Phosphofructokinase from the Liver Fluke *Fasciola hepatica*

I. Activation by Adenosine 3′,5′-Phosphate and by Serotonin

DEBORAH BENNETT STONE[1] AND TAG E. MANSOUR

Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California 94304

(Received October 25, 1966)

SUMMARY

Phosphofructokinase from the liver fluke *Fasciola hepatica* has been isolated in an inactive form by a procedure involving differential centrifugation. The inactive enzyme could be activated by a combination of adenosine 3′,5′-phosphate (cyclic 3′,5′-AMP) and a thermostable fraction obtained from the incubation of a heavy particulate fraction with ATP and Mg^{++}. Analysis of the thermostable fraction demonstrated that Mg^{++}, ADP, and inorganic phosphate in this fraction could account for enzyme activation in the presence of cyclic 3′,5′-AMP. Nucleoside triphosphates were found to be nearly as effective as ADP while inorganic phosphate could be replaced by sulfate anions or hexose phosphates. Activation of the fluke enzyme was found to be readily reversible by dialysis. Sucrose gradient ultracentrifugal analysis of the enzyme before and after activation gave an $S_{20,w}$ of 5.5 S for the inactive enzyme and 12.8 S for the activated enzyme, suggesting the involvement of a monomer–polymer system. Analysis of the rate of activation data suggests that the activation process behaves like a first-order reaction. Serotonin (5-hydroxytryptamine) when incubated with the heavy particulate fraction and ATP/Mg^{++} resulted in the formation of a thermostable fraction which can activate inactive phosphofructokinase in the absence of cyclic 3′,5′-AMP. The evidence indicates that serotonin activates this enzyme through the action of cyclic 3′,5′-AMP.

INTRODUCTION

It was originally reported by Cori in 1942 that phosphofructokinase plays an important role in the regulation of glycolysis (1). This finding was based on measurement of cell levels of hexose phosphates in resting frog skeletal muscle as well as after electric stimulation. Recently it was reported that glycolysis is increased in the liver fluke *Fasciola hepatica* by serotonin (5-hydroxytryptamine) (2). Evidence based on an increase in cell levels of fructose-1,6-di-P, and a decrease in the levels of the hexose monophosphates by serotonin implicated phosphofructokinase as the key enzyme responsible for the increase in the rate of glycolysis in this organism (3). More direct evidence was found when it was shown that serotonin added directly to cell-free homogenates from the flukes could activate phosphofructokinase as well as total glycolysis (3, 4). Attempts were made to clarify the mechanism of the serotonin effect. It was reported before that adenylcyclase in the liver fluke can be stimulated by serotonin, but not by catecholamines, leading to an increase in the synthesis of cyclic 3′,5′-AMP[2] (5). The cyclic nucleotide was

[1] Present address: Cardiovascular Research Institute, University of California School of Medicine, San Francisco, California 94122.

[2] The following abbreviation is used: cyclic 3′,5′-AMP, adenosine 3′,5′-phosphate.

reported to activate phosphofructokinase from the liver fluke (4). It therefore appeared likely that the effect of serotonin on liver fluke phosphofructokinase like that of catecholamines on phosphorylase kinase in mammals (6) is mediated through cyclic 3′,5′-AMP. Since the presence of serotonin in the liver fluke has been demonstrated (7, 8) it was suggested that serotonin plays a hormonal role in the regulation of glycolysis in this organism. The enzyme from the liver fluke appears to be as yet the only phosphofructokinase that can be stimulated by a hormonal agent like serotonin.

The present paper is an extension of a previous investigation to study the nature of serotonin and cyclic 3′,5′-AMP activation of the fluke phosphofructokinase (4). A method will be described to isolate the inactive enzyme. Requirements for enzyme activation and the nature of the activated enzyme will be described. It will also be reported that serotonin acts on the particulate fraction of the fluke where the adenylcyclase is located. Furthermore cyclic 3′,5′-AMP acts on a cell fraction different from that where serotonin acts. The findings indicate that the effect of serotonin is mediated through cyclic 3′,5′-AMP. A preliminary note of this investigation was reported (9).

