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Some Properties of Hepatic Glycerol Kinase and their Relation to the Control of Glycerol Utilization

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1. Glycerol kinase (EC 2.7.1.30) is shown to catalyse a non-equilibrium reaction in rat liver; and, as it is the first enzyme in the pathway metabolizing glycerol, its properties may be pertinent to the metabolic regulation of glycerol uptake and utilization by this tissue. 2. The properties of hepatic glycerol kinase were studied by using a radiochemical technique to measure the enzyme activity. When the concentration of ATP is low the activity of glycerol kinase is inhibited by high concentrations of glycerol; but when the concentration of ATP is high there is no inhibition and the double-reciprocal plot is linear, providing a K_m for glycerol of $3{\cdot}16 \times 10^{-6}$M. Glycerol kinase is activated by high ATP concentrations provided that the concentration of the second substrate (glycerol) is high; at low concentrations of glycerol ATP does not activate the enzyme so that the double-reciprocal plot is linear, providing a K_m for ATP of $5{\cdot}8 \times 10^{-5}$M. It is suggested that these kinetics may be explained by a model similar to that described by Ferdinand (1966) for phosphofructokinase. 3. Hepatic glycerol kinase is inhibited by ADP and AMP, and raising the Mg^{2+} concentration increases the inhibition by these two compounds; this suggests that ADP–Mg^{2+} and AMP–Mg^{2+} complexes are the inhibitory species. The physiological significance of these inhibitions may be to prevent phosphorylation of glycerol when the hepatic ATP concentration is low. It is suggested that this inhibition may provide an approach to the problem of measurement of rates of lipolysis by glycerol release in tissues that contain glycerol kinase (e.g. liver, kidney, muscle, adipose tissue). 4. Hepatic glycerol kinase is inhibited by L-3-glycerophosphate competitively with respect to glycerol. The physiological significance of this inhibition may be that factors that change the intracellular concentration of L-3-glycerophosphate could change glycerol uptake by the tissue. Thus it is suggested that thyroxine treatment or feeding rats on a diet high in glycerol, which increase the activity of glycerophosphate oxidase in liver and kidney cortex respectively, lead to an increased glycerol uptake through a decrease in the concentration of glycerophosphate in these tissues. It is known that ethanol administration decreases glycerol uptake by liver, and this can be explained by the increased concentration of L-3-glycerophosphate causing inhibition of glycerol kinase.

Glycerol is metabolized in mammals primarily by the liver and kidney (Borchgrevink & Havel, 1963). The results of the preceding paper (Robinson & Newsholme, 1969) suggest that under conditions of starvation or a low-carbohydrate diet glycerol uptake is regulated by the plasma concentration of glycerol. It is possible, however, that under other conditions uptake could be regulated by the liver or kidney tissues themselves; thus it has been observed that feeding with a diet containing 25% of glycerol increases the uptake of glycerol by kidney-cortex slices in comparison with the normal fed control (Robinson & Newsholme, 1969). The two possible sites for control of glycerol uptake are the transport into the tissue and the further metabolism within the tissue. The available evidence strongly suggests that glycerol transport is not a limiting process in glycerol utilization (see Cahill, Ashmore, Renold & Hastings, 1959; Larsen, 1963; Shafrir & Gorin, 1963). Therefore the metabolism of glycerol within the tissues must regulate glycerol uptake.

A rational approach to the study of regulation of

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metabolic pathways has been described by Newsholme & Gevers (1967), and this approach was followed in the present investigation. Glycerol kinase (EC 2.7.1.30) has been shown to catalyse a non-equilibrium reaction in liver (see the Results section); and, as it is the first enzyme in the metabolism of glycerol in this tissue (see Bublitz & Kennedy, 1954*b*; Hoberman & D'Adamo, 1960), its catalytic activity must regulate glycerol utilization. Therefore factors that could modify the catalytic activity of this enzyme could be important in the regulation of glycerol utilization. The properties of the enzyme were therefore investigated in an attempt to identify such factors, and to provide a basis for formulating a theory of control of glycerol kinase activity. The results of the investigation are reported in this paper. A preliminary report of some of this work has been published (Robinson & Newsholme, 1967*b*).

While this paper was in preparation the work of Grunnet & Lundquist (1967) appeared; these workers carried out a similar study with partially purified glycerol kinases from rat liver and *Candida mycoderma*. They used coupled enzymic assays depending on the formation of either ADP or L-3-glycerophosphate to measure the activity of glycerol kinase; the validity of this method as a satisfactory assay depends on purification of the glycerol kinase so that there are no enzymes present that could compete effectively with the commercial pyruvate kinase for ADP. The results of Grunnet & Lundquist (1967) are generally similar to those reported in the present paper, but they do not report the kinetics of glycerol kinase at low concentrations of both substrates. One important difference, however, is that these workers do not find L-3-glycerophosphate inhibition of rat liver glycerol kinase under somewhat similar conditions to those used in the present investigation (see the Results section).

