Some Properties of Fructose 1,6-Diphosphatase of Rat Liver and their Relation to the Control of Gluconeogenesis

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1. Fructose 1,6-diphosphatase has been purified tenfold from rat liver. The final preparation was not contaminated by either glucose 6-phosphatase or phosphofructokinase. The properties of the enzyme have been investigated in an attempt to define factors that could be of revelance to metabolic control of fructose 1,6-diphosphatase activity. 2. The metal ions Fe^{2+}, Fe^{3+} and Zn^{2+} inhibited the activity of fructose 1,6-diphosphatase even in the presence of an excess of mercaptoethanol; other metal ions tested had no effect. The inhibition produced by Zn^{2+} was reversed by EDTA, but that produced by either Fe^{2+} or Fe^{3+} was not reversible. 4. The enzyme has a very low K_m for fructose 1,6-diphosphate (2·0 μM). Concentrations of fructose 1,6-diphosphate above 75 μM inhibited the activity; however, even at very high fructose 1,6-diphosphate concentrations only 70% inhibition was obtained. 5. The activity was also inhibited by low concentrations of AMP, which lowered $V_{max.}$ and increased K_m for fructose 1,6-diphosphate. Evidence is presented that suggests that AMP can be defined as an allosteric inhibitor of fructose 1,6-diphosphatase. 6. The inhibitions by both fructose 1,6-diphosphate and AMP were extremely specific. Also, the degree of inhibition was not affected by the presence of intermediates of glycolysis, of the tricarboxylic acid cycle, of amino acid metabolism or of fatty acid metabolism. 7. It is suggested that the intracellular concentrations of AMP and fructose 1,6-diphosphate could be of significance in controlling the activity of fructose 1,6-diphosphatase in the liver cell. The possible relationship between these intermediates and the control of gluconeogenesis is discussed.

Fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11), discovered by Gomori (1943), is taken to be one of the enzymes that control the overall rate of gluconeogenesis (Pogell & McGilvery, 1952; Mokrasch, Davidson & McGilvery, 1956; Krebs, 1963, 1964). The properties of this enzyme, especially those that influence its activity, are consequently of special interest and they were systematically investigated. During this investigation it was found that excess of substrate and low concentrations of AMP inhibit the enzyme, and the characteristics of these inhibitions suggest that they play a role in controlling the enzyme activity, and therefore in the regulation of gluconeogenesis. A preliminary account of some of the experiments has been published (Newsholme, 1963).

MATERIALS AND METHODS

Chemicals. Glucose 6-phosphate (sodium salt), FDP* (tetracyclohexylammonium salt), 6-phosphogluconate, ribose 5-phosphate, DL-glycerophosphate, DL-glyceraldehyde 3-phosphate (diethylacetal barium salt), cyclic-3,5-AMP, ATP, ADP, AMP, IMP, UMP, GMP, phosphoenolpyruvate (tricylohexylammonium salt), 1,3-diphosphoglycerate (tricyclohexylammonium salt), NAD, $NADH_2$, NADP, tris, adenine, adenosine, sodium pyruvate and triethanolamine hydrochloride were obtained either from Sigma Chemical Co. or from C. F. Boehringer und Soehne G.m.b.H. Palmitic acid and 2-mercaptoethanol were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Acetyl-CoA was prepared by the method described by Ochoa (1955). In one experiment (Table 7) $NADH_2$ was prepared from Sigma NAD by the method of Rafter & Colowick (1955) as adapted by Dalziel (1962*a*).

Sodium acetoacetate and oxaloacetic acid were prepared in this Laboratory by Mr T. Gascoyne, by the methods of Krebs & Eggleston (1949) and Wohl & Claussner (1907) respectively. Sephadex G-25 was obtained from Pharmacia Ltd., West Ealing, London. DL-β-Hydroxybutyrate (sodium salt) and cysteine hydrochloride were obtained from British Drug Houses Ltd. and used without further purification. Palmitoyl-CoA was kindly provided by Mr N. Kuhn, of this Department, and Dr P. B. Garland, of the Biochemistry Department, University of Cambridge.