MATERIALS AND METHODS

Materials. Fasciola hepatica were obtained from the bile ducts of infected cattle at a local slaughterhouse. The worms were transported and maintained in the laboratory as described previously (2). Bovine serum was obtained from Grand Island Biological Company. The enzymes, aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, phosphoglucose isomerase, and glucose-6-P dehydrogenase, were purchased from Boehringer Mannheim Corporation through Cal-Bio-Chem, Inc. Cyclic 3′,5′-AMP, glucose-6-P, and fructose-1,6-di-P were obtained from Sigma Chemical Company; glucose-1-P, from Nutritional Biochemical Corporation. ATP, ADP, AMP and all other nucleotides were obtained from P-L Biochemicals, Inc. All nucleotide solutions were neutralized with KOH and standardized by determining the OD_{260} of a suitable dilution. Fructose-6-P barium (Boehringer Mannheim Corporation) was converted to the dipotassium salt by passage through a Dowex 50 column and neutralization with KOH. Solutions of fructose-6-P dipotassium were standardized enzymically through the phosphoglucose isomerase and glucose-6-P dehydrogenase reactions (10).

Preparation of cell fractions from the liver fluke. Phosphofructokinase was isolated from liver fluke homogenates by a procedure involving differential centrifugation. Liver flukes, in batches of 20–25, were incubated at 37° for 1 hr under nitrogen in 50 ml of a saline medium of the following composition: NaCl, 120 mM; KCl, 4 mM; $CaCl_2$, 0.9 mM; $MgSO_4$, 1.2 mM; sodium phosphate buffer, 40 mM, pH 7.7; glucose, 5.6 mM. This step was found to be necessary for stabilization of phosphofructokinase activity. After anaerobic incubation the flukes were rinsed in distilled water, blotted on filter paper, weighed, and homogenized in an all-glass homogenizer in cold 10 mM glycylglycine buffer, pH 7.5 (1 g wet weight in 5 ml). The homogenate was centrifuged at 80,000 *g* for 30 min. The sedimented material (80-sediment) was resuspended to the original volume of the homogenate in cold glycylglycine buffer and rehomogenized. The supernatant fluid (80-supernatant) was further centrifuged at 150,000 *g* for 3 hr. The final supernatant fluid (150-supernatant) was decanted, and the small pellet of sedimented material (150-sediment) was suspended in one-tenth the original volume of buffer. All fractions were stored at −15°. The 150-sediment was found to lose little activity when kept frozen for periods of 2 months or more. Other fractions were always used within 48 hr of preparation.

A thermostable fraction was prepared from the 80-sediment by the following procedure: 0.4 ml 80-sediment was incubated at 30° for 45 min in a final volume of 1.3 ml containing potassium glycylglycine buffer, 77 mM, pH 7.5; ATP, 15 mM; and $MgCl_2$, 12 mM. The reaction was stopped by placing the reaction tube in a boiling

water bath for 3 min. The boiled incubate was then chilled on ice and centrifuged at 14,000 rpm for 15 min. The supernatant fluid isolated after centrifugation is referred to as the thermostable fraction.

Activation of 150-sediment phosphofructokinase. Phosphofructokinase from the 150-sediment was found to be essentially inactive when assayed under a variety of conditions (see Results). Because of this property, the enzyme isolated in the 150-sediment fraction will be referred to as "inactive" phosphofructokinase. The inactive enzyme can be assayed in the presence of 0.16 M ammonium sulfate. "Inactive" phosphofructokinase could be activated by incubation with the thermostable fraction and cyclic 3′,5′-AMP. Activation was carried out in a final volume of 0.10 ml containing 0.02 ml 150-sediment, 0.065 ml thermostable fraction, and 10^{-4} M cyclic 3′,5′-AMP. This activation mixture was incubated for 12 min at 30°. Enzyme which has been treated in this manner will be referred to as "activated" phosphofructokinase.

Assay of phosphofructokinase activity. Phosphofructokinase activity was determined in cell fractions of liver fluke homogenates by measurement of the rate of fructose-1,6-di-P formation. Two procedures were used. The first (assay procedure A) was used to assay enzyme activity in crude cellular fractions. Aliquots of these fractions were incubated at 30° for 30 min in a total volume of 0.5 ml solution containing, at final concentration, potassium glycylglycine buffer, 50 mM, pH 7.5; ATP, 3.2 or 10 mM; $MgCl_2$, 8 mM; and fructose-6-P, 12 mM. The phosphofructokinase reaction was started by adding 0.1 ml of the homogenate or an equivalent volume of the cell fraction containing the enzyme. The reaction was stopped by adding an equal volume of 8% perchloric acid. After centrifugation in the cold, the supernatant fluid was decanted and neutralized with 4.3 N KOH. The potassium perchlorate residue formed was separated by centrifugation in the cold and discarded. The amount of fructose-1,6-di-P in aliquots of the supernatant was determined enzymically though the aldolase-triosephosphate isomerase-glycerophosphate dehydrogenase system.