MATERIALS AND METHODS

Chemicals, enzymes and animals. Glucose 6-phosphate, tris, NADH, NAD^+, $NADP^+$, ATP, ADP, AMP, creatine phosphate, fructose 6-phosphate (barium salt), ribose 5-phosphate (barium salt), phosphoenolpyruvate (tricyclohexylammonium salt), fructose 1,6-diphosphate (tetracyclohexylammonium salt) and all the enzymes used in this work were obtained from the Boehringer Corp. (London) Ltd., London W.5. The barium salts were converted into sodium salts before use. L-3-Glycerophosphate was obtained from Calbiochem Ltd., London W.1. [1-^{14}C]Glycerol (specific radioactivity 23·6 μc/μmole) was obtained from The Radiochemical Centre, Amersham, Bucks. L-3-[^{14}C]-Glycerophosphate was prepared as described by Newsholme, Robinson & Taylor (1967). 2-Mercaptoethanol was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and Sephadex G-25 was purchased from Pharmacia Ltd., London W. 13.

Female Wistar rats weighing 200 g. were used throughout.

Glycerol kinase preparations. Two preparations of liver glycerol kinase were used for the kinetic studies.

(*a*) 80000*g* liver supernatant. The liver of a freshly killed rat was flushed through with homogenizing medium [1% (w/v) KCl–1mM-EDTA] by means of a 25ml. syringe inserted into the hepatic portal vein; this removed most of the blood from the liver. The liver was passed through a Fisher mincer and homogenized in 4 vol. of the homogenizing medium in a stainless-steel overhead-drive homogenizer. The homogenate was centrifuged at 80000*g* for 1hr. at 0° (Beckman Spinco model L centrifuge, no. 40 rotor) and the supernatant decanted. Assays were performed on the fresh supernatant or after storage at −70°. Small volumes of supernatant could be stored at −70° for up to 3 weeks with loss of less than 20% of the original activity. This preparation had low adenosine triphosphatase activity (13% of glycerol kinase activity) but high adenylate kinase activity (500% of glycerol kinase activity). The preparation did not metabolize L-3-glycerophosphate as measured both radiochemically and enzymically (see below). Before assay the supernatant was passed through a column of Sephadex G-25 equilibrated with the incubation medium (minus substrates). Preliminary studies had established that at least 90% of the glycerol kinase of liver was present in the 80000*g* supernatant; the latter is therefore a satisfactory preparation with which to study the properties of hepatic glycerol kinase.

(*b*) Partially purified glycerol kinase. Glycerol kinase was partially purified by following the method of Bublitz & Kennedy (1954*a*), except that the dialyses were replaced by gel filtration through Sephadex G-25 and the heat treatment was amended to 60min. at 60°. This procedure decreased adenylate kinase activity to 10% of the glycerol kinase activity. Before assay the protein was dissolved in incubation medium (minus substrates) and passed through a column of Sephadex G-25 in the same medium.

For assay of maximal tissue activities of glycerol kinase crude homogenates were used. Both liver and kidney cortex were homogenized in 4vol. of 1% KCl–1mM-EDTA and diluted a further 1:5 in incubation medium (minus substrates) before assay.

Assay of glycerol kinase. Glycerol kinase activity was assayed by the radiochemical method of Newsholme *et al.* (1967), which is based on the conversion of [^{14}C]glycerol into L-3-[^{14}C]glycerophosphate and adsorption of the latter on DEAE-cellulose-paper disks. The same procedure as described by Newsholme *et al.* (1967) was adopted, except that for kinetic experiments the incubation medium contained only 100mM-tris and various concentrations of ATP, $MgSO_4$ and glycerol at pH7·5. The final specific radioactivity of glycerol was 23·6 or 8 μc/μmole. Maximal activity measurements (with crude homogenates of liver and kidney) were made in media containing 100mM-tris, 1mM-EDTA, 25mM-NaF, 20mM-mercaptoethanol, 10mM-creatine phosphate, 0·16mg. of creatine phosphokinase/ml., 6mM-ATP, 4mM-$MgSO_4$ and 3mM-[^{14}C]glycerol (final specific radioactivity 2·0 μc/μmole), at pH7·5.

Samples of enzyme solution (20 μl.) were incubated with 100 μl. of incubation medium at 25° for various times and the reaction was inhibited with 100 μl. of ethanol. Samples (20 μl.) were pipetted on to DEAE-cellulose-paper disks, which were washed on top of two unused DEAE-cellulose-paper disks with 200ml. of water. The disks were dried and

counted in a Beckman model LS200 liquid-scintillation spectrometer. A time-course, usually of 1, 2 and 3min., at 25° was performed for each assay. This was linear and was therefore considered to represent the initial rate of the enzyme activity. Controls were made either with boiled enzyme or in the absence of ATP: there was no detectable activity under either of these conditions.

Assay of contaminating enzyme activities. Enzymic sampling assays were used to measure the activities of non-specific adenosine triphosphatases, adenylate kinase and enzymes metabolizing L-3-glycerophosphate. A sample (0·2ml.) of the enzyme preparation was incubated with 1·0ml. of the appropriate incubation medium (as for glycerol kinase assay but with the changes described below) and the reaction stopped with 0·3ml. of 20% (w/v) $HClO_4$. The precipitated protein was removed by centrifugation and the supernatant was neutralized with $KHCO_3$.

