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The Subcellular Distribution of Rat Liver L-Alanine–Glyoxylate Aminotransferase in Relation to a Pathway for Glucose Formation Involving Glyoxylate

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1. The distribution of L-alanine–glyoxylate aminotransferase activity between subcellular fractions prepared from rat liver homogenates was investigated. The greater part of the homogenate activity (about 80%) was recovered in the 'total-particles' fraction sedimented by high-speed centrifugation and the remainder in the cytosol fraction. 2. Subfractionation of the particles by differential sedimentation and on sucrose density gradients revealed a specific association between the aminotransferase and the mitochondrial enzymes glutamate dehydrogenase and rhodanese. 3. The aminotransferase activities in the cytosol and the mitochondria are due to isoenzymes. The solubilized mitochondrial enzyme has a pH optimum of 8.6, an apparent K_m of 0.24mM with respect to glyoxylate and is inhibited by glyoxylate at concentrations above 5mM. The cytosol aminotransferase shows no distinct pH optimum (over the range 7.0–9.0) and has an apparent K_m of 1.11mM with respect to glyoxylate; there is no evidence of inhibition by glyoxylate. 4. The mitochondrial location of the bulk of the rat liver L-alanine–glyoxylate aminotransferase activity is discussed in relation to a pathway for gluconeogenesis involving glyoxylate.

With L-alanine as donor transamination of glyoxylate is promoted by liver homogenates of many animal species (Rowsell *et al.*, 1966, 1969*b*) including man (Thompson & Richardson, 1967); activities with L-glutamate are considerably lower (Rowsell *et al.*, 1966; Taktak, 1967; Thompson & Richardson, 1967) and other amino acids are still less effective, at least with rat or rabbit liver (E. V. Rowsell & J. A. Carnie, unpublished work). Kidney is the only other tissue in which we have observed L-alanine–glyoxylate aminotransferase activity and, on a whole-organ basis, the contribution is much less than that of liver (Rowsell *et al.*, 1969*a*). These observations *in vitro* suggest that quantitatively the most important transamination reaction involving glyoxylate in animals is with L-alanine in the liver.

The reverse reaction with glycine and pyruvate is barely detectable in crude liver homogenates (Rowsell *et al.*, 1969*b*), and not at all with L-alanine–glyoxylate aminotransferase purified from human liver (Thompson & Richardson, 1967). It seems likely therefore that the enzyme does operate to convert glyoxylate into glycine, a process that undoubtedly can occur *in vivo* in the rat (Weinhouse & Friedman, 1951; Weissbach & Sprinson, 1953) and in man (Gerritsen *et al.*, 1969).

L-Alanine–glyoxylate aminotransferase activity is elevated in rat liver during the neonatal period or after glucagon injection in the adult, and liver activity is relatively high in carnivorous animals (Rowsell *et al.*, 1969*b*). It was suggested the enzyme might have a glucogenic role *in vivo* by initiating a process of glucose formation from glyoxylate via glycine, and two possible pathways were indicated (Scheme 1). Circumstantial evidence favouring one of these (*b*) was noted, namely an elevation also of L-serine–pyruvate aminotransferase activity under conditions associated with enhanced gluconeogenesis. Direct evidence that L-serine is largely converted into glucose without the intermediate formation of pyruvate has been reported (Lardy *et al.*, 1969). Our principal concern in the present paper is with the subcellular location in rat liver of the enzymes implicated in route (*b*).

The conversion of glycine into L-serine, CO_2 and NH_3 is catalysed principally in the mitochondria (Kawasaki *et al.*, 1966; Sato *et al.*, 1969). Observations in this laboratory (A. H. Al-Tai & K. Snell, unpublished work) show a wholly particulate location for L-serine–pyruvate aminotransferase, which promotes the conversion of serine into hydroxypyruvate in route (*b*), whereas serine dehydratase, which would direct metabolism along route (*a*), is entirely in the cytosol (Wahbi, 1966). No systematic investigation of the subcellular site of D-glycerate dehydrogenase is on record. However, Vandor & Tolbert (1970) describe a NADH-specific enzyme (not lactate dehydrogenase) catalysing the reduction of hydroxypyruvate to glycerate located in mitochondrial and peroxisomal subfractions of rat

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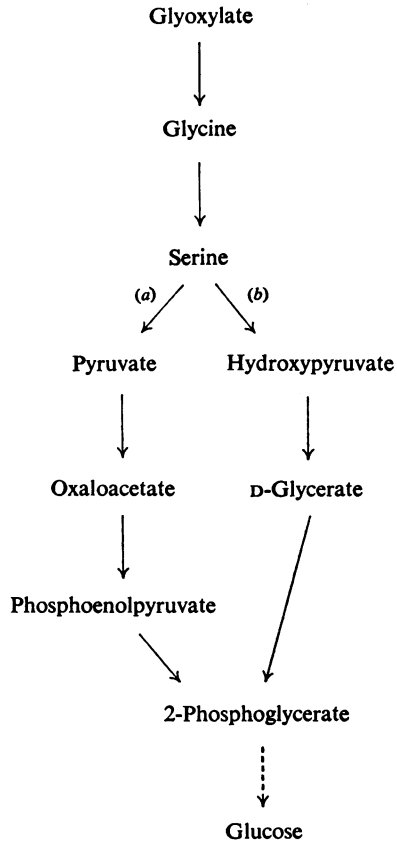


Scheme 1. *Possible metabolic pathways for the conversion of glyoxylate into glucose*

liver. D-Glycerate kinase, forming 2-phosphoglycerate, has been reported to be located in the mitochondria (Lamprecht *et al.*, 1959). All the remaining enzymes necessary for a pathway to glucose 6-phosphate are largely confined to the cytosol (Bucher & McGarrahan, 1956; Kennedy & Lehninger, 1949), whereas glucose 6-phosphatase, catalysing glucose liberation, is a microsomal enzyme (de Duve *et al.*, 1955).