In assay procedure B, phosphofructokinase activity was measured spectrophotometrically by coupling the enzyme to aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase. This procedure was used to assay purified enzyme preparations. The reaction mixture (0.5 ml) had a final concentration of potassium glyclyglycine buffer, 50 mM, pH 7.5; fructose-6-P, 4 mM; ATP, 0.6 mM; $MgCl_2$, 0.6 mM; albumin, 0.01%; DPNH, 0.12 mM; cysteine, 14 mM; aldolase, 25 μg (0.25 unit); triosephosphate isomerase, 0.5 μg (1.0 unit); glycerophosphate dehydrogenase, 5 μg (0.18 unit); and an appropriate dilution of phosphofructokinase. Phosphofructokinase preparations were diluted in a solution that had a final concentration of Tris chloride buffer, 10 mM, pH 8.0; albumin, 0.01%; and GSH, 10 mM; with a final pH of 8.0. The reaction was started by the addition to the reaction mixture of 0.10 ml of the diluted phosphofructokinase. The rate of disappearance of DPNH was measured with an Eppendorf photometer in a light path of 1 cm at a wavelength of 334 mμ for 5 min. Blank values for DPNH oxidation in the absence of fructose-6-P were routinely determined. The blank values rarely exceeded 0.010–0.015 optical density units; higher blank values for DPNH oxidation were obtained only in the presence of extremely high ATP levels (10 mM). All enzyme activities were determined after subtracting the blank values. A unit of phosphofructokinase is the amount of enzyme that catalyzes the formation of 1 μmole of fructose-1,6-di-P per minute.

Other methods. AMP, ADP, and ATP were measured enzymically with test combinations obtained from Boehringer Mannheim Corporation. ATP was assayed with the phosphoglycerate kinase–glyceraldehyde-3-phosphate dehydrogenase system (10). ADP and AMP (following conversion to ADP by myokinase) were assayed by the pyruvate kinase–lactic dehydrogenase system (10). Inorganic phosphate was measured by the procedure of Fiske and

SubbaRow (11). Proteins were determined by the method of Lowry *et al.* (12). Sucrose gradient centrifugation was carried out by the method of Martin and Ames (13) as modified before for heart phosphofructokinase (14).

RESULTS

Phosphofructokinase in Different Cell Fractions from the Liver Fluke

Phosphofructokinase in homogenates from the liver fluke was found to be mostly inactive when assayed in the presence of concentrations of fructose-6-P as high as 10 mM and under conditions described above under Materials and Methods, assay procedure A. The inactivity of the enzyme was not due to inhibition by ATP or Mg^{++} during assay. This was indicated by the fact that a change in the concentration of ATP from 2 to 10 mM or the Mg^{++} from 1 to 10 mM did not significantly affect enzyme activity.

The effect of several agents which were previously reported to activate phosphofructokinase was tested on the homogenate. These included serotonin (3, 4), cyclic 3′,5′-AMP (3, 4), and ammonium sulfate (14, 15). As reported before, 10^{-4} M serotonin or 10^{-4} M cyclic 3′,5′-AMP caused a marked activation of the enzyme in the fluke homogenate (4). Furthermore, ammonium sulfate at a concentration of 0.16 M caused a greater increase in enzyme activity. Maximum stimulation[3] of enzyme activity by ammonium sulfate was observed only at concentrations as high as 0.16 M.

Attempts were made to isolate the enzyme in a partially purified form. The fact that ammonium sulfate added to the reaction mixture can activate the enzyme was used as a means to assay for the inactive enzyme in different cell fractions. This distribution of phosphofructokinase in different cell fractions after differential centrifugation is summarized in Table 1. Following centrifugation at 80,000 *g* for 30 min, phosphofructokinase was found predominantly in the supernatant fluid (80-supernatant). Further centrifugation of the 80-supernatant at 150,000 *g* for 3 hr resulted in appearance of the enzyme activity in the sedimented material (150-sediment). The 150-sediment had a specific activity of 142 units per gram of protein and contained 93% of the original homogenate activity. This represented a 9-fold increase in the specific activity. Both the final supernatant fraction (150-supernatant) and the 80-sediment contained insignificant phosphofructokinase activity.