Non-specific breakdown of ATP (adenosine triphosphatase activity) was measured after incubation as above in medium minus glycerol by assaying ADP (Adam, 1963) in the neutralized supernatant. Interconversion of adenine nucleotides (adenylate kinase activity) was measured after incubation in medium containing 1mM-AMP instead of glycerol by assaying ADP in the neutralized supernatant. Metabolism of L-3-glycerophosphate was measured by determining the concentration of this compound (see Hohorst, 1963) after it had been incubated in medium minus glycerol. Metabolism of L-3-glycerophosphate was also estimated by a radiochemical assay in which L-3-[^{14}C]-glycerophosphate replaced [^{14}C]glycerol in the medium: the reaction medium was sampled on to DEAE-cellulose-paper disks and washed and counted as in the radiochemical assay for glycerol kinase. Any metabolism of L-3-glycerophosphate to a product not retained on the DEAE-cellulose was indicated by loss of radioactivity.

Expression of results. Activities are expressed either as total radioactivity (c.p.m.) incorporated into L-3-glycerophosphate during the incubation (e.g. 3min.), or as μmoles of L-3-glycerophosphate formed during the incubation by the quantity of extract added to the incubation tube. The latter value depends on a knowledge of the amount of glycerol in the incubation tube, the total radioactivity (c.p.m.) in the incubation tube (i.e. the specific radioactivity of the glycerol) and the radioactivity (c.p.m.) incorporated into L-3-glycerophosphate (see, e.g. Newsholme, Rolleston & Taylor, 1968).

RESULTS

Mass-action ratio for glycerol kinase. The contents of ATP, ADP, L-3-glycerophosphate and glycerol were measured in 'freeze-clamped' rat livers, and glycerol was measured in serum from blood obtained from the hepatic portal vein; experiments were performed under ether anaesthesia (for description of methods see Start & Newsholme, 1968). The intracellular concentrations of ATP, ADP and L-3-glycerophosphate (Table 1) were calculated from the content/g. fresh wt. of liver on the assumption that they are found only in the intracellular water (72% of the total water; Hohorst, Kreutz & Bücher, 1959). The intracellular concentration of glycerol (Table 1) was calculated assuming that the concentration in the extracellular space was the same as in the serum and then apportioning the total amount of glycerol in the 'freeze-clamped' liver between the intra- and extracellular spaces. The mass-action ratio for the glycerol kinase reaction (calculated from the data in Table 1) is 1·12, whereas the apparent equilibrium constant for this reaction is 1102 (Table 1). As glycerol kinase is the first enzyme involved in the metabolism of glycerol in liver, the fact that it catalyses a non-equilibrium reaction strongly suggests that its catalytic activity regulates glycerol uptake and utilization; and therefore its properties may be of some importance in the metabolic regulation of glycerol utilization.

Effect of Mg^{2+} concentration. It was found that maximal activity of glycerol kinase was not obtained at a constant Mg^{2+}/ATP concentration ratio; thus 2mM-Mg^{2+} produced maximal activities at both 0·02mM- and 1·0mM-ATP (i.e. the range of ATP concentrations used in this study) (see Table 2). Therefore 2mM-Mg^{2+} was used in the routine assay of glycerol kinase.

Glycerol kinase activity as a function of glycerol concentration. The effect of glycerol concentration on glycerol kinase activity was found to depend on

Table 1. *Mass-action ratio and equilibrium constant of the glycerol kinase reaction*

The concentrations of intermediates were determined from freeze-clamped livers, and are expressed as means ±S.D. with the numbers of animals in parentheses. Serum glycerol was assayed in blood taken from the hepatic portal vein with the rat under anaesthesia. K' was calculated from values for $\Delta G'$ (Burton, 1961) by using the formula: $\Delta G' = -RT \ln K'$.

Intermediate	Concn. in intracellular fluid (mM)	Mass-action ratio (τ) $\left(\frac{[\text{L-3-glycerophosphate}][\text{ADP}]}{[\text{glycerol}][\text{ATP}]}\right)$	Apparent equilibrium constant (K')	$\frac{\tau}{K'}$
Glycerol	0·23±0·09 (4)			
ATP	4·66±0·55 (4)			
L-3-Glycerophosphate	0·92±0·38 (4)	1·12	1102	$1·015 \times 10^{-3}$
ADP	1·30±0·22 (4)			
Glycerol in serum	0·132 (2)			

Table 2. *Effect of the concentration of* Mg^{2+} *on glycerol kinase activity at* 0·02mM- *and* 1mM-*ATP concentrations*

Partially purified glycerol kinase was assayed radiochemically. The incubation medium contained 1mM-glycerol.

