Biochem. J. (1973) **132**, 55–60
Printed in Great Britain

The Effect of Glycerol and Dihydroxyacetone on Hepatic Adenine Nucleotides

By H. F. WOODS* and H. A. KREBS
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, and the Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(*Received* 7 *August* 1972)

1. The changes in the metabolite content in the isolated perfused rat liver and in the perfusion medium were measured after loading the liver with glycerol or dihydroxyacetone. 2. Glycerol was rapidly taken up by livers from fed and starved rats; glucose, lactate and pyruvate were discharged into the medium. The [lactate]/[pyruvate] ratio in the medium rose from 10 to 30 and in the tissue from 9.6 to 36.6. 3. The most striking effects of glycerol loading were: (i) the accumulation in the liver of α-glycerophosphate, which increased from 0.13 to 8.45 μmol/g at 40 min; (ii) the decrease in the concentration of adenine nucleotides to 70% of the control value at 40 min. 4. The P_i content of the tissue also fell, from 4.25 to 2.31 μmol/g at 10 min, but the sum of the phosphates measured rose from the normal value of 13.8 to 18.8 μmol/g at 40 min, because of an uptake of P_i from the medium. 5. Omission of phosphate from the standard perfusion medium increased the depletion of adenine nucleotides on glycerol loading. 6. Dihydroxyacetone was more rapidly metabolized than glycerol. Again glucose, lactate and pyruvate were the main products. The [lactate]/[pyruvate] ratio remained below 10. 7. Dihydroxyacetone caused an increase of the fructose 1-phosphate content from 0.23 to 0.39 μmol/g at 10 min. The adenine nucleotide content of the tissue was not significantly decreased by dihydroxyacetone loading. 8. The rate of removal of both glycerol and dihydroxyacetone was about 60% greater in the livers from fed than in those from starved animals. 9. The results extend previous findings by Burch *et al.* (1970), who administered glycerol and dihydroxyacetone intraperitoneally.

When the liver is loaded with fructose the sum of the concentrations of the adenine nucleotides in the tissue rapidly falls (Mäenpää *et al.*, 1968; Raivio *et al.*, 1969; Burch *et al.*, 1969; Woods *et al.*, 1970). This depletion of adenine nucleotides is connected with the high rate of phosphorylation of fructose, which causes a rapid decrease in the concentrations of ATP and of P_i. Since ATP is an inhibitor of 5′-nucleotidase and P_i is an inhibitor of AMP deaminase, the decrease in the concentrations of ATP and P_i results in an increased rate of the reactions that cause an irreversible degradation of AMP.

The present work was primarily undertaken to test whether glycerol and dihydroxyacetone, which, like fructose, are rapidly phosphorylated in the liver, can cause similar changes in the adenine nucleotide content. Glycerol loading of the isolated perfused rat liver was found to have such effects whereas dihydroxyacetone loading did not.

* Present address: Medical Research Council Clinical Pharmacology Unit, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

Materials and Methods

Animals and diets

Female Wistar rats each weighing about 200 g were obtained from Carworth (Europe) Ltd., Alconbury, Hunts., U.K., and were fed on a standard small-animal diet (Spillers Mills Ltd., Gainsborough, Lincs., U.K.), water being provided *ad libitum*.

Reagents

Glycerol of analytical grade was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and dihydroxyacetone from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Nucleotides, coenzymes and crystalline enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

Liver perfusion

The method of liver perfusion was that described by Hems *et al.* (1966). The composition of the basal

perfusion medium was as previously described (Woods *et al.*, 1970), except in some experiments where a medium containing minimal amounts of P_i was needed. This was prepared by omitting the phosphate from the basal medium of Krebs & Henseleit (1932), by dialysing the bovine albumin against a phosphate-free medium and by washing the erythrocytes five times with 10vol. of the phosphate-free Krebs & Henseleit (1932) medium. The only phosphate present in this medium was that contained in the washed erythrocytes and amounted to 0.5mM. The standard medium contained 1.92 (S.E.M. ± 0.08)mM-P_i (11 observations).