TABLE 1

Phosphofructokinase activity in cell fractions of the liver fluke

Cell fractions were isolated from a batch of 55 liver flukes (3.11 g) as described under Materials and Methods. Aliquots of the different fractions were assayed for phosphofructokinase activity by assay procedure A (see Materials and Methods) in the presence of 3.2 mM ATP and 0.16 M $(NH_4)_2SO_4$.

Cell fraction	Units		Protein (mg/ml)	Specific activity (units/g protein)	Recovery (%)
	Per ml	Total			
Homogenate	0.35	5.22	21.6	16	100
Sediment, 80,000 *g*	0.07	1.11	14.7	5	21
Supernatant, 80,000 *g*	0.48	5.80	10.6	46	111
Sediment, 150,000 *g*	4.03	4.83	28.3	142	93
Supernatant, 150,000 *g*	0.03	0.42	8.0	4	8

[3] The term "stimulation" will be used to indicate an increase in enzyme activity by an agent during measurement of catalytic activity of the enzyme.

Activation of Phosphofructokinase in the 150-Sediment

Phosphofructokinase in the 150-sediment like that in the original homogenate was found to be inactive. Varying the pH of the assay reaction mixture from 6.7 to 8.5 caused no change in the activity of the "inactive" enzyme. Furthermore, no change in activity over an ATP range of 0.02 to 10 mM in the presence of 4 mM fructose-6-P was observed. Experiments were carried out to compare the effect of different activators on the 150-sediment enzyme with that in the homogenate (Table 2). In these experiments fluke homogenate had low enzyme activity which was stimulated 4–5 times by serotonin or cyclic 3′,5′-AMP. In contrast, the 150-sediment showed no activity when assayed alone or in the presence of these two activators. The absence of activating action by serotonin or by cyclic 3′,5′-AMP on 150-sediment phosphofructokinase suggested that a cellular component which was removed during differential centrifugation was essential for enzyme activation. It was later found that enzyme activation by these two agents could be restored when the 80-sediment was combined with the 150-sediment. Under these conditions a small amount of enzyme activity was observed even in the absence of any activator (Table 2). This low activity could be further stimulated by either serotonin or cyclic 3′,5′-AMP. Phosphofructokinase activity in the 80-sediment alone was negligible and could not be significantly stimulated by either of the activators. The stimulatory action of ammonium sulfate on the 150-sediment enzyme is shown in Table 2 for comparison.

The results reported above indicate that the 80-sediment contains a factor or factors essential for activation of 150-sediment phosphofructokinase by serotonin or cyclic 3′,5′-AMP. This conclusion was not surprising in the case of serotonin since results from previous work suggested that the effect of serotonin could be mediated through its effect in increasing the synthesis of cyclic 3′,5′-AMP by the heavy particulate fraction (5). This point will be dealt with in greater detail below. The participation of the 80-sediment in the activation effect of cyclic 3′,5′-AMP was further investigated.

TABLE 2

Effect of serotonin, cyclic 3′,5′-AMP, and $(NH_4)_2SO_4$ on phosphofructokinase in different cell fractions

Fractions were assayed for phosphofructokinase activity by assay procedure A (see Materials and Methods) in the presence of 10 mM ATP and the indicated additions. Phosphofructokinase activity of the cell fractions is expressed as units per gram of fluke (wet weight).

Additions to assay	Cell fraction			
	Homogenate	150-Sediment	150-Sediment + 80-sediment	80-Sediment
None	0.38	0	0.63	0.11
Serotonin, 10^{-4} M	1.53	0	1.69	0.15
Cyclic 3′,5′-AMP, 10^{-4} M	1.91	0	1.87	0.20
$(NH_4)_2SO_4$, 0.16 M	4.08	3.01	—[a]	—[a]

[a] Phosphofructokinase was not determined.

Separation of the Processes of Enzyme Activation and Assay

Attempts were made to investigate whether the increase in liver fluke phosphofructokinase observed when cyclic 3′,5′-AMP was added to the reaction mixture was due to a true conversion of the inactive form of the enzyme to an active form (i.e., direct activation) or whether it was due to a kinetic effect on the enzyme dur-

ing catalysis of the phosphofructokinase reaction. In order to avoid misinterpretation, the term "activation" will be used here to indicate an increase in enzyme activity following preincubation of the concentrated inactive enzyme with various components. Under these conditions a direct effect of the activating components on the enzyme during assay was always excluded since the enzyme was assayed after several hundredfold dilution. These components did not affect the enzyme in the assay reaction mixture after such dilution. Thus an effect of the concentrations of cyclic 3′,5′-AMP carried over with the enzyme in the assay mixture was excluded.