Concn. of Mg^{2+} (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of extract)	
	0·02mM-ATP	1mM-ATP
0	3140	3500
0·01	5720	5280
0·02	6020	—
0·04	7520	—
0·08	8550	—
0·1	8420	9500
0·2	9200	10200
1·0	9250	13450
2·0	9570	13450
4·0	7920	12900
20·0	—	12850

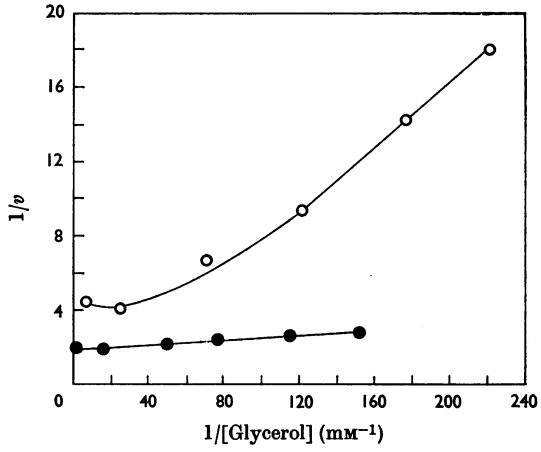

Fig. 1. Double-reciprocal plots of glycerol kinase activity against glycerol concentration at two concentrations of ATP. Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 2mM-Mg^{2+}. The activity, v, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of extract. ○, 0·02mM-ATP; ●, 1mM-ATP.

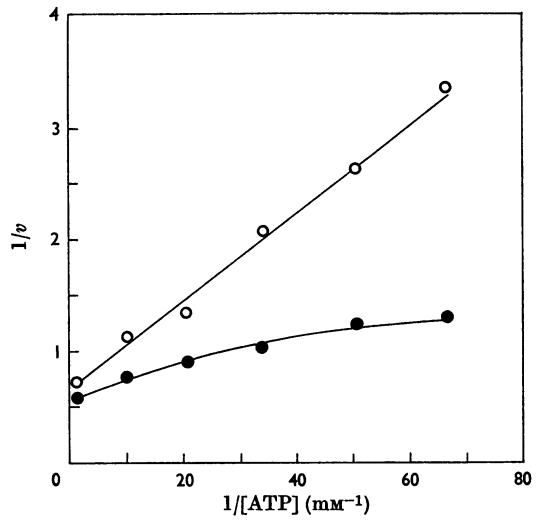

Fig. 2. Double-reciprocal plots of glycerol kinase activity against ATP concentration at two concentrations of glycerol. Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 2mM-Mg^{2+}. The activity, v, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of extract. ○, 0·01mM-Glycerol; ●, 0·33mM-glycerol.

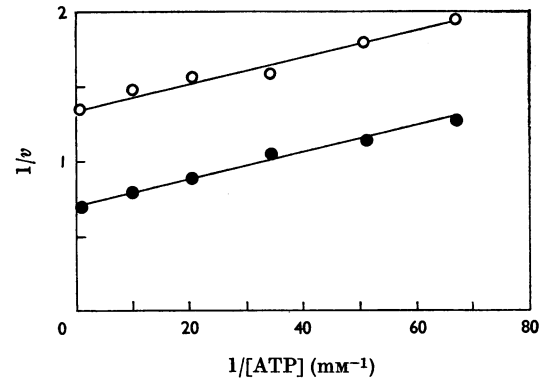

Fig. 3. Double-reciprocal plots of glycerol kinase activity against ATP concentration at two concentrations of Mg^{2+}. Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 0·33mM-glycerol. The activity, v, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of extract. ○, 0·02mM-Mg^{2+}; ●, 0·2mM-Mg^{2+}.

the concentration of the second substrate (ATP) (see Fig. 1). At 1mM-ATP a double-reciprocal plot of activity against concentration of glycerol was linear, giving a K_m for glycerol of $3{\cdot}16 \times 10^{-6}$M. However, at 0·02mM-ATP the plot was concave upwards at high glycerol concentrations (see Fig. 1).

Glycerol kinase activity as a function of ATP concentration. This relationship was also dependent on the fixed concentration of the second substrate (in this case glycerol). At 0·33mM-glycerol the reciprocal plot of activity against ATP concentration was non-linear, being concave downwards at high ATP concentrations; whereas at 0·01mM-glycerol the

plot was linear giving a K_m for ATP of $5{\cdot}8 \times 10^{-5}$M (Fig. 2).

The effect of ATP concentration on activity was also dependent on the concentration of Mg^{2+}: at 0·02mM- and 0·2mM-Mg^{2+}, and 0·33mM-glycerol, reciprocal plots of activity against ATP concentration were linear (Fig. 3).

Inhibition of glycerol kinase by ADP and AMP. As reported by Bublitz & Kennedy (1954*a*) glycerol kinase is inhibited by ADP (see Table 3). The inhibition is dependent on the concentration of Mg^{2+} (Table 4); with increasing Mg^{2+} concentration the inhibition by ADP was increased. Ca^{2+} also potentiated the inhibition by ADP (Table 5) and was more effective than an equimolar concentration of Mg^{2+} (compare Tables 4 and 5). The effect of ATP on the inhibition by ADP is shown as a double-reciprocal plot in Fig. 4. The kinetics of the inhibition are obviously complex, but the inhibition appears to be predominantly non-competitive at low ATP concentrations and of a more competitive nature at high ATP concentrations. ADP inhibits uncompetitively with glycerol (Fig. 5).