Thus it appears that the enzymes governing the initial steps from glycine in route (*b*) may have a common, mitochondrial, site of action, and it became of interest to investigate the subcellular distribution of L-alanine–glyoxylate aminotransferase, the first enzyme of the suggested pathway leading from glyoxylate to glucose, for which no systematic studies have been reported.

A preliminary account of the present work has been published (Snell *et al.*, 1970).

Experimental

Methods

Preparation of homogenates. Fed male rats (200–300g) of an inbred black-hooded strain were stunned with a blow to the head and bled to death by severing the blood vessels of the neck. Liver was quickly removed into a tared beaker in ice and, after weighing, was minced well with scissors and transferred, by using homogenizing medium, to the glass tube of a Potter-type homogenizer. The tissue was homogenized with the aid of a Teflon pestle rotating at 1400rev./min, in 10s bursts with rest periods of 30s for 4–5min, keeping the homogenizer tube in ice. Homogenates were filtered through two thicknesses of surgical gauze and adjusted to a concentration of 10% (w/v). The medium used for homogenization was unbuffered 0.4M-sucrose except for the preparation of nuclear fractions [experiment (2) below], when 0.4M-sucrose containing 0.2mM-$CaCl_2$ was used.

Subcellular fractional. All centrifugation procedures were at 2–4°C in a Spinco model L ultracentrifuge and all fractions were stored on ice until required for enzyme analysis. The SW 25 rotor was employed in the preparation of nuclear fractions and for density-gradient centrifugation; for other procedures the 40 angle rotor was used. Sedimented fractions were not washed before assay unless otherwise stated.

Differential sedimentation fractionation. In separate experiments the following fractionations were made. (1) The homogenate was centrifuged at 100000*g* for 75min to give a clear supernatant cytosol fraction and a sedimented total-particles fraction. (2) A nuclear fraction was sedimented at 550*g* for 2min leaving a supernatant containing the remainder of the subcellular particulate material together with the cytosol. The sedimented nuclear fraction was washed twice by resuspension in fresh medium and recentrifugation under the same conditions as above. (3) The homogenate was centrifuged at 6500*g* for 10min to sediment a crude mitochondrial fraction (M_1) leaving a supernatant containing other particulate material together with the cytosol. (4) The homogenate was centrifuged at 26700*g* for 10min to sediment a particulate fraction and the supernatant was recentrifuged at 105000*g* for 100min to sediment a microsomal fraction, now leaving the cytosol as supernatant. (5) The homogenate was centrifuged at 550*g* for 2min and the sedimented nuclear fraction was discarded. The supernatant was recentrifuged at 6500*g* for 10min to sediment a crude mitochondrial fraction (M_2). The supernatant from this fractionation was then centrifuged at 105000*g* for 100min to sediment a microsomal fraction, leaving the cytosol as the final supernatant.

Sedimented fractions were resuspended in the

original homogenizing medium with the aid of a Teflon homogenizer operated by hand.

Density-gradient fractionation. Separation of mitochondria, lysosomes and peroxisomes was attempted by using discontinuous sucrose density gradients. A 10% (w/v) homogenate was centrifuged at 550*g* for 2min and the sedimented nuclear fraction was discarded. The supernatant was recentrifuged at 26700*g* for 10min to sediment a particulate fraction for application to a density gradient. A portion of this particulate fraction (resuspended as indicated below) was retained for enzyme analysis.

Method (*a*): preparation of a lysosomal fraction. Lysosomal fractions were prepared from rats injected intraperitoneally with Triton WR-1339 (a gift from Hilton Davis Chemicals Ltd., Newcastle upon Tyne, U.K.) at a dose of 85mg/100g body weight in 0.9% (w/v) NaCl 4 days before being killed. The Triton is preferentially taken up by the liver lysosomes *in vivo*; in consequence there is a specific decrease in their equilibrium density in sucrose density gradients (from about 1.22 to about 1.10g/ml), allowing the lysosomes to be separated from the mitochondria and peroxisomes, which equilibrate at higher densities (>1.19g/ml) (Wattiaux *et al.*, 1963).

A particulate fraction (equivalent to 9g of original tissue) was prepared as above and resuspended in 45ml of 54% (w/v) sucrose with the aid of a Teflon homogenizer operated by hand. The resuspended fraction was incorporated into a discontinuous density gradient based on that described by Leighton *et al.* (1968) as follows: 14ml of the resuspended particulate fraction in 54% (w/v) sucrose (density 1.20g/ml) was layered at the bottom of a centrifuge tube followed by a layer of 11ml of 40% (w/v) sucrose (1.15g/ml) and finally a layer of 5ml of 15% (w/v) sucrose (1.06g/ml). The gradient was then centrifuged at 47900*g* for 2h. It was expected that the buoyant Triton-filled lysosomes would collect at the interface of the top two sucrose layers, and a fraction was collected with the aid of a hypodermic syringe equipped with a gauge 1 needle inserted laterally just beneath this point.