Preliminary experiments indicated that preincubation of the inactive enzyme (150-sediment) with 80-sediment, ATP/Mg^{++} and cyclic 3′,5′-AMP for 30 min resulted in enzyme activation. When either the 80-sediment or the ATP/Mg^{++} was omitted no enzyme activation was observed. The participation of the 80-sediment with ATP/Mg^{++} as activating components suggested the formation of a factor or factors which are essential for enzyme activation. Indeed, it was later found that preincubation of 80-sediment with ATP/Mg^{++} for about 45 min yielded a supernatant fluid which with cyclic 3′,5′-AMP could activate the enzyme. This supernatant fluid was found to retain its full activity in activating the enzyme with cyclic 3′,5′-AMP after boiling for 3 min. This boiled supernatant fluid isolated after incubation of the 80-sediment with ATP/Mg^{++} will be referred to as the "thermostable fraction." Details for the isolation of this fraction are described under Materials and Methods.

Based on the above experiments a sequence of three steps was carried out for activation and assay of fluke phosphofructokinase. In *step I*, the 80-sediment was incubated with ATP and Mg^{++} to produce the thermostable fraction. The maximal time of incubation for formation of the thermostable fraction was found to be 45 min. In *step II*, the 150-sediment containing concentrated, inactive phosphofructokinase was activated by incubation with the thermostable fraction and cyclic 3′,5′-AMP. After activation, the enzyme was diluted and assayed spectrophotometrically (*step III*). Further experimental details of the system are given under Materials and Methods.

Results of a typical experiment are shown in Table 3. Prior to activation the phosphofructokinase activity of the 150-sediment was 0.36 unit/ml. Incubation with glycylglycine buffer alone, glycylglycine and 10^{-4} M cyclic 3′,5′-AMP or thermostable fraction alone produced no significant activation. However, when the incubation was carried out with both the thermostable fraction and cyclic 3′,5′-AMP, a 6-fold increase in phosphofructokinase activity resulted. The activation components were diluted 200-fold in the final spectrophotometric assay. At such dilution, cyclic 3′,5′-AMP and the thermostable fraction had no effect on assay levels of the enzyme. This is demonstrated by the finding that when the inactive enzyme from the 150-sediment fraction was diluted and assayed in the presence of a 200-fold dilution of thermostable fraction and cyclic

TABLE 3

Activation of Phosphofructokinase

Phosphofructokinase was activated by incubation of 0.02 ml 150-sediment with the indicated components in a final volume of 0.10 ml for 12 min at 30°. After incubation the mixture was diluted 200-fold and assayed by assay procedure B, as described under Materials and Methods. The thermostable fraction was prepared by incubation of the 80-sediment with ATP and Mg^{++} (see Materials and Methods). Prior to activation the enzyme had an activity of 0.36 units/ml 150-sediment.

Activation components	Phosphofructokinase activity (units/ml 150-sediment)
Glycylglycine buffer, 50 mM, pH 7.5	0.31
Glycylglycine + cyclic 3′,5′-AMP, 10^{-4} M	0.23
Thermostable fraction, 0.065 ml	0.41
Thermostable fraction + cyclic 3′,5′-AMP	2.15

3′,5′-AMP an activity of only 0.38 units/ml resulted.

The relationship between the concentration of cyclic 3′,5′-AMP and its effect in activating phosphofructokinase is summarized in Fig. 1. As little as 10^{-6} M cyclic 3′,5′-AMP caused some enzyme activation

Properties of the Thermostable Fraction

The nature of the factor(s) isolated in the thermostable fraction (see above, step I) which was essential for phosphofructokinase activation was investigated. Acidification of the fraction with 4% perchloric acid followed by neutralization with KOH