The characteristics of the inhibition of glycerol kinase by AMP (Table 6) are very similar to those of the inhibition by ADP. Increasing the Mg^{2+} concentration increases the inhibition (Table 7). Mn^{2+} was more effective, but Ca^{2+} less effective, than Mg^{2+} in potentiating the AMP inhibition (Table 8). The effects of varying the concentrations of ATP and glycerol on the inhibition by AMP are qualitatively similar to the effects on the inhibition by ADP.

The similarity of the inhibition of glycerol kinase by ADP and AMP (the only difference being the effectiveness of Ca^{2+} in potentiating the inhibitions) raised the question whether a common effector could be responsible in both cases. Interconversion of ADP and AMP during the assay procedure (through adenylate kinase) was excluded by assaying ADP and AMP in the incubation medium at the end of the incubation. The conversion of ADP into AMP, and vice versa, was not detectable. The effects of common contaminants of commercial

Table 3. *Effect of ADP on glycerol kinase activity*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP, 2mM-Mg^{2+} and 0·17mM-glycerol.

Concn. of ADP (mM)	Glycerol kinase activity (c.p.m incorporated into product/3min./20μl. of extract)	Inhibition by ADP (%)
0	10700	—
0·10	7500	30
0·25	5800	46
0·5	4400	59
1·0	3400	69
2·5	2800	74
5·0	2300	79

Table 4. *Effect of* Mg^{2+} *concentration on the inhibition of glycerol kinase activity by ADP*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP and 0·17mM-glycerol.

Concn. of Mg^{2+} (mM)	Concn. of ADP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of extract)	Inhibition by ADP (%)
0·2	—	7700	
0·2	1·0	5750	25
2·0	—	9750	
2·0	1·0	3360	66
20·0	—	9360	
20·0	1·0	1650	82

Table 5. *Effect of* Ca^{2+} *on the inhibition of glycerol kinase activity by ADP*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP and 0·17mM-glycerol.

Concn. of Mg^{2+} (mM)	Concn. of Ca^{2+} (mM)	Concn. of ADP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of extract)	Inhibition by ADP (%)
—	2·0	—	4240	
—	2·0	1·0	360	91·5
0·2	2·0	—	10800	
0·2	2·0	1·0	1390	86

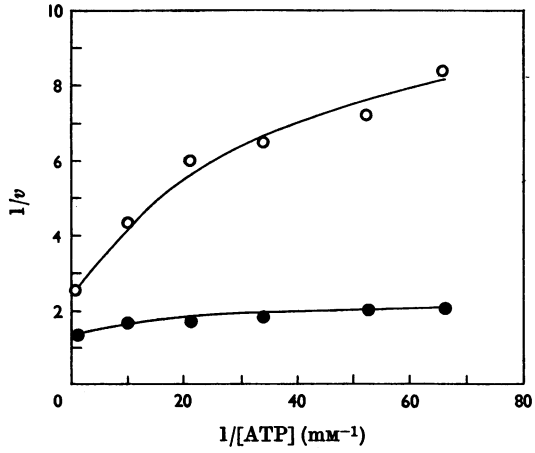

Fig. 4. Double-reciprocal plots of glycerol kinase activity against ATP concentration in the presence and absence of ADP. Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 2mM-Mg^{2+} and 0·17mM-glycerol. The activity, v, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of extract. ●, In the absence of ADP; ○, in the presence of 0·25mM-ADP.

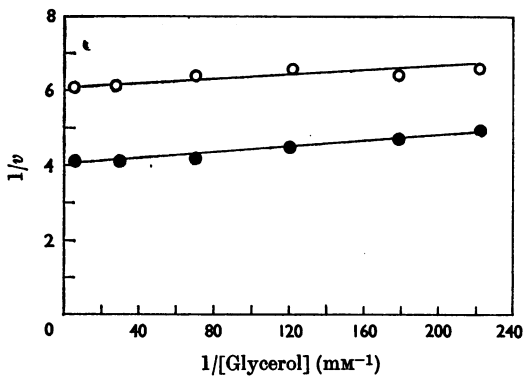

Fig. 5. Double-reciprocal plots of glycerol kinase activity against glycerol concentration in the presence and absence of ADP. Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP and 2mM-Mg^{2+}. The activity, v, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of extract. ●, In the absence of ADP; ○, in the presence of 0·1mM-ADP.

preparations of ADP and AMP are shown in Table 9. They all produced much lower inhibitions than did comparable concentrations of ADP and AMP, and therefore could not be responsible for the observed inhibitions.

Inhibition of glycerol kinase by L-3-*glycerophosphate.* Hepatic glycerol kinase was inhibited by L-3-glycerophosphate and the inhibition was competitive with glycerol (Fig. 6); the K_i for L-3-glycerophosphate is $5{\cdot}8 \times 10^{-4}$M. The effect of L-3-glycerophosphate on a double-reciprocal plot of activity against ATP concentration is shown in Fig. 7; the kinetics appear to be complex. Similar inhibitions have also been observed with the partially purified enzyme. Grunnet & Lundquist (1967) reported that the glycerol kinase from rat liver was not inhibited by L-3-glycerophosphate. The reason for the inability of Grunnet & Lundquist (1967) to observe L-3-glycerophosphate inhibition of rat liver glycerol kinase is not known, but it is pointed out that the conditions of assay are different, e.g. the coupled assay requires the presence of high concentrations of phosphoenolpyruvate, NADH and probably ammonium sulphate; it seems possible that one of these compounds may modify the glycerol kinase so that it is no longer sensitive to glycerophosphate.