Method (*b*): preparation of a peroxisomal fraction. A particulate fraction (equivalent to 9g of original tissue) was prepared as above from a normal rat and resuspended in 11ml of 48% (w/v) sucrose. A 3ml sample was layered on to a discontinuous density gradient built as follows from the bottom of the tube: 2.5ml of 68% (w/v) sucrose (density 1.255g/ml), 2.5ml of 60% (w/v) sucrose (1.225g/ml), 2.5ml of 55% (w/v) sucrose (1.204g/ml), 7.5ml of 53% (w/v) sucrose (1.200g/ml), 5.0ml of 51% (w/v) sucrose (1.191g/ml) and 5.0ml of 48% (w/v) sucrose (1.178g/ml). The gradient was centrifuged at 47900*g* for 1h. It was anticipated that the peroxisomes would collect at the interface with the bottom sucrose layer of density 1.255g/ml, since their reported equilibrium density in sucrose gradients is 1.24g/ml (Beaufay *et al.*, 1964), and a fraction was isolated from the gradient at this point by slicing the tube. [A Beckman tube slicer was used as described by Turner (1964).] Another fraction (designated X) was sliced out of the upper part of the gradient at the interface of the 48% and 51% sucrose layers, where a large proportion of the liver preparation appeared to have accumulated.

Method (*c*): A resuspended particulate fraction was layered on to a discontinuous density gradient [as in method (*b*) above]. After centrifugation for 1h at 47900*g* the whole gradient was divided into four fractions by slicing the centrifuge tube at three arbitrary points.

Characterization of subcellular fractions. All subcellular fractions prepared in the present work were analysed with respect to the activities of 'marker enzymes', taking the recovery of a particular activity in any fraction as a quantitative index of the recovery of the relevant subcellular component. The marker enzymes employed were: glutamate dehydrogenase and rhodanese (mitochondrial), acid phosphatase (lysosomal), urate oxidase (peroxisomal), glucose 6-phosphatase and arylsulphatase C (microsomal), lactate dehydrogenase (cytosol); DNA was used as a marker for nuclei. In all experiments the unfractionated homogenate was assayed for the activities of the various enzymes and the volume of every separated fraction was measured so that each enzyme activity assayed could be expressed, for the complete fraction, as a percentage of the equivalent whole homogenate activity. Table 1 shows the whole homogenate activities for all the enzymes assayed in the present work.

Enzyme assays. Glutamate dehydrogenase (EC 1.4.1.2) was assayed in the direction of glutamate synthesis essentially by the method of Beaufay *et al.* (1959) with minor modifications in the incubation mixture. This contained 2μmol of 2-oxoglutarate, 0.4μmol of NADH, 150μmol of NH_4Cl, 135μmol of potassium phosphate buffer, pH7.5, and sonicated enzyme preparation in a total volume of 3.0ml. Fractions were sonicated before assay by using a MSE–Mullard 60W ultrasonic drill at maximum output for 6min at 2°C. Sucrose at high concentrations reversibly inhibits glutamate dehydrogenase activity as measured in the direction of 2-oxoglutarate amination (Yielding, 1970) (e.g. 0.5M-sucrose inhibits by 28%) and fractions were diluted before assay so that at the final sucrose concentration in the assay system (never more than 6mM) there was negligible enzyme inhibition. Lactate dehydrogenase (EC 1.1.1.27) was assayed in the direction of pyruvate reduction essentially as described by Schwartz & Bodansky (1966), again with fractions sonicated as detailed above. Acid phosphatase (EC 3.1.3.2) was assayed as described by Gianetto & de Duve (1955); the enzyme was activated either by sonication (as

Table 1. *Whole homogenate activities of enzymes in rat liver*

Activities are expressed as units/g of liver ± S.E.M. (no. of observations). DNA concentrations were 2010 ± 26 (3) μg of DNA/g of liver.

Enzyme	Units	Activity
L-Alanine–glyoxylate aminotransferase	μmol of pyruvate formed/min	3.61 ± 0.20 (17)
Glutamate dehydrogenase	μmol of NADH utilized/min	39.5 ± 7.3 (14)
Rhodanese	μmol of thiocyanate formed/min	327 ± 66 (5)
Acid phosphatase	μmol of P_i formed/min	4.01 ± 0.45 (12)
Urate oxidase	μmol of urate utilized/min	1.62 ± 0.27 (12)
Glucose 6-phosphatase	μmol of P_i formed/min	8.33 ± 0.94 (6)
Arylsulphatase C	μmol of nitrophenol formed/min	0.21 ± 0.01 (6)
Lactate dehydrogenase	μmol of NADH utilized/min	320 ± 24 (11)

above) or by the inclusion of Triton X-100 (final concn. 0.1%) in the incubation system. Sucrose is known to inhibit acid phosphatase activity (Hinton *et al.*, 1969) and fractions were diluted so that the final sucrose concentration in the assay system (<0.1M) gave negligible inhibition of enzyme activity. Arylsulphatase C (EC 3.1.6.1) was assayed by the method of Dodgson *et al.* (1953) by using *p*-nitrophenyl sulphate as substrate and measuring the liberated *p*-nitrophenol within 15min of the end of the 60min incubation to minimize any conversion into *p*-aminophenol (see Milsom *et al.*, 1968). Rhodanese (EC 2.8.1.1) was assayed by the method of Sorbo (1955) after activation of the enzyme in the fractions by Triton X-100 (final concn. 0.03%). Urate oxidase (EC 1.7.3.3) was assayed as described by Leighton *et al.* (1968). Glucose 6-phosphatase (EC 3.1.3.9) was assayed by the method of Swanson (1950) but with tris–maleate, pH6.5, as buffer (Gomori, 1955). P_i liberated in the phosphatase assays was measured either as described by Fiske & SubbaRow (1925) or, when Triton derivatives were present in the fractions, by the method of Eibl & Lands (1969).