Analytical methods

The methods of freeze-clamping and extracting the livers, of sampling the medium and of determining metabolites were as described by Woods *et al.* (1970). Glycerol and dihydroxyacetone were determined enzymically by the methods of Wieland (1963*a*,*b*).

Results

Changes in the perfusion medium after loading with glycerol and dihydroxyacetone

The changes in the concentrations of metabolites in the perfusion medium are given in Table 1 and the rates derived from these results are shown in Table 2. When 10mM-glycerol was added to the medium the rate of removal of glycerol by the liver of well-fed rats was approximately constant until the concentration of glycerol had fallen to below 1mM. The main products appearing in the medium were glucose, lactate and pyruvate. The increments over the formation of these products in the control livers were less than the amounts of glycerol removed (10.7 μmol/ml removed, 7.6 μmol of C_3 equivalents/ml recovered after 120min). This calculation is based on the assumption that the added glycerol does not affect the formation of glucose, lactate and pyruvate from endogenous glycogen, and the discrepancy suggests that glycerol either decreases the degradation of glycogen or that some of the added glycerol is converted into glycogen.

The results obtained when dihydroxyacetone was added were very similar except that the rate of disappearance of the substrate was much more rapid; most of it had disappeared after 45min. After 120min the glucose concentration was the same as in the control to which no substrate had been added, and lactate and pyruvate were the only products the concentrations of which were elevated over those of the control. This again indicates that the added substrate alters the metabolism of liver glycogen.

With livers from starved rats, however, the rates of removal of glycerol and dihydroxyacetone were

Table 1. *Metabolite concentrations in the perfusion medium after loading with glycerol and dihydroxyacetone*

The rats were well fed. The concentrations (mM) are expressed as means ± S.E.M. for three observations with glycerol and four observations under the other conditions. The initial volume of the medium was 150ml and sampling decreased this by 0.5ml every 15min.

Time (min)	Concn. of metabolites in perfusion medium (mM)											
	Glycerol added				Dihydroxyacetone added				No substrate added			
	Substrate	Glucose	Lactate	Pyruvate	Substrate	Glucose	Lactate	Pyruvate	Glucose	Lactate	Pyruvate
0	11.2 ±0.36	0.28±0.06	0.19±0.03	0.06±0.03	9.02±0.22	0.37±0.01	0.31±0.03	0.02±0.006	0.27±0.26	0.26±0.02	0.06±0.01
15	9.59±0.71	2.78±0.15	0.95±0.06	0.05±0.01	5.25±0.18	3.62±0.41	2.01±0.28	0.35±0.06	2.44±0.26	0.83±0.08	0.13±0.05
30	8.12±0.33	4.08±0.13	1.37±0.19	0.05±0.006	2.49±0.16	4.01±0.35	3.30±0.36	0.47±0.06	3.44±0.27	1.30±0.04	0.15±0.06
45	6.13±0.27	5.23±0.26	1.99±0.28	0.06±0.02	0.46±0.11	4.29±0.33	4.14±0.51	0.53±0.07	4.08±0.34	1.59±0.06	0.15±0.08
60	4.62±0.42	6.27±0.34	2.84±0.44	0.11±0.03	<0.04	4.85±0.26	4.61±0.41	0.61±0.07	4.85±0.36	1.87±0.05	0.19±0.08
90	1.55±0.52	7.85±0.53	4.28±0.63	0.18±0.05	<0.04	5.58±0.22	4.48±0.29	0.71±0.09	5.18±0.30	1.91±0.16	0.22±0.12
120	0.51±0.09	8.03±0.36	4.97±1.08	0.47±0.13	<0.04	5.85±0.19	4.54±0.51	0.72±0.10	5.91±0.60	2.00±0.24	0.25±0.12
Change in 120min (μmol/ml)	−10.7	+7.75	+4.78	+0.41	−9.0	+5.5	+4.3	+0.7	+5.6	+1.7	+0.2

Table 2. *Initial rates of metabolic changes in the perfused liver of well-fed rats after the addition of glycerol or dihydroxyacetone*

The initial rates, expressed as μmol/min per g wet wt., were calculated from the values in Table 1. They refer to the first 45 or 60min for the changes in lactate, pyruvate and substrate. For glucose formation in the presence of dihydroxyacetone they refer to the first 15min, when the rate was linear.