Table 6. *Effect of AMP on glycerol kinase activity*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP, 2mM-Mg^{2+} and 0·17mM-glycerol.

Concn. of AMP (mM)	Glycerol kinase activity (c.p.m incorporated into product/3min./20μl. of extract)	Inhibition by AMP (%)
0	4660	
0·1	3800	19
0·2	3250	30
0·5	2150	54
1·0	1590	66
2·5	890	81
5·0	600	87

Effect of other metabolic intermediates on glycerol kinase activity. The following intermediates had no effect on glycerol kinase activity: glucose, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, triose phosphates (generated from fructose diphosphate by the action of aldolase) and citrate.

Effect of various diets on glycerol kinase and glycerophosphate oxidase activities of liver and kidney cortex. Robinson & Newsholme (1969) showed that starvation (48hr.) or a casein–margarine diet did not affect the rate of glycerol uptake by liver and kidney-cortex slices *in vitro*. As expected these diets did not affect glycerol kinase activities (Table 10). The diet containing 25% of glycerol did increase glycerol utilization by kidney-cortex slices, but no effect could be demonstrated for liver slices. There was, however, no effect of this 25%-glycerol diet on the activity of glycerol kinase in

Table 7. *Effect of* Mg^{2+} *concentration on the inhibition of glycerol kinase activity by AMP*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP and 0·17mM-glycerol.

Concn. of Mg^{2+} (mM)	Concn. of AMP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of extract)	Inhibition by AMP (%)
2·0	—	4660	
0·5	0·2	4300	8
1·0	0·2	3880	17
2·0	0·2	3220	31
4·0	0·2	2260	52
7·5	0·2	1650	65
10·0	0·2	1380	71

Table 8. *Effect of* Mn^{2+} *and* Ca^{2+} *on the inhibition of glycerol kinase activity by AMP*

Partially purified glycerol kinase was assayed radiochemically; the incubation medium contained 1mM-ATP and 1mM-glycerol in Expt. 1 and 1mM-ATP and 0·17mM-glycerol in Expt. 2.

Expt. no.	Concn. of Mn^{2+} (mM)	Concn. of Mg^{2+} (mM)	Concn. of Ca^{2+} (mM)	Concn. of AMP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of extract)	Inhibition by AMP (%)
1	2·0	—	—	—	21600	
	2·0	—	—	0·2	7200	67
	—	2·0	—	—	24300	
	—	2·0	—	0·2	17600	27
2	—	—	2·0	—	4240	
	—	—	2·0	0·5	3760	11
	—	0·2	2·0	—	10800	
	—	0·2	2·0	0·5	7550	30

Table 9. *Effect of contaminants and breakdown products of AMP and ADP on glycerol kinase activity*

The 80000*g* supernatant preparation was assayed radiochemically; the incubation medium contained 0·04mM-ATP, 2mM-Mg^{2+} and 1 mM-glycerol.

Addition	Concn. (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./20μl. of supernatant)
—	—	7100
Adenosine	6·0	6670
Adenine	6·0	5620
Ribose 5-phosphate	6·0	5030
ADP	6·0	850
AMP	6·0	830

either the liver or kidney cortex (Table 10). It seemed possible that the increased rate of glycerol uptake could be explained by a change in the activity of L-3-glycerophosphate oxidase, which could modify glycerol kinase activity through an effect on L-3-glycerophosphate. The results in Table 10 show that in both tissues the 25%-glycerol

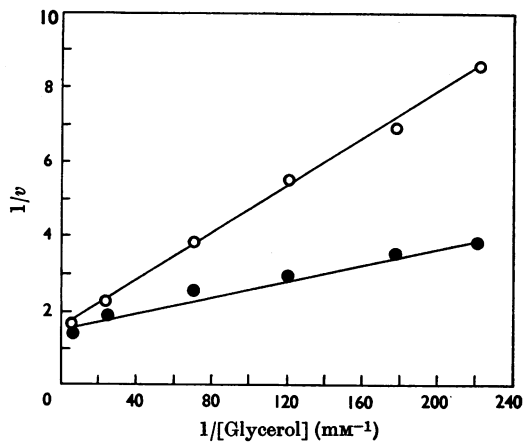

Fig. 6. Double-reciprocal plots of glycerol kinase activity against glycerol concentration in the presence and absence of L-3-glycerophosphate. The 80000*g* liver supernatant was assayed radiochemically; the incubation medium contained 1mM-ATP and 2mM-Mg^{2+}. The activity, *v*, is expressed as nmoles of L-3-glycerophosphate formed/3min./20μl. of supernatant. ●, In the absence of L-3-glycerophosphate; ○, in the presence of 1mM-L-3-glycerophosphate.

diet increased the activity of glycerophosphate oxidase, but the increase was much greater in kidney cortex.

DISCUSSION

The double-reciprocal plots of hepatic glycerol kinase activity against the concentration of the two substrates are linear only under certain conditions.