Glutamate dehydrogenase, lactate dehydrogenase and urate oxidase were assayed at 30°C, rhodanese at 25°C and all other enzymes at 37°C. Activities were calculated from linear progress curves and it was established in all cases that initial rates of reaction were proportional to enzyme concentration over the range employed.

DNA was extracted and determined by the method of Webb & Levy (1955) by using calf thymus DNA as standard.

L-Alanine–glyoxylate aminotransferase assay. The aminotransferase was assayed by the colorimetric method of Rowsell *et al.* (1969*a*), or by the more sensitive method in which pyruvate formed is determined in the presence of tris by using lactate dehydrogenase and excess of NADH as described by Rowsell *et al.* (1969*b*). Preliminary experiments established that neither sucrose nor sonication had any effect on enzyme activity.

Preparation of mitochondrial and cytosol L-alanine–glyoxylate aminotransferases. Rat liver homogenates (10%, w/v) were centrifuged at 100000*g* for 30min; the sedimented-particles fraction was used as a source of the mitochondrial enzyme and the supernatant as a source of the cytosol enzyme. The mitochondrial enzyme was solubilized by 2min Ultra-Turrax (Janke und Kunkel A.-G., Staufen i. Br., Germany) treatment, at 0°C in 15s periods with 15s intervals; the mitochondrial fragments were removed by centrifugation at 105000*g* for 80min and the supernatant was found to contain 88–95% of the aminotransferase activity present originally in the particulate fraction. In experiments where the mitochondrial enzyme was solubilized in 0.1M-phosphate buffer, pH7.4, the cytosol, in 0.4M-sucrose, was dialysed overnight against 0.1M-phosphate buffer, pH7.4; otherwise both enzymes were in 0.4M-sucrose before dilution in the assay mixture.

Michaelis constant calculation. Assuming Michaelis–Menten kinetics for the range of substrate concentrations tested, results from each experiment were fitted to the double-reciprocal plot by the iterative least-squares method with the aid of a computer program (prepared by the Department of Biochemistry, University of Edinburgh, for British Olivetti Ltd., London, U.K.). From an input of initial rates and substrate concentrations the values provided by computer analysis included the apparent Michaelis constant, the slope of the straight line and the intercept on the ordinate axis.

Materials

NADH, sodium pyruvate and lactate dehydrogenase (from rabbit muscle) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

Table 2. *Enzyme activities of subcellular fractions isolated by differential sedimentation*

Fractions were prepared by differential sedimentation from rat liver homogenates as described in the Experimental section [experiments (1)–(5)]. Enzyme activities are given as percentages of the original whole-homogenate activities. N.D., not determined.

Expt.	L-Alanine–glyoxylate aminotransferase	Glutamate dehydrogenase	Lactate dehydrogenase	Acid phosphatase	Urate oxidase	Glucose 6-phosphatase	Aryl-sulphatase C	DNA
(1)								
Total-particles fraction	80	98	13	90	96	82	99	N.D.
Cytosol fraction	20	0	92	2	3	0	3	N.D.
Recovery	100	98	105	92	99	82	102	
(2)								
Nuclear fraction	10	8	4	10	5	4	3	85
Supernatant fraction	92	90	101	83	90	94	90	10
Recovery	102	98	105	93	95	98	93	95
(3)								
Crude mitochondrial fraction (M_1)	75	90	10	50	40	N.D.	30	N.D.
Supernatant fraction	25	7	90	50	60	N.D.	65	N.D.
Recovery	100	97	100	100	100		95	
(4)								
Particulate fraction	78	105	9	N.D.	N.D.	55	48	N.D.
Microsomal fraction	7	0	11	N.D.	N.D.	48	56	N.D.
Cytosol fraction	22	0	76	N.D.	N.D.	0	7	N.D.
Recovery	107	105	96			103	111	
(5)								
Crude mitochondrial fraction (M_2)	65	93	8	56	44	40	33	N.D.
Microsomal fraction	8	2	10	30	50	44	52	N.D.
Cytosol fraction	20	0	80	3	0	6	5	N.D.
Recovery	93	95	98	89	94	90	90	

Triton X-100 and *p*-nitrophenyl sulphate were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and 2-oxoglutaric acid from Mann Research Labs. Inc., New York, N.Y., U.S.A. Other chemicals were of analytical standard, obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Subcellular fractions prepared by differential sedimentation