	Initial rate (μmol/min per g wet wt.)	
Metabolic change	Glycerol added (three observations)	Dihydroxyacetone added (four observations)
Substrate removed	2.24±0.21	3.96±0.28
Glucose formed	2.10±0.16	4.05±0.61
Lactate formed	0.84±0.07	1.46±0.20
Pyruvate formed	0.03±0.007	0.24±0.04

Table 3. *Rate of metabolic changes in the perfused liver of rats starved for 48h*

The substrates were added after 38min of perfusion and the first sample was taken 2min later. The rates were calculated from a plot of total metabolite in the medium against time and refer to the subsequent 60min of perfusion. The rates are expressed as μmol/min per g wet wt. and are the mean of three experiments.

	Rate (μmol/min per g wet wt.)							
Period after addition (min)	Glycerol added				Dihydroxyacetone added			
	Substrate	Glucose	Lactate	Pyruvate	Substrate	Glucose	Lactate	Pyruvate
0–15	−1.50	0.65	0.02	<0.01	−3.15	1.45	0.05	0.08
15–30	−1.78	0.59	−0.34	<0.01	−1.33	0.98	0.57	−0.02
30–45	−1.34	0.76	0.30	<0.01	−1.73	1.15	0.34	0.08
45–60	−1.49	0.68	0.23	<0.01	−2.28	0.65	0.24	0.09

low, being about 60% of those in liver of fed rats (Table 3), and the amounts of glucose, lactate and pyruvate formed were reasonably equivalent to those of substrates removed. These results support the previous conclusion that discrepancies in the balance are connected with the presence of glycogen.

Content of intermediary metabolites in the freeze-clamped perfused rat liver after glycerol loading

The most striking changes occurred in the contents of the adenine nucleotides, α-glycerophosphate and P_i (Table 4). Within 10min of the addition of glycerol, the sum of the adenine nucleotides had fallen to 78% of the original value, and to 70% after 40min. This fall was mainly due to a loss of ATP. The P_i content fell from 4.25μmol/g to 2.13μmol/g at 10min and then rose within 40min to 3.49μmol/g. The sum of phosphates measured rose from 13.8 to 18.8μmol/g at 40min, owing to an uptake of phosphate by the liver from the perfusion medium (see below). The content of α-glycerophosphate rose from 0.13 to 8.45μmol/g after 40min. The content of triose phosphates, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate, as well as those of phosphoenolpyruvate, the phosphoglycerates and pyruvate all decreased, but in absolute terms the changes were small. The lactate content rose.

When a perfusion medium containing minimal amounts of P_i was used, the depletion of adenine nucleotides was substantially greater. Evidently the phosphate deposited in the form of phosphorylated intermediates is normally replenished by the uptake of P_i from the medium, and in the absence of this source the resynthesis of ATP is restricted.

Content of intermediary metabolites in the freeze-clamped perfused rat liver after dihydroxyacetone loading

In contrast to glycerol, dihydroxyacetone caused no depletion of adenine nucleotides and no accumulation of phosphorylated intermediates (Table 5).

The small changes in the content of adenine nucleotides and P_i were of the same order as those found on aerobic perfusion of livers from well-fed rats when no substrate was added (Woods, 1970). There were small decreases in the contents of fructose 1,6-diphosphate, glucose 6-phosphate, fructose 6-phosphate, phosphoenolpyruvate and 2-phosphoglycerate. The content of fructose 1-phosphate rose somewhat as would be expected if equilibrium existed in the fructose 1-phosphate aldolase (EC 4.1.2.7) system because of the increase in the content of dihydroxyacetone phosphate. There were small increases in the contents of 3-phosphoglycerate and α-glycerophosphate. The metabolite ratios shown in Table 5 were all of the order expected for equilibrium.