Enzymes. The commercial enzymes used for determination of FDP, AMP, glucose 6-phosphate and fructose

* Abbreviations: FDP, fructose 1,6-diphosphate; FDPase, fructose 1,6-diphosphatase.

6-phosphate were obtained from C. F. Boehringer und Soehne G.m.b.H.

Preparation of fructose 1,6-*diphosphatase*. The liver from a freshly killed male Wistar rat was weighed, cut into small pieces and homogenized with 3 vol. of 0·15 M-KCl–5 mM-cysteine hydrochloride solution in a manually operated Potter-Elvehjem homogenizer. The pH of the resultant suspension was lowered to 5·2 by the addition of HCl, and this was then incubated at 37° for 10 min. The homogenate was centrifuged at 30000*g* for 45 min. at 0°. The supernatant, which contained the enzyme, was neutralized and small volumes were stored at −10° until required. This procedure resulted in complete separation of FDPase from glucose 6-phosphatase and phosphofructokinase, and in a tenfold purification.

Assay of activity. The FDPase activity was assayed in two ways. First, the formation of hexose monophosphate (glucose 6-phosphate and fructose 6-phosphate) was measured after short periods of incubation of the substrate (FDP) with the extract. A 0·1 ml. sample of extract was incubated with 0·9 ml. of incubation medium at 30° for 3–5 min. The incubation medium comprised 100 mM-tris–maleate, 50 mM-$MgSO_4$, 0·1 mM-EDTA and varying concentrations of FDP at pH 7·5. The reaction was stopped by addition of 1 ml. of 6% perchloric acid. The precipitate was removed by centrifugation and perchloric acid was precipitated as the potassium salt by addition of potassium hydrogen carbonate at 0°. The hexose monophosphates present in the supernatant were assayed by enzymic methods, by following the reduction of NADP at 340 mμ with phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (Newsholme & Randle, 1961). Control experiments showed that extract incubated without FDP produced no hexose monophosphate, and that standard glucose 6-phosphate added to the assay system could be quantitatively recovered as hexose monophosphate (this method is referred to below as the sampling technique). Secondly, the activity was measured by following the reduction of NADP by glucose 6-phosphate dehydrogenase at 340 mμ in a Zeiss spectrophotometer model PMQ2 at room temperature, or a Gilford recording spectrophotometer at 25°, when 0·05 ml. of diluted extract was added to 1·95 ml. of incubation medium contained in the cuvette. The diluted extract was prepared by adding 1 ml. of the original extract to 9 ml. of 50 mM-tris–HCl buffer, pH 7·5, containing 20 mM-mercaptoethanol. In this assay the incubation medium comprised 50 mM-tris–HCl, 25 mM-$MgSO_4$, 20 mM-mercaptoethanol, 0·2 mM-NADP, 10 μg. each of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase and usually 0·1 mM-FDP at pH 7·5 (Taketa & Pogell, 1963). In some experiments the substrate was omitted from the incubation medium and the reaction was then initiated by addition of the substrate. When very low concentrations of substrate were employed greater sensitivity was achieved by measuring the fluorescence of $NADPH_2$ by using the method and apparatus as described by Dalziel (1962*b*). This method is referred to below as the continuous assay technique. Factors that inhibited the activity of FDPase in this assay system did not change the rate or the final value of reduction of NADP when fructose 6-phosphate was added to the system. It was assumed that these factors were inhibiting the activity of FDPase. In all cases tested factors that influenced FDPase in one assay system had a similar effect in the other system. In the continuous assay the final dilution of the original tissue was 1:1600 (protein concentration approx. 1·5 μg./ml.); in the sampling technique the final dilution was 1:40 (protein concentration approx. 600 μg./ml.). Under conditions of the continuous assay, the activity of non-specific phosphatase, as measured with *p*-nitrophenyl phosphate as substrate, was less than 3% of FDPase activity.