The activities of L-alanine–glyoxylate aminotransferase and various marker enzymes were measured in subcellular fractions prepared by differential sedimentation from rat liver homogenates and representative results are given in Table 2. High-speed supernatants (cytosol fraction) contained low activities of the particle-marker enzymes but consistently about 20% of the total homogenate aminotransferase activity; the remainder (about 80%) of the aminotransferase activity was recovered along with the particle-marker enzymes in the 'total particles' fraction. Nuclear fractions contained intact nuclei as judged by phase-contrast microscopy, and 83–85% of the total homogenate DNA; some contamination with the particle-marker enzymes was apparent, and a small proportion of the aminotransferase activity (about 10%) was also recovered in these fractions. Crude mitochondrial preparations with good recoveries of glutamate dehydrogenase (≥90%) and lesser amounts of the other particle-marker enzymes contained the bulk of the particulate aminotransferase activity (about 70% of the total homogenate activity). In microsomal fractions approx. 50% recoveries were observed for glucose 6-phosphatase and arylsulphatase C activities; they were heavily contaminated with peroxisomal and lysosomal marker enzymes but contained negligible glutamate dehydrogenase activity and only low amounts of aminotransferase (7–8%).

The distribution of the bulk of the particulate aminotransferase activity in these fractions followed closely that shown by glutamate dehydrogenase activity; no significant correlation with the distribution of any other particle-marker was apparent.

Subcellular fractions prepared by density-gradient fractionation

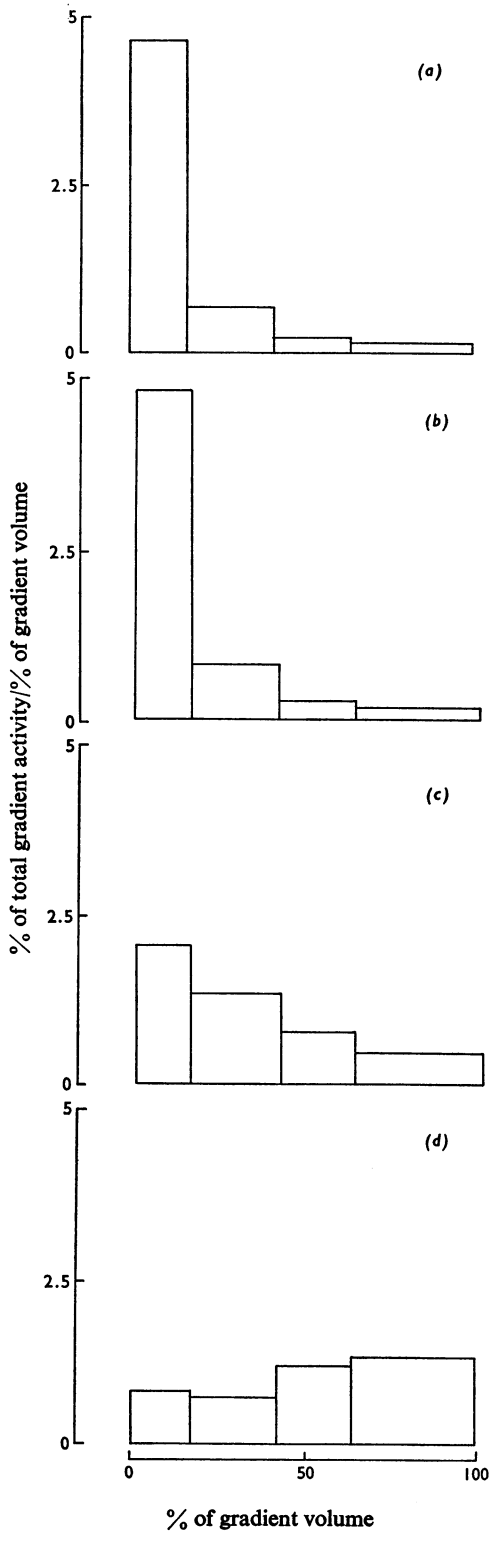
A particulate fraction prepared by differential centrifugation was further resolved by using discontinuous sucrose density gradients.

In this way lysosome fractions were prepared from rats pretreated with Triton WR-1339 and typical enzyme recoveries are shown in Table 3. These fractions contained about 30% of the total homogenate acid phosphatase activity, with low recoveries

Table 3. *Enzyme activities of subcellular fractions isolated by density-gradient centrifugation*

The procedures for the separation of lysosomes or peroxisomes from other subcellular components are described in methods (*a*) and (*b*) respectively. Fraction X, method (*b*), was isolated at a point in the gradient (density 1.178–1.191 g/ml) of lower density than the peroxisomal fraction (1.225–1.255 g/ml). Enzyme activities are given as percentages of the original whole homogenate activities. N.D., not determined.

Method	Fraction	L-Alanine–glyoxylate aminotransferase	Glutamate dehydrogenase	Lactate dehydrogenase	Rhodanese	Acid phosphatase	Urate oxidase
(*a*)	Particulate fraction before gradient fractionation	66	74	N.D.	N.D.	64	76
	Lysosomal fraction	3	2	N.D.	N.D.	33	3
(*b*)	Particulate fraction before gradient fractionation	70	N.D.	6	68	70	82
	Gradient fraction X	25	N.D.	N.D.	18	24	13
	Peroxisomal fraction	6	N.D.	N.D.	6	2	21



(<3%) of the other particle-marker enzymes [in agreement with the data of Leighton *et al.* (1968)] and of the L-alanine–glyoxylate aminotransferase activity.