It is probable that the differences in the effects of glycerol and dihydroxyacetone on the content of the intermediary metabolites are connected with the rate of disposal of the primary product of glycerol and dihydroxyacetone metabolism. Both substances are phosphorylated, but whereas dihydroxyacetone phosphate can be readily disposed of by the reactions of gluconeogenesis and glycolysis, α-glycerophosphate requires dehydrogenation to the triose phosphate

Table 4. *Effect of glycerol on the metabolite content of perfused rat liver*

Livers from well-fed rats were perfused with a medium containing glycerol (initial concn. 10mM). The livers were freeze-clamped at intervals after the start of perfusion. The perfusion medium used in the series marked by an asterisk contained a low concentration of phosphate, the only phosphate present being that in the erythrocytes. The control values refer to livers freeze-clamped *in vivo* after cervical dislocation. The results are means±S.E.M. Where no S.E.M. values are given only one or two measurements were made.

	Metabolite content (μmol/g wet wt.)				
	Livers not perfused	Perfused livers			
Time of perfusion (min) ...	0	10	10*	40	40*
No. of observations ...	4	3	2	3	3
ATP	2.22±0.07	1.62±0.09	1.39	1.39±0.04	0.86±0.13
ADP	0.78±0.08	0.71±0.03	0.75	0.70±0.11	0.55±0.01
AMP	0.26±0.05	0.24±0.03	0.15	0.20±0.03	0.30±0.03
Total adenine nucleotides	3.27±0.12	2.56±0.04	2.29	2.29±0.17	1.72±0.11
P_i	4.25±0.25	2.13±0.56	1.90	3.49±0.23	2.96±0.49
Free glucose	7.22±0.77	2.79±0.23	3.66	4.47±0.41	5.56
Glycerol	0.13±0.01	5.69	4.86	4.14	—
Lactate	0.79±0.09	1.16±0.30	0.92	1.58±0.11	1.19±0.12
Pyruvate	0.08±0.01	0.05±0.002	0.05	0.04±0.002	0.06±0.01
Fructose 1,6-diphosphate	0.02±0.002	0.02±0.002	0.01	0.01±0.003	0.01
Glucose 6-phosphate	0.25±0.03	0.17±0.001	0.09	0.20±0.03	0.14
Fructose 6-phosphate	0.06±0.01	0.04±0.006	0.03	0.05±0.005	0.04
Glucose 1-phosphate	<0.01	<0.01	<0.01	<0.01	<0.01
Phosphoenolpyruvate	0.16±0.02	0.03±0.006	0.02	0.04±0.01	0.02
2-Phosphoglycerate	0.04±0.01	0.03±0.01	0.02	0.02±0.01	0.03
3-Phosphoglycerate	0.26±0.06	0.06±0.01	0.07	0.08±0.01	0.07
α-Glycerophosphate	0.13±0.10	4.77±0.42	3.57	8.45±1.28	5.20±1.01
Glyceraldehyde 3-phosphate	0.006±0.0002	0.004±0.0001	0.005	0.005±0.0006	0.005
Dihydroxyacetone phosphate	0.04±0.003	0.04±0.003	0.02	0.05±0.002	0.03
[Lactate]/[pyruvate]	9.60±0.96	22.4±5.02	27.50	36.6±2.71	34.33
[Fructose 6-phosphate]/[glucose 6-phosphate]	0.25±0.02	0.25±0.03	0.33	0.25±0.03	0.29
[2-Phosphoglycerate]/[3-phosphoglycerate]	0.20±0.06	0.57±0.24	0.29	0.29±0.15	0.43
[α-Glycerophosphate]/[dihydroxyacetone phosphate]	3.55±0.40	130±14.9	192	167±28.3	282
[ATP][AMP]/[ADP]^2	0.97±0.17	0.77±0.08	0.37	0.60±0.12	0.78