RESULTS

Metal ions and fructose 1,6-*diphosphatase activity*. The activity of the enzyme varied according to the magnesium sulphate (or magnesium chloride) concentrations in the test system (Table 1). Maximum activity was observed at 5 mM, and concentrations above this produced some inhibition of activity. In the absence of Mg^{2+} there was no activity. In the presence of 5 mM-Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+} at concentrations of 0·1 mM had no effect; Fe^{2+} and Zn^{2+} at the same concentration inhibited FDPase by approx. 70% (Table 2). When EDTA (0·05 mM) was added to the extract before the metal ion, no inhibition was observed. Addition of EDTA (0·05 or 0·5 mM) after the metal ion had no effect on the inhibition produced by either Fe^{2+} or Fe^{3+}; however, 0·05 mM-EDTA removed most of the inhibitory action of Zn^{2+}. EDTA could prevent inhibition by Fe^{2+} and Fe^{3+} if it was added to the cuvette before the extract, but it was ineffectual if these metal ions had been in contact with the enzyme. Thus inhibitions by Fe^{2+} and Fe^{3+} were not readily reversed, whereas that by Zn^{2+} was easily reversed. The enzyme was partially protected from Fe^{2+} and Fe^{3+} by the presence of substrate; but the inhibition increased with time of incubation, probably because the substrate became less protective with time. The effect of Zn^{2+} was very rapid and the enzyme was not protected by the presence of the substrate. The effect of increasing the concentration of the metal ions is shown in Table 3; 50% inhibition of activity was obtained at concentrations of 0·5 μM-Zn^{2+} and 17·0 μM-Fe^{3+}. In general the activity of FDPase in liver extracts was increased by addition of low concentrations of EDTA (Table 2); this is probably

Table 1. *Effect of magnesium sulphate concentration on fructose* 1,6-*diphosphatase activity*

Activity was measured by the continuous assay technique and is expressed in μmoles of fructose 6-phosphate produced/g. of wet tissue/hr.

Concn. of $MgSO_4$ (mM)	Activity
0·25	198
0·5	334
1·0	416
5·0	456
10·0	383
25·0	352

Table 2. *Effect of metal ions on the activity of fructose* 1,6-*diphosphatase at* 5 mM-Mg^{2+}

Activity was measured by the continuous assay technique and is expressed in μmoles of fructose 6-phosphate produced/g. of wet tissue/hr. The extract was preincubated for 10 min. with the metal ion, and the reaction initiated by addition of substrate. When EDTA (50 μM) was added before the metal ion, the extract was preincubated for 5 min. with EDTA, then the metal ion was added, and the reaction was initiated 10 min. later; when EDTA was added after the metal ion, the reaction was initiated 5 min. after addition of EDTA.

Addition (0·1 mM)	Activity: No EDTA added	Activity: EDTA added before metal ion	Activity: EDTA added after metal ion
Control	284	341	284
$CaCl_2$	275	—	—
$CuSO_4$	288	—	—
$MnCl_2$	301	—	—
$CoCl_2$	287	—	—
$FeSO_4$	96	308	90
$ZnSO_4$	66	345	288
$FeCl_3$	106	323	98
Ferric citrate	81	351	—

Table 3. *Effect of concentration of ferric citrate and of zinc sulphate on the activity of fructose* 1,6-*diphosphatase at* 5 mM-Mg^{2+}

Activity of FDPase was measured by the continuous assay technique and is expressed in μmoles of fructose 6-phosphate produced/g. of wet tissue/hr. The extract was preincubated for 10 min. with the metal ion and the reaction was initiated by addition of substrate (0·1 mM). Ferric citrate was dissolved in tris–HCl buffer, pH 7·5 These two experiments have been done with different batches of extract.

Concn. of ferric citrate (μM)	Activity	Inhibition (%)	Concn. of $ZnSO_4$ (μM)	Activity	Inhibition (%)
0·0	344		0·0	173	
2·5	313	9	0·05	147	15
5·0	234	32	0·5	79	54
10·0	205	40	1·0	66	62
25·0	123	64	5·0	19	89
50·0	93	72	10·0	16	91
100·0	98	71			

related to the metal ion inhibition which is described above. McGilvery (1964) has also observed an increase in activity of FDPase by addition of EDTA or cysteine.