Peroxisomal fractions were prepared from normal rats and, as shown in Table 3, urate oxidase was recovered to a greater extent than the other particle-marker enzymes, with a recovery of the aminotransferase activity (4–6%) similar to that of the latter enzymes. Fractions (designated X) isolated at a point further up the gradient (at a sucrose density of about 1.185g/ml) contained greater recoveries of the aminotransferase activity and of the mitochondrial and lysosomal marker enzymes but lesser recoveries of urate oxidase, as compared with the peroxisomal fractions. The results indicate that very little, if any, aminotransferase activity is associated with lysosomes or peroxisomes.

Fig. 1 shows the distribution of enzyme activities throughout a discontinuous sucrose density gradient after the application of a particle preparation from normal rat liver and centrifugation. The results are plotted as described by de Duve (1967); the area of each column in the histogram represents the total enzyme activity in each fraction and the height represents the relative concentration of enzyme activity. A close correspondence between the distribution profile for the aminotransferase and that for rhodanese (the mitochondrial marker) is apparent, and these distributions clearly differ from those observed for urate oxidase or acid phosphatase.

Altogether the results obtained by using differential sedimentation and density-gradient fractionation indicate that the greater part, if not all, of the particulate L-alanine–glyoxylate aminotransferase activity is associated with the mitochondria. The results also showed consistently that a small proportion (about 20%) of the total homogenate aminotransferase activity is recovered in the cytosol fraction. It was recognized that the appearance of activity in the cytosol fraction might be the result of mitochondrial leakage. However, this explanation is

Fig. 1. *Distribution of enzyme activities after centrifuging a particulate fraction on a discontinuous sucrose density gradient*

See method (*c*) for fractionation procedure. The columns in each histogram from left to right correspond to fractions from top to bottom of the centrifuge tube; the abscissa shows the percentage of the total volume (28ml) occupied by each fraction and ordinates give a measure of relative concentration in the enzyme activity indicated. Recoveries of enzyme activities were 90–98% of those applied to the gradient. (*a*) L-Alanine–glyoxylate aminotransferase; (*b*) rhodanese; (*c*) acid phosphatase; (*d*) urate oxidase.

not supported by the results on glutamate dehydrogenase distribution (see Table 2), and it was necessary to consider the alternative possibility of a genuine dual subcellular localization of the aminotransferase, with activity in each compartment due, perhaps, to a separate isoenzyme. Some properties of the cytosol and mitochondrial enzymes were examined to investigate this point.

Properties of mitochondrial and cytosol L-*alanine–glyoxylate aminotransferase*

While the present work was in progress it was observed that whole-homogenate L-alanine–glyoxylate aminotransferase activity was inhibited by substrate glyoxylate. Optimum activity was obtained by using an assay concentration of 5 mM-glyoxylate; at 20 mM-glyoxylate, the concentration used as a routine in the aminotransferase assay (see Rowsell *et al.*, 1969*a*), 17% inhibition of activity was observed. The effect of varying the glyoxylate concentration between 5 and 20 mM on the activity of the solubilized mitochondrial enzyme (in 0.1 M-phosphate buffer, pH 7.4) and on the cytosol enzyme is shown in Fig. 2. The mitochondrial enzyme activity was inhibited with increasing glyoxylate concentration, whereas the cytosol enzyme showed a slight increase in activity. Because of the inhibitory effect of 20 mM-glyoxylate subsequent L-alanine–glyoxylate aminotransferase assays in this work were conducted by using 5 mM-glyoxylate substrate concentration.

In each case the apparent Michaelis constant with respect to glyoxylate was determined by using glyoxylate concentrations of 5 mM and less at a fixed alanine concentration of 20 mM. The double-reciprocal plots obtained (Fig. 3) indicate K_m 1.11 mM for the cytosol enzyme and 0.24 mM for the solubilized mitochondrial enzyme.

The effect of pH on solubilized mitochondrial enzyme and cytosol enzyme activities is shown in Fig. 4. The mitochondrial enzyme has a sharp peak of activity at about pH 8.6 and the cytosol enzyme has a constant activity over the pH range examined.

Discussion

Present results show that about 80% of the L-alanine–glyoxylate aminotransferase activity in cell-free homogenates of rat liver is recovered with the combined sedimentable particles, with the remainder in the cytosol fraction. From further fractionation studies it became clear that the activity in the particles fraction is largely, perhaps entirely, associated with the mitochondria.

The activities in the mitochondrial and cytosol fractions are due, it seems, to isoenzymes, differing with respect to pH-dependence, apparent Michaelis constant towards glyoxylate and in the inhibitory

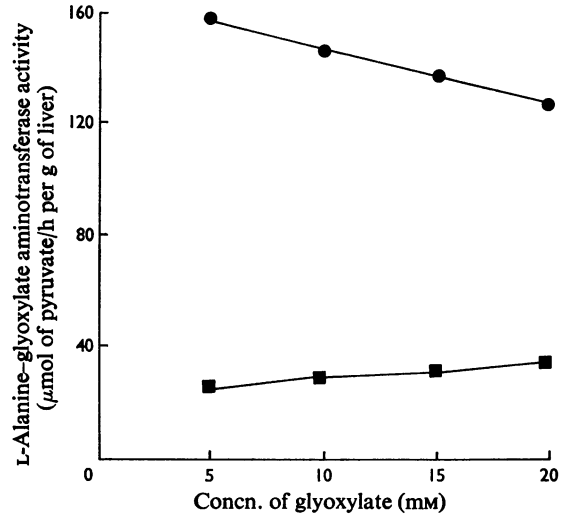


Fig. 2. *Effect of glyoxylate concentration above 5 mM on the mitochondrial and cytosol* L-*alanine–glyoxylate aminotransferase activities*

The mitochondrial (●) and cytosol (■) preparations were obtained in 0.1 M-potassium phosphate buffer, pH 7.4, as described in the text. L-Alanine concentration was fixed at 20 mM.