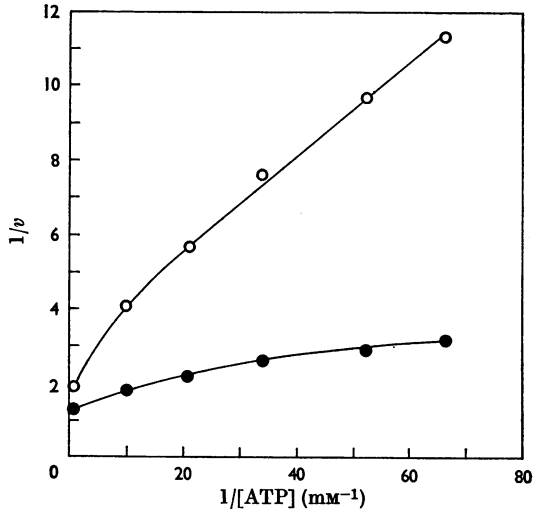

Fig. 7. Double-reciprocal plots of glycerol kinase activity against ATP concentration in the presence and absence of L-3-glycerophosphate. The 80000*g* liver supernatant was assayed radiochemically; the incubation medium contained 2mM-Mg^{2+} and 0·17mM-glycerol. The activity, *v*, is expressed in nmoles of L-3-glycerophosphate formed/3min./20μl. of supernatant. ●, In the absence of L-3-glycerophosphate; ○, in the presence of 10mM-L-3-glycerophosphate.

The double-reciprocal plot of activity against glycerol concentration is linear at an ATP concentration of 1mM, which provides a K_m for glycerol of $3{\cdot}16 \times 10^{-6}$M. However, at 0·02mM-ATP the reciprocal plot is curved upwards; this could be explained by glycerol inhibition at higher concentrations and glycerol activation at lower concentrations. Grunnet & Lundquist (1967) did not carry out kinetics at such a low concentration of ATP and consequently report only linear kinetics with respect to variation in the glycerol concentration. The double-reciprocal plot of activity against ATP concentration is curved downwards at high ATP concentrations, provided that the glycerol concentration is high (0·33mM). When the glycerol concentration is low (0·01mM) this plot is linear, giving a K_m for ATP of $5{\cdot}8 \times 10^{-5}$M. This linear plot does not appear to have been reported by Grunnet & Lundquist (1967). To some extent the properties of this enzyme are similar to those of phosphofructokinase: thus one substrate (glycerol) is an inhibitor and the other substrate (ATP) is an activator; but the effects of the two substrates are less exaggerated than with phosphofructokinase as they can only be observed with double-reciprocal plots.

The explanation for the non-linear behaviour of the enzyme may be very similar to that discussed for phosphofructokinase by Ferdinand (1966). This would necessitate a number of assumptions: the mechanism of glycerol kinase catalysis involves the formation of a ternary complex; one pathway leading to the formation of the ternary complex (the one involving the enzyme–ATP complex as an intermediate) is kinetically preferred; and the formation of the ternary complex limits the rate of catalysis. Thus with a high initial concentration of glycerol most of the enzyme would exist in the

Table 10. *Effect of diet on the glycerol kinase and glycerophosphate oxidase activities of liver and kidney cortex*

Glycerol kinase was assayed (at 26°) radiochemically in crude homogenates of liver and kidney cortex. Glycerophosphate oxidase was assayed (at 28°) by coupling the enzyme activity to the reduction of a tetrazolium dye and was adapted from the assay for succinate dehydrogenase (Pennington, 1961) and the assay for succinic acid (Clark & Porteous, 1964). The results are expressed as means with the numbers of observations in parentheses.

		Glycerol kinase activity (μmoles of L-3-glycerophosphate formed/hr./g. wet wt.)			
Tissue	Diet of animals ...	Normal	25% glycerol for 13–18 days	Starved	Casein–margarine for 6 days
Kidney cortex		172 (2)	131 (2)	174 (2)	175 (3)
Liver		120 (3)	112 (2)	117 (2)	125 (3)
		Glycerophosphate oxidase activity (μmoles of L-3-glycerophosphate oxidized/hr./g. wet wt.)			
Kidney cortex		30·6 (2)	96 (2)	—	—
Liver		10·2 (2)	22·5 (2)	—	—

enzyme–glycerol form so that at low concentrations of ATP the slowest pathway leading to the ternary complex would be followed. As the concentration of ATP was increased the formation of the enzyme–ATP complex would be favoured so that the catalysis could take place via the kinetically preferred pathway; this would give rise to the phenomenon of ATP activation. This would explain why non-linear kinetics were not observed at a low concentration of glycerol, as sufficient free enzyme would exist for ATP to form the enzyme–ATP complex so that the preferred pathway would be always available. Similarly, substrate inhibition by high concentrations of glycerol, when the ATP concentration was low, could be explained by conversion of most of the enzyme into the enzyme–glycerol form; and it would explain the absence of complex kinetics when the ATP concentration was kept high, since again the kinetically preferred pathway would be available. This explanation is an alternative to the action of ATP as a positive modifier of the enzyme, as suggested by Grunnet & Lundquist (1967). However, there is as yet no means of clearly deciding between these two possibilities.