Substrate inhibition of fructose 1,6-*diphosphatase.* The activity of FDPase is known to be inhibited by high concentrations of the substrate (Krebs, 1963; Weber, 1964). The phenomenon was investigated further; in Fig. 1 the effects of increasing the substrate concentration from 0·5 to 10 mM are shown. The inhibition of activity did not exceed approx. 70% of the rate obtained at the optimum substrate concentration. The lowest substrate concentration required for inhibition was found to be dependent on the Mg^{2+} concentration (Table 4). At 5 mM-Mg^{2+} optimum activity was obtained at a substrate concentration of 50 μM, and 10% and 20% inhibitions of the activity were observed at 75 and 100 μM respectively; at 0·5 and 25 mM-Mg^{2+} inhibition by substrate was observed at a concentration of 250 μM.

Fructose 1,6-*diphosphatase activity and compounds related to fructose* 1,6-*diphosphate.* The effect of glucose 6-phosphate (and therefore fructose 6-phosphate, as the preparation contains phosphoglucose isomerase), glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, fructose 1-phosphate and inorganic phosphate at concentrations approximately ten times the physiological were investigated. No inhibition of activity was produced by any of these compounds. A slight increase in activity was, in fact, observed with each compound, dihydroxyacetone phosphate having the largest effect.

Inhibition of fructose 1,6-*diphosphatase by adenosine monophosphate.* The activity of FDPase was inhibited by low concentrations of AMP; this inhibition was not affected by increasing the concentration of FDP (Fig. 1). Reciprocal plots of activity against FDP concentration give K_m 2·0 μM for FDP and $V_{max.}$ 360 μmoles/g. of wet tissue/hr. in the absence of AMP (Fig. 2). The presence of AMP

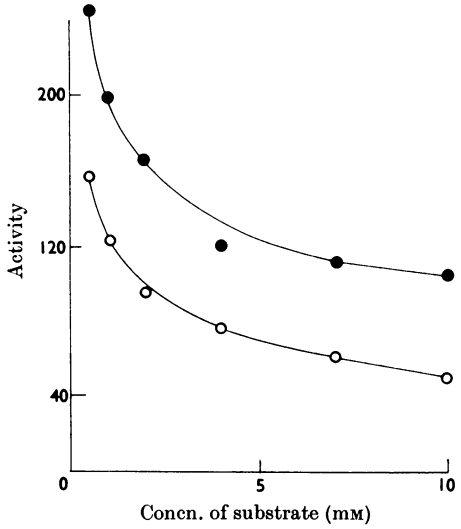

Fig. 1. Effect of varying concentrations of substrate on FDPase activity in the presence and absence of AMP. Activity is expressed as μmoles of product formed/g. of wet tissue/hr., as measured by the sampling technique at a magnesium sulphate concentration of 50mM: ●, in the absence of AMP; ○, in the presence of 0·2mM-AMP.

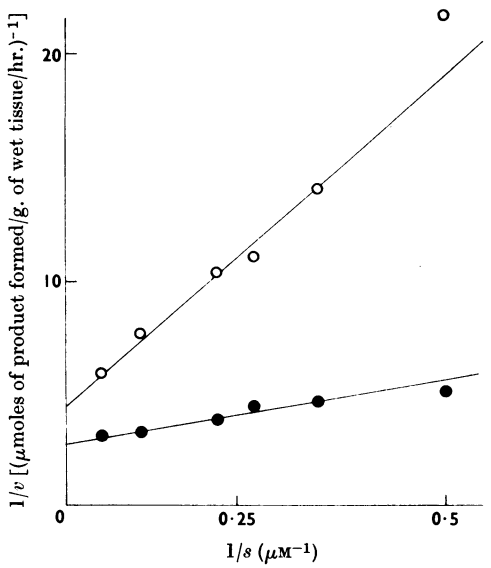

Fig. 2. Lineweaver–Burk plots of FDPase activity in the presence and absence of AMP. Activity was measured by the continuous assay technique at a magnesium sulphate concentration of 25mM: ●, in the absence of AMP; ○, in the presence of 0·2mM-AMP.