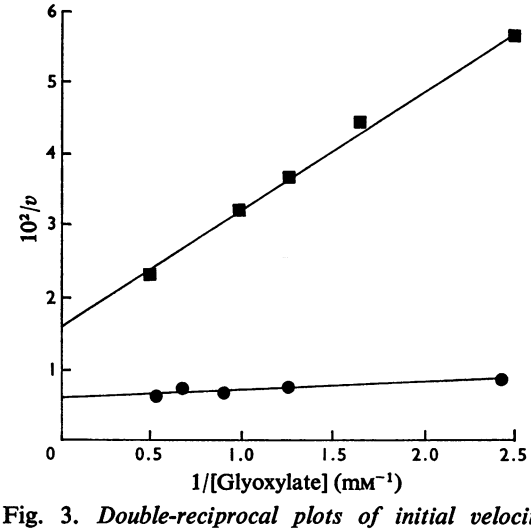


Fig. 3. *Double-reciprocal plots of initial velocity against glyoxylate concentration for the mitochondrial and cytosol* L-*alanine–glyoxylate aminotransferase activities*

The mitochondrial (●) and cytosol (■) preparations were obtained in 0.4 M-sucrose, as described in the text. L-Alanine concentration was fixed at 20 mM. Initial velocity (v) is given as μmol of pyruvate/h per g of liver. Straight lines were drawn through the experimental points and K_m values determined according to computer analysis.

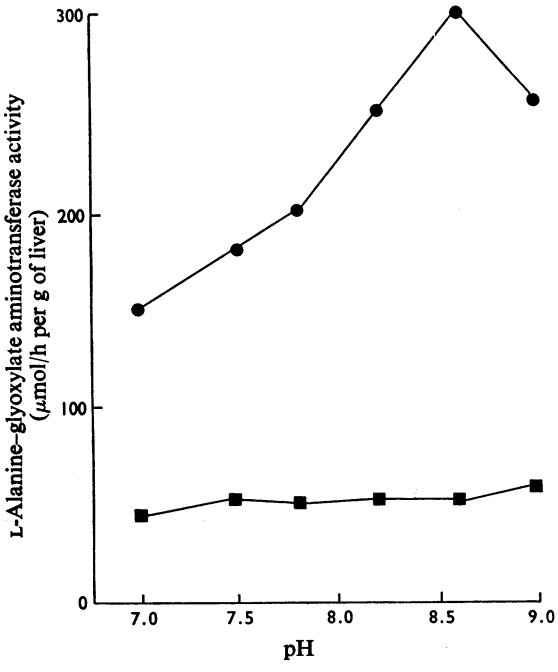


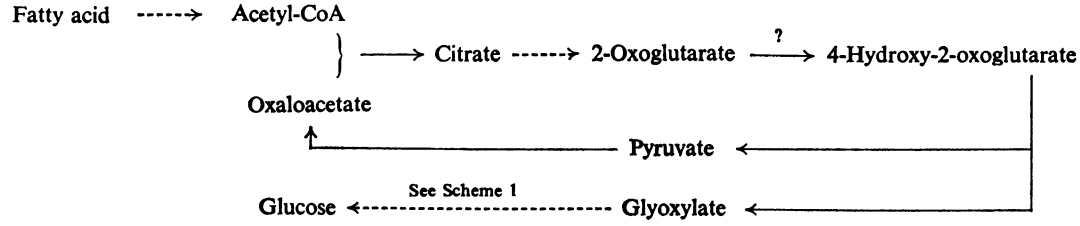
Fig. 4. *Effect of pH on the mitochondrial and cytosol* L-*alanine–glyoxylate aminotransferase activities*

The mitochondrial (●) and cytosol (■) preparations were obtained in 0.4M-sucrose, as described in the text. Incubations were with 20mM-L-alanine, 5mM-glyoxylate and 50mM-buffer to give the required pH value: KH_2PO_4–NaOH buffer, pH7.0–7.8; boric acid–KCl–NaOH buffer, pH8.2–9.0.

response to high glyoxylate concentrations. Similar findings, with regard to subcellular distribution, have been made with a number of other rat liver aminotransferases: reactions with pyruvate and L-phenylalanine, L-leucine or L-methionine, and with 2-oxoglutarate and L-tyrosine are promoted by both the mitochondrial and cytosol fractions (Rowsell *et al.*, 1963) and in some cases, where this distribution is observed, activities for the two fractions can be attributed to distinct isoenzymes, namely with L-aspartate–2-oxoglutarate aminotransferase (Boyd, 1961), L-alanine–2-oxoglutarate aminotransferase (Swick *et al.*, 1965) and L-histidine–pyruvate aminotransferase (Spolter & Baldridge, 1964). The distribution of L-aspartate–2-oxoglutarate aminotransferase in the mitochondria and cytosol has a function, it is suggested, in the transport of oxaloacetate across the mitochondrial membrane (Lardy *et al.*, 1965), but the significance of this dual location for other aminotransferases (including the L-alanine–glyoxylate enzyme) remains obscure.