Table 5. *Effect of dihydroxyacetone on the metabolite content of perfused rat liver*

Livers from well-fed rats were perfused with a medium containing initially 10mM-dihydroxyacetone. The livers were freeze-clamped either at 10 or at 40min after the addition of the substrate. Results are means±S.E.M. Where no S.E.M. values are given only one or two measurements were made. The contents of fructose 1-phosphate and dihydroxyacetone in the control (non-perfused) livers were 0.23μmol/g wet wt. (two observations), and 0.23 (S.E.M. ±0.05)μmol/g wet wt. (four observations) respectively. The other control values are given in Table 4.

Metabolite	Content (μmol/g wet wt.) after 10min	Content (μmol/g wet wt.) after 40min
ATP	2.30±0.08	2.05±0.10
ADP	0.80±0.11	0.70±0.06
AMP	0.22±0.09	0.26±0.04
Total adenine nucleotides	3.32±0.19	3.01±0.18
P_i	4.40±0.67	3.78±0.20
α-Glycerophosphate	0.92±0.22	0.44±0.11
Dihydroxyacetone	1.88±0.35	0.58±0.09
Glucose	5.03±1.57	6.02±1.63
Lactate	1.93±0.18	3.48±0.20
Pyruvate	0.25±0.03	0.31±0.06
Fructose 1,6-diphosphate	0.03±0.01	0.02±0.01
Glucose 6-phosphate	0.22±0.01	0.10±0.01
Fructose 6-phosphate	0.06±0.01	0.03±0.003
Fructose 1-phosphate	0.39	0.34
Glucose 1-phosphate	<0.01	<0.01
Phosphoenolpyruvate	0.11±0.02	0.10±0.03
2-Phosphoglycerate	0.05±0.01	0.03±0.01
3-Phosphoglycerate	0.31±0.04	0.18±0.01
Glyceraldehyde 3-phosphate	0.004	0.004
Dihydroxyacetone phosphate	0.11±0.03	0.07±0.02
[Lactate]/[pyruvate]	7.86±0.28	11.97±2.28
[Fructose 6-phosphate]/[glucose 6-phosphate]	0.27±0.03	0.35±0.02
[2-Phosphoglycerate]/[3-phosphoglycerate]	0.16±0.02	0.17±0.03
[α-Glycerophosphate]/[dihydroxyacetone phosphate]	8.23±1.30	7.29±2.90
$[ATP][AMP]/[ADP]^2$	0.84±0.13	1.07±0.04

oxidation level. This appears to be the rate-limiting step. The information on substrate removal indicates that dihydroxyacetone is phosphorylated more rapidly than glycerol.

The values for the ratios [fructose 6-phosphate]/[glucose 6-phosphate] and [2-phosphoglycerate]/[3-phosphoglycerate] were of the order expected for equilibrium. The addition of glycerol caused a major rise in the value of the [α-glycerophosphate]/[dihydroxyacetone phosphate] ratio, and this was not paralleled by the equivalent rise of the [lactate]/[pyruvate] ratio. This implies that equilibrium was not maintained in the α-glycerophosphate dehydrogenase and lactate dehydrogenase systems, which share a common pool of NAD. The formation of α-glycerophosphate was evidently too rapid in relation to the capacity of the α-glycerophosphate dehydrogenase. Similar observations were made by Burch *et al.* (1970) after an intraperitoneal glycerol load.