Table 4. *Effect of substrate concentration on the activity of fructose* 1,6-*diphosphatase at three magnesium sulphate concentrations*

Activity was measured by the continuous assay technique, and is expressed as μmoles of fructose 6-phosphate produced/g. of wet tissue/hr.

Concn. of substrate (mM)	Activity		
Concn. of $MgSO_4$ (mM) ...	0·5	5·0	25
0·02	281	361	244
0·04	320	396	273
0·05	355	434	289
0·075	370	385	296
0·1	368	346	303
0·25	344	280	259
0·5	284	207	231

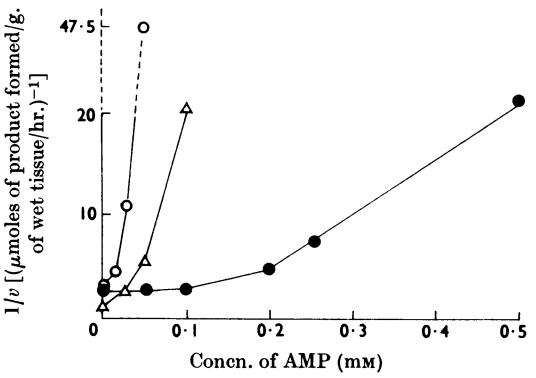

Fig. 3. Reciprocal plot of activity of FDPase against the AMP concentration. Activity was measured by the continuous assay technique at ●, 25mM-, △, 5mM-, and ○, 0·5mM-magnesium sulphate concentrations.

changed both of these values: the K_m was increased to 6·7 and $V_{max.}$ decreased to 225. In Fig. 3 the effect of increasing the AMP concentration on the reciprocal of activity is shown at three different Mg^{2+} concentrations; the inhibition was increased as the concentration of magnesium sulphate was decreased. The plots obtained are not linear, which suggests unusual inhibition kinetics (Dixon & Webb, 1958).

Fructose 1,6-*diphosphatase activity and compounds related either structurally or metabolically to adenosine monophosphate.* Adenine, adenosine, ribose 5-phosphate, cyclic-3′,5′-AMP, ADP-ribose, ADP, ATP and other mononucleotides produced either no inhibition or much less than a comparable concentration of AMP (Table 5). Although ADP lowered the activity by about 27% this can be correlated to a small AMP contamination, which has been verified enzymically and chromatographically.

Table 5. *Effect of metabolic analogues and degradation products of adenosine monophosphate on the activity of fructose 1,6-diphosphatase*

In Expt. 1 activity was measured by the sampling technique at a substrate concentration of 5 mM and a Mg^{2+} concentration of 50 mM; in Expt. 2 the continuous assay technique was employed at a substrate concentration of 0·1 mM and a Mg^{2+} concentration of 25 mM. In the latter case the reaction was initiated by addition of substrate. In the last horizontal row of Expt. 2, 50 μg. of adenylate kinase was added before the preincubation.

Expt. no.	Addition	Concn. (mM)	Activity (μmoles of fructose 6-phosphate/g. of wet tissue/hr.)
1	Control	1·0	435
	AMP	1·0	54
	Adenine	2·0	446
	Adenosine	2·0	443
	Ribose 5-phosphate	2·0	453
	IMP	1·0	418
2	Control	—	320
	AMP	0·3	82
	ADP-ribose	1·0	320
	Cyclic-3,5-AMP	1·0	331
	UMP	1·0	378
	GMP	1·0	341
	ATP	2·5	322
	ADP	2·5	238
	ATP (2·5 mM) + AMP (0·3 mM)	—	73
	ATP (2·5 mM) + AMP (0·3 mM)	—	268

Table 6. *Reversal of adenosine monophosphate inhibition by treatment with Sephadex G-25*

The extract was incubated for 1 min. with either water (control) or AMP (1 mM), cooled to 0° and a sample was passed through a Sephadex G-25 column kept at 0°. Activities were measured by the sampling technique at a substrate concentration of 5 mM and a Mg^{2+} concentration of 50 mM. Because treatment with Sephadex produces some dilution of the extract, the activities are based on protein concentrations, and expressed as μmoles of fructose 6-phosphate formed/min./g. of protein.