Effects of ADP and AMP. Hepatic glycerol kinase is inhibited by both ADP and AMP; assuming an Mg^{2+} concentration of 2–5mM, the concentrations of ADP and AMP normally found in aerobic 'freeze-clamped' liver (approx. 1·3mM and 0·2mM respectively) would be expected to cause partial inhibition of glycerol kinase (see Tables 3 and 7). The physiological significance of these inhibitions may be the regulation of glycerol kinase activity in concert with the regulation of gluconeogenesis by the adenine nucleotide concentrations in the liver (and kidney cortex) (for discussion see Newsholme & Gevers, 1967). The most important function of plasma glycerol is to provide precursor for glucose formation in these tissues, and if gluconeogenesis has been inhibited by a lowering of the 'energy status' of the cell (i.e. a decrease in ATP, and increases in ADP and particularly AMP) it would be sensible to inhibit the conversion of glycerol into L-3-glycerophosphate, particularly as an increased rate of glycolysis (due to the lowering of the energy status) may require the conversion of dihydroxyacetone phosphate into L-3-glycerophosphate to reoxidize glycolytically produced NADH for the maintenance of glycolysis.

The inhibition of glycerol kinase by AMP and ADP could provide a possible experimental approach to the problem of the measurement of rates of lipolysis in tissues that possess glycerol kinase activity (e.g. liver, kidney, heart muscle, some skeletal muscles, adipose tissue; see Robinson & Newsholme, 1967*a*). The main problem is that in such tissues glycerol release probably represents a balance between glycerol produced by lipolysis and glycerol removed by the action of glycerol kinase. If the lipase activity is unaffected by conditions that lower the tissue ATP concentration (e.g. anoxia, cell poisons, uncoupling agents), and Vaughan (1962) has some evidence that this might be the case in adipose tissue, then glycerol kinase could be inhibited and measurement of glycerol release by the tissue under these particular conditions should provide an indication of lipolysis.

Effect of L-3-*glycerophosphate.* The K_i of glycerol kinase for L-3-glycerophosphate is $5{\cdot}8 \times 10^{-4}$M whereas the concentration in liver from a normal fed animal is about 0·9mM; therefore glycerophosphate should cause partial inhibition of glycerol kinase. The physiological significance of this inhibition may be to relate glycerol uptake by liver (and kidney) to the intracellular concentration of L-3-glycerophosphate, so that factors that can modify this concentration can modify the uptake of glycerol through changes in glycerol kinase activity. Two important factors regulating the concentration of L-3-glycerophosphate are the extramitochondrial $NADH/NAD^+$ concentration ratio (through the reaction catalysed by glycerophosphate dehydrogenase) and the activity of glycerophosphate oxidase. There is some experimental evidence that both these factors can modify glycerol uptake by liver or kidney cortex.

Glycerol uptake by kidney-cortex slices was increased by feeding rats on a diet containing 25% of glycerol (Robinson & Newsholme, 1969); under these conditions there was no detectable increase in glycerol kinase activity in kidney extracts, but glycerophosphate oxidase activity was increased about threefold (Table 10). A smaller increase in glycerophosphate oxidase activity was observed with liver extracts, but no change in glycerol uptake was observed. Treatment of rats with thyroxine has been shown to increase the rate of gluconeogenesis from glycerol in the perfused liver (Freedland & Krebs, 1967); under these conditions there was no change in the glycerol kinase activity of liver extracts (J. Robinson & E. A. Newsholme, unpublished work). However, there is a well-documented increase in glycerophosphate oxidase activity of liver under these conditions (Lee, Takemori & Lardy, 1959), which could thus explain the effect of thyroxine on gluconeogenesis from glycerol.

The metabolism of glycerol by the whole animal is inhibited by ethanol administration (Lundquist, Tygstrup, Winkler & Jensen, 1965), and ethanol inhibits gluconeogenesis from glycerol by the perfused liver (Krebs, 1968). Under both these conditions the hepatic content of L-3-glycerophosphate is increased (Zakim, 1965; Krebs, 1968), probably through an increase in the extramitochondrial $NADH/NAD^+$ concentration ratio. Thus the effect of ethanol on glycerol uptake can be explained

simply by a raised concentration of glycerophosphate causing inhibition of glycerol kinase activity.

K_m of glycerol kinase and K_m for glycerol uptake. The K_m of partially purified glycerol kinase for glycerol is 3×10^{-6}M and for glycerol kinase in the 80000*g* supernatant is 7×10^{-6}M. However, the K_m for glycerol uptake by kidney-cortex slices is $1{\cdot}0 \times 10^{-3}$M and is probably of a similar order for liver slices (see Robinson & Newsholme, 1969). This discrepancy is difficult to explain at the present time; if it were due to L-3-glycerophosphate inhibition of glycerol kinase the intracellular concentration of glycerophosphate should be at least two orders of magnitude greater than the observed K_i of the enzyme for L-3-glycerophosphate. The total hepatic concentration of L-3-glycerophosphate as measured in 'freeze-clamped' livers is only about twice the K_i value. It suggests that there may be other factors, which are as yet unknown, involved in the regulation of glycerol kinase activity.

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