Discussion

Depletion of adenine nucleotides after glycerol loading

Burch *et al.* (1970) have described the loss of total adenine nucleotides after injecting intraperitoneally a large dose of glycerol. The experiments reported in the present paper show a depletion of hepatic adenine nucleotides on perfusion of the isolated liver with 10mM-glycerol. Thus glycerol has in this respect the same effect as fructose (Woods *et al.*, 1970). A common characteristic of fructose and glycerol is the accumulation of a phosphorylated intermediate in the liver caused by the high activity of fructokinase and glycerokinase respectively. As discussed previously (Woods *et al.*, 1970), a fall of the concentrations of ATP and of P_i leads to an activation of hepatic AMP-degrading enzymes and this causes a virtually irreversible loss of AMP through the formation of IMP and adenosine. Dihydroxyacetone loading did not lead

to depletion of adenine nucleotides or to a formation of phosphorylated intermediates. Xylitol, on the other hand, can also cause an accumulation of α-glycerophosphate (up to 12mM) and a depletion of hepatic adenine nucleotides (Woods, 1972).

Accumulation of α-glycerophosphate

The accumulation of α-glycerophosphate to concentrations of 8.5mM after glycerol loading indicates that the formation of α-glycerophosphate is more rapid than is dehydrogenation. This is somewhat unexpected, because the activity of hepatic α-glycerophosphate dehydrogenase is higher under the assay conditions than that of glycerokinase (Burch *et al.*, 1970), and the K_m value of α-glycerophosphate dehydrogenase for α-glycerophosphate is about 0.1mM (Young & Pace, 1958; van Eys *et al.*, 1959). However, the assay conditions are very different from the physiological conditions and the assay was carried out in the direction of α-glycerophosphate formation.

According to Burch *et al.* (1970) the hepatic accumulation of α-glycerophosphate is increased when rats are kept on a riboflavin-deficient diet, i.e. when the activity of the mitochondrial (flavin-linked) α-glycerophosphate dehydrogenase is decreased to about 25% of the normal value. On the other hand, administration of tri-iodothyronine, which increases mitochondrial α-glycerophosphate dehydrogenase activity (Lee & Lardy, 1965), prevents an accumulation of α-glycerophosphate on glycerol loading (Williamson *et al.*, 1969). These observations support the view (Burch *et al.*, 1970) that under physiological conditions a major part of the α-glycerophosphate arising from glycerol is oxidized by the mitochondrial α-glycerophosphate dehydrogenase. This does not apply to all conditions (see Williamson *et al.*, 1971; Carnicero *et al.*, 1972).

We acknowledge the competent technical assistance of Mr. R. D. Carter. The work was supported by grants from the Medical Research Council and the U.S. Public Health Service (Grant no. AM 11748).

References

Burch, H. B., Max, P., Chyu, K. & Lowry, O. H. (1969) *Biochem. Biophys. Res. Commun.* **34**, 619–626

Burch, H. B., Lowry, O. H., Meinhardt, L., Max, P. & Chyu, K. (1970) *J. Biol. Chem.* **245**, 2092–2102

Carnicero, H. H., Moore, C. L. & Hoberman, H. D. (1972) *J. Biol. Chem.* **247**, 418–426

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lee, Y. P. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1427–1436

Mäenpää, P. H., Raivio, K. O. & Kekomäki, M. P. (1968) *Science* **161**, 1253–1254

Raivio, K. O., Kekomäki, M. P. & Mäenpää, P. H. (1969) *Biochem. Pharmacol.* **18**, 2615–2624

van Eys, J., Nuenke, B. J. & Patterson, M. K. (1959) *J. Biol. Chem.* **234**, 2308–2313

Wieland, O. (1963*a*) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 211–214, Academic Press, London

Wieland, O. (1963*b*) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 244–245, Academic Press, London

Williamson, D. H., Veloso, D., Ellington, E. V. & Krebs, H. A. (1969) *Biochem. J.* **114**, 575–584

Williamson, J. R., Jakob, A. & Refino, C. (1971) *J. Biol. Chem.* **246**, 7632–7641

Woods, H. F. (1970) D.Phil. Thesis, University of Oxford

Woods, H. F. (1972) *Acta Med. Scand.* **30**, *Suppl.* 30, 87–103

Woods, H. F., Eggleston, L. V. & Krebs, H. A. (1970) *Biochem. J.* **119**, 501–510

Young, H. L. & Pace, N. (1958) *Arch. Biochem. Biophys.* **75**, 125–141