Additions	Sephadex treatment	Activity
Control	−	67·0
Control	+	68·0
AMP (1 mM)	−	2·2
AMP (1 mM)	+	50·0

Removal of adenosine monophosphate inhibition. The inhibition of AMP can be reversed by removal of AMP from the experimental system. An extract was incubated with AMP and the latter was subsequently removed by passing the extract down a column of Sephadex G-25 (Table 6) (bed vol. 15 ml.; void vol. 10 ml.).

After this treatment approx. 75% of the control activity was observed, although no AMP could be detected in the extract. The AMP was measured by enzymic assay (Adam, 1963). This failure to obtain complete reversal of activity is possibly due to binding of AMP to the FDPase molecule.

A high concentration (10 mM) of ATP partially reversed the effect of AMP, when FDPase was assayed by the sampling technique; under these conditions the concentration of AMP fell on incubation owing to the action of adenylate kinase, which is present in the extract. No such effect of ATP was observed in the continuous assay technique (in which the extract concentration is much lower and adenylate kinase is too low to measure), but if commercial adenylate kinase was added to the system some relief of AMP inhibition was obtained (Table 5).

Lag period for adenosine monophosphate inhibition. The time-courses of FDPase activity shown in Fig. 4 were constructed by using the sampling technique for very short time-intervals. When the reaction was initiated by addition of extract the curves with substrate alone (at either inhibitory or non-inhibitory concentrations) were linear and, when extrapolated, passed through the origin. However, in the presence of AMP the linear plot, when extrapolated, cut the ordinate above the origin. This indicated that under these conditions the initial rate of the reaction was greater than the final inhibited rate, which is given by the slope of the plot, and that the maximum inhibition was not achieved immediately on mixing the enzyme with AMP but developed within a short time-interval (approx. 11 sec.). This is borne out by the observation that no such lag occurred when the extract was preincubated with AMP and the reaction initiated by addition of sub-

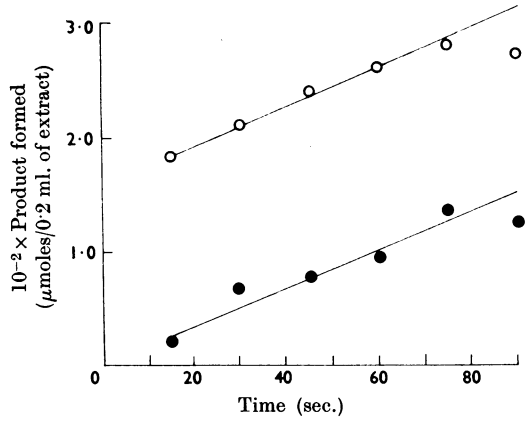

Fig. 4. Progress curve for FDPase. ○, At zero time the substrate and AMP (0·3 mM) were added to the extract; ●, at zero time the substrate was added, after the extract had been preincubated for 60 sec. with AMP (0·3 mM). The Mg^{2+} concentration in each case was 50 mM.

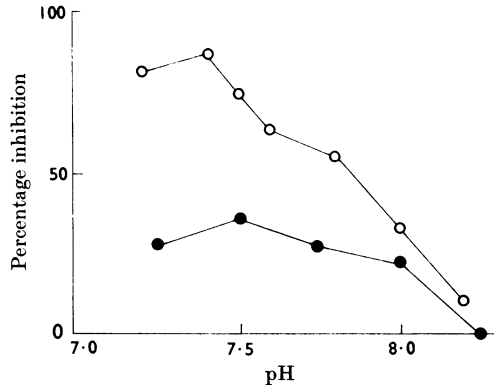

Fig. 5. pH profile of AMP and substrate inhibition of FDPase. The pH was varied with tris–HCl buffer and the activity was measured by the continuous assay technique. Experimental details are given in the text. ○, 0·3 mM-AMP and 0·1 mM-FDP; ●, 1·0 mM-FDP.