It has been reported that rat liver peroxisomal fractions contain L-alanine–glyoxylate aminotransferase and L-glutamate–glyoxylate aminotransferase activities (Vandor & Tolbert, 1970). No indication is given of the recoveries of the peroxisomal and mitochondrial fractions selected for assay, but we calculate that the L-glutamate–glyoxylate activity for their peroxisomal fraction represents about 30% of total homogenate activity (as measured, with similar assay incubation conditions, by Rowsell *et al.*, 1966, and Taktak, 1967). Moreover a high specific activity (per mg of protein) was measured for the peroxisomal fraction, as compared with the mitochondrial fraction. It appears, therefore, that a substantial proportion, at least, of the L-glutamate–glyoxylate aminotransferase enzyme is located in the peroxisomes. However, we calculate a recovery of L-alanine–glyoxylate aminotransferase activity for their peroxisomal fraction of <2% total homogenate (as measured under similar conditions in the present work), and this is of doubtful significance: peroxisomal fractions prepared in the present work contained L-alanine–glyoxylate aminotransferase (6% of the total homogenate activity, see Table 3), but the mitochondrial marker, rhodanese, was recovered to the same extent. We note, further, that in Vandor & Tolbert's (1970) experiments L-glutamate was a much more effective donor than DL-alanine with glyoxylate (sixfold with their peroxisomal fraction, fourfold with their mitochondrial fraction), whereas with whole rat liver homogenate L-alanine is a better donor (2–3-fold) than L-glutamate (Rowsell *et al.*, 1966; Taktak, 1967) and we surmise that the fractions richest in L-alanine–glyoxylate aminotransferase were not selected for assay in their investigation. The presence of a small percentage of the total L-alanine–glyoxylate aminotransferase in the peroxisomes cannot be ruled out, but from the present work the bulk of the particulate activity appears to be mitochondrial.

Given a supply of substrates the mitochondria are equipped, apparently, to synthesize glycine under the influence of L-alanine–glyoxylate aminotransferase. Also, the observation that the neonatal maximum in rat liver L-alanine–glyoxylate aminotransferase activity can be attributed entirely to an increase in the mitochondrial fraction (Snell & Walker, 1971) may be taken as evidence that it is the enzyme in this particular location that is functionally linked, in some way, with gluconeogenesis. Considered in conjunction with observations that indicate a mitochondrial site also for the enzymes governing the initial steps of gluconeogenesis from glycine (see the introduction), these findings give further justification for the suggested metabolic route proceeding from glyoxylate, via glycine, serine and hydroxypyruvate to glucose [Scheme 1, (*b*)]. The operation of the whole sequence of reactions would be facilitated with the relevant enzymes sited in the same organelle, each reaction product generated within a small volume of



Scheme 2. *Possible route for the conversion of fatty acids into glucose*

the cell becoming available at a relatively high concentration as substrate for the subsequent reaction.

The major problem posed by the whole hypothesis concerns the function of the postulated sequence of reactions [Scheme 1 (*b*)]: for a role in gluconeogenic metabolism some source of glyoxylate is required and the only well-authenticated original sources in animals are allantoic acid, the product of purine catabolism in amphibia, fishes and some marine invertebrates (see, e.g., Laskowski, 1951), and, more generally, 4-hydroxy-2-oxoglutarate, the product of L-hydroxyproline catabolism (see, e.g., Meister, 1965). In the context of the present work it is notable that the rat liver aldolase which promotes the scission of 4-hydroxy-2-oxoglutarate to form pyruvate and glyoxylate is also a mitochondrial enzyme (Maitra & Dekker, 1964). Both products are potentially gluconeogenic, pyruvate via oxaloacetate and phosphoenolpyruvate, and glyoxylate, we would suggest, via Scheme 1 (*b*), and both processes would be facilitated by the liberation of their substrates within the mitochondria. Even so, as Krebs (1964) suggests, the quantities of glyoxylate arising from L-hydroxyproline breakdown must be small, and we are giving active consideration to other conceivable pathways of glyoxylate formation, e.g. from 2-oxoglutarate via metabolic hydroxylation to 4-hydroxy-2-oxoglutarate, having in mind, if that should be shown to occur, that it would point to the possibility of a route for the conversion of fatty acids into glucose (see Scheme 2). We are prompted to look in this direction by experimental findings that suggest that in the suckling mammal (rat and man), at least, fatty acids may serve as substrates for gluconeogenesis (Hahn & Koldovsky, 1966). It is also noted that it is during the suckling period in the rat that maximum liver activity is observed for 4-hydroxy-2-oxoglutarate aldolase (J. A. Carnie, E. V. Rowsell & M. Dabbaghian, unpublished work), L-alanine–glyoxylate aminotransferase, L-serine–pyruvate aminotransferase (Rowsell *et al.*, 1969*b*), D-glycerate dehydrogenase (Johnson *et al.*, 1964), pyruvate carboxylase (Yeung *et al.*, 1967), another mitochondrial enzyme, and fatty acid oxidation with tissue slices or mitochondrial preparations (Drahota *et al.*, 1966).

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