl chromophore relative to the protein. This effect has been observed in dimethylaminonaphthalenesulphonyl-labelled aminopolystyrene (Gottlieb & Wahl, 1963).

In the treatment of the results of the quenching of fluorescence (Green, 1964) the theoretical curves were calculated for a molecule of three sub-units. The quenching efficiency for a single dinitrophenyl residue/four sub-units is much lower ($q_1/q_0 = 0.25$ rather than < 0.1). The calculated curve, e^{-Mn} , in

Fig. 2 of Green (1964) was based on the assumption that only unsubstituted avidin would contribute to the fluorescence and it now lies below the experimental points, since the monosubstituted DNP-avidin contributes appreciably. The new calculated curve for quenching of fluorescence by the dinitrophenylhydrazide of biotin lies rather closer to the experimental points than did the curve calculated for the molecule of three sub-units but it still deviates from them in the same direction. The main conclusions are therefore unchanged.

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