strate. In contrast with this the inhibition by substrate was maximal, as far as could be ascertained, as soon as the reaction was started. The presence of this lag means that the sampling technique yielded inaccurate values for the reduction in activity by AMP, unless the extract was preincubated with AMP. The activity observed is higher than if the AMP were fully inhibitory from zero time. Moreover, the extent of this error depends on the activity of the enzyme in the absence of AMP, which in turn depends on the substrate concentration. Therefore, if the substrate concentration is in the inhibitory range, lowering the concentration increases the activity and a greater quantity of the product of FDPase is formed in the lag period; thus the extent of AMP inhibition is less than the true value. This explains a discrepancy between results reported in this paper and some in the preliminary report (Newsholme, 1963), in which it was reported that AMP was a less effective inhibitor at lower substrate concentrations. In the latter case the extract was not preincubated with AMP, so that the FDPase activity during the lag period would interfere with the measurement of AMP inhibition, as described above.

Effect of pH *on fructose* 1,6-*phosphate and adenosine monophosphate inhibitions.* There was a decrease in the inhibitory action of both compounds between pH 7·2 and 8·5, and the effect of pH was qualitatively the same in each case (Fig. 5).

Effect of metabolic intermediates on activity of fructose 1,6-*diphosphatase.* Recently evidence has been obtained that the activity of certain rate-limiting enzymes can be modified by the presence of specific metabolic intermediates, e.g. the effect of citrate on the activity of phosphofructokinase (Passonneau & Lowry, 1963). It was therefore important to test whether the activity of FDPase, or the inhibition by either substrate or AMP, could be changed by the presence of metabolic intermediates. The effect of a large number of metabolic intermediates on the activity of FDPase was therefore investigated. The activity was measured by both the sampling technique and the continuous assay technique in the presence and absence of inhibitory concentrations of FDP and AMP. Thus conditions of both high and low extract concentrations were provided in these experiments.

The results show that none of these compounds tested modified the inhibition by either FDP or AMP, nor did they change the activity of the enzyme. Table 7 is a list of the intermediates investigated in this manner.

DISCUSSION

Several of the properties of FDPase are of relevance to the concept that this enzyme plays a key role as a pacemaker in gluconeogenesis (see Krebs, 1963, 1964; Weber, 1964; Taketa & Pogell, 1963). The K_m for FDP is very low (2·0 μM) and hence maximal activity is reached at substrate concentrations that occur physiologically in the liver. When the substrate concentration was increased above a certain optimum, inhibition of activity occurred; but even at very high concentrations the inhibition never exceeded 70%. Non-specific phosphatase activity was too low to explain this residual activity. This suggests that either the enzyme–inhibitor complex retains some activity, or a form of the

Table 7. *Intermediates of metabolism which failed to modify the activity of fructose* 1,6-*diphosphatase, or the inhibitions by adenosine monophosphate and fructose* 1,6-*diphosphate*

The extract was usually preincubated for 3 min. at room temperature with the compound under investigation and the reaction was initiated by addition of the substrate. Where necessary the pH of the intermediate was adjusted to 7·5 before use. The intermediates have been tested on (1) the activity of FDPase at concentrations of the substrate that were not inhibitory (0·1 mM), by using the continuous assay technique, (2) the activity in the presence of inhibitory concentrations of AMP (0·3 mM), by using both assay systems, and (3) the activity in the presence of inhibitory concentrations of FDP (5 mM), by using both assay systems. Experimental details are given in the text.

Additions	Concn. (mM)
DL-α-Glycerophosphate	10·0
Glucose	5·0
Phosphoenolpyruvate	2·5
6-Phosphogluconate	10·0
Pyruvate	25·0
NAD	3·0
$NADH_2$	5·0
Fumarate	5·0
Citrate	5·0
Malate	4·0
Oxaloacetate	5·0
Succinate	5·0
α-Oxoglutarate	5·0
L-Alanine	10·0
L-Aspartate	10·0
Casein digest	2·5 mg./ml.
L-Glutamate	10·0
L-Phenylalanine	1·0
Valine	10·0
Urea	10·0
Acetate	5·0
Acetoacetate	5·0
Acetyl-CoA	1·0
Butyrate	10·0
DL-β-Hydroxybutyrate	5·0
Palmitoyl-CoA	0·4

enzyme exists that is not susceptible to substrate inhibition. Evidence has been reported that suggests that liver tissue may contain more than one form of FDPase (Pogell & McGilvery, 1952; Mokrasch *et al.* 1956; Byrne, 1964; Luppis, Traniello, Wood & Pontremoli, 1964); whether one form is less susceptible to substrate inhibition has not been investigated. The lowest concentration of substrate required for inhibition was dependent on the Mg^{2+} concentration. At 5 mM-Mg^{2+} the susceptibility to excess of substrate was maximal and inhibition occurred at 75 μM-FDP. The reason for this relation between FDP inhibition and Mg^{2+} is not known. Extensive studies on the effect of pH and Mg^{2+} concentration on FDPase activity have been carried out by McGilvery (1964), who suggested that a chelation complex between FDP and Mg^{2+} might be the actual substrate for FDPase. Although no obvious connexion exists between this work and the FDP inhibition and Mg^{2+} effects, it may offer a point for further investigation.

AMP at very low concentrations is a highly specific inhibitor of FDPase. AMP has the property of an allosteric effector (Monod, Changeux & Jacob, 1963); it is not structurally similar to the substrate and the kinetics observed are characteristic of mixed inhibition. Moreover, Taketa & Pogell (1964) have reported that the FDPase molecule binds more than one molecule of AMP (probably three), and that these binding sites are all spatially separated from the catalytic site. This is strong evidence that AMP is an allosteric effector of FDPase. Fig. 3 shows that as the Mg^{2+} concentration is lowered the inhibition by AMP is increased. This may be related to the complex formed between AMP and Mg^{2+} (Walaas, 1958), which would lower the effective concentration of AMP and reduce the degree of inhibition of FDPase. To decide the degree of inhibition of FDPase inside the liver cell knowledge of both the AMP and Mg^{2+} concentrations would be required. The intracellular concentration of AMP can be calculated as 0·17 mM [from the data on ATP and ADP concentrations of Hohorst, Kreutz & Bücher (1959) and the equilibrium constant for adenylate kinase (Eggleston & Hems, 1952)], and, assuming a Mg^{2+} concentration 5–10 mM, this would be sufficient to produce partial inhibition of FDPase.

The inhibition of FDPase activity by AMP and by FDP are the two obvious properties that may afford some insight into the metabolic control of FDPase and hence of gluconeogenesis; these two agents can be described as possible regulatory effectors of FDPase. The AMP concentration of the liver cell is related to the ATP concentration through the transphosphorylation reaction catalysed by adenylate kinase, so that changes in the ATP concentration produce much larger changes in that of AMP (for a full discussion see Krebs, 1964). The inhibition of FDPase activity by AMP can be interpreted as a system for increasing the activity when the ATP concentration of the cell increases, and decreasing the activity in response to a fall in the ATP concentration. The intracellular concentration of ATP, through this mechanism, could be a factor regulating the rate of glucose formation in the liver. The inhibition by FDP suggests that lowering the intracellular concentration would lead to an increase in glucose formation; conversely, increasing the concentration would depress glucose formation. The concentration of FDP is linked to the $NADH_2$/NAD ratio via the aldolase and glycerophosphate-dehydrogenase reactions, such that an increase in this ratio would lead to a fall in the FDP concentration.

Thus this ratio could be an important controlling factor in gluconeogenesis. Evidence for this mechanism of control has been obtained with a pigeon-liver homogenate (Krebs, 1964). Another factor that could be of importance in changing the intracellular concentration of FDP is the activity of phosphofructokinase; this has been investigated by Underwood & Newsholme (1965).

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