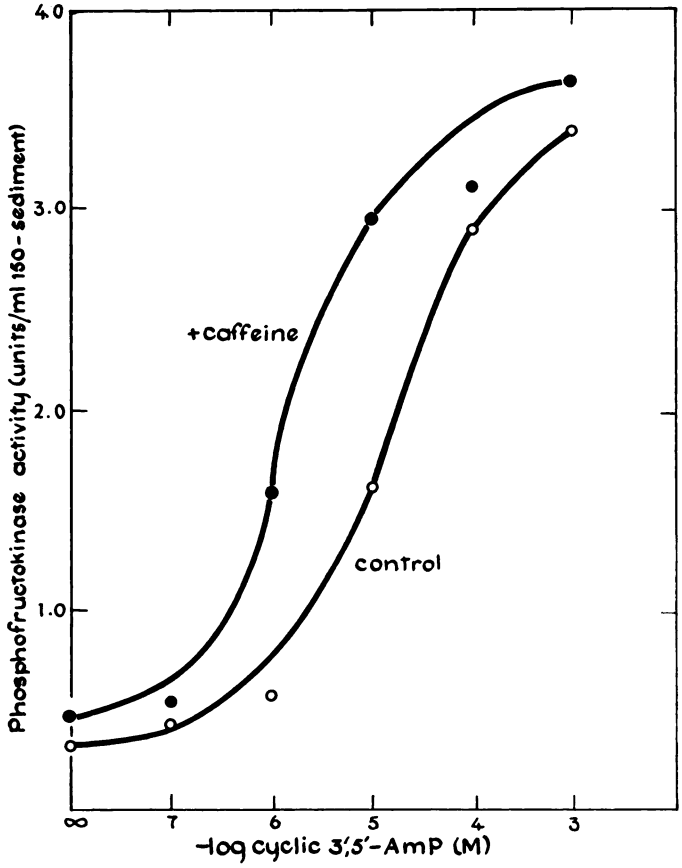

FIG. 1. *Effect of cyclic 3′,5′-AMP concentration on phosphofructokinase activation in the presence and absence of caffeine*

Activation mixtures containing 0.02 ml 150-sediment, 0.065 ml thermostable fraction, and from 0 to 10^{-3} M cyclic 3′,5′-AMP in a final volume of 0.10 ml were incubated for 12 min at 30°. The activated enzyme was diluted 200-fold and assayed by assay procedure B as described under Materials and Methods. Activation was carried out in the presence and absence of 13 mM caffeine.

in the absence of caffeine. The same concentration of cyclic 3′,5′-AMP caused a greater degree of activation when tested in the presence of caffeine, an inhibitor of cyclic 3′,5′-AMP phosphodiesterase (16). This suggests that there is diesterase activity in the 150-sediment.

caused no change in the ability of the thermostable fraction to activate phosphofructokinase in the presence of cyclic 3′,5′-AMP. Dialysis of the fraction against 10 mM glycylglycine buffer, pH 7.5, for 3 hr resulted in a 60% loss in this activity indicating that the effect is due to a dialyzable

TABLE 4

Adenine nucleotide and inorganic phosphate content of the thermostable fraction

Thermostable fractions were prepared from four separate 80-sediment preparations by the method described under Materials and Methods. Fractions were assayed for AMP, ADP, ATP, and inorganic phosphate by methods described under Materials and Methods. The total adenine nucleotide content was calculated from the OD_{260} of a suitable dilution at neutral pH (E_{max} = 15,400). Results are expressed as micromoles per milliliter of thermostable fraction.

Component	Experiment number				Average
	I	II	III	IV	
AMP	2.58	3.59	4.31	2.65	3.28
ADP	5.40	4.59	5.64	5.83	5.37
ATP	5.53	5.85	4.34	6.44	5.54
AMP + ADP + ATP	13.5	14.0	14.3	14.9	14.2
Total nucleotide content (OD_{260})	13.1	13.6	13.1	14.8	13.7
Inorganic phosphate	9.6	11.6	12.8	8.4	10.6

fraction. Treatment of the thermostable fraction with Dowex 50 resulted in 90% loss of activity; full activity was restored upon addition of $MgCl_2$. Treatment of the thermostable fraction with Dowex 1 also resulted in complete loss of its effect in activating phosphofructokinase. The effect of the Dowex 1-treated thermostable fraction was not restored by the addition of ATP. These results indicate that the active factor(s) of the thermostable fraction was an anionic compound of small molecular weight and that Mg^{++} was essential for activation.

Since ATP was essential for production of the thermostable fraction, but was unable to replace the factor(s) removed by Dowex 1, it seemed possible that one or more metabolites of ATP might be the active factor(s) of the thermostable fraction. An analysis of four separate thermostable fractions for ATP, ADP, and AMP was carried out and is summarized in Table 4. The thermostable fraction was found to contain all three adenine nucleotides in concentrations of approximately 3–6 mM. In each case the total amount of AMP, ADP, and ATP determined by enzymic assay could account for the total adenine nucleotide content calculated from the absorbancy of the solutions at 260 mμ. Fractionation of the thermostable fraction on paper and thin layer chromatography and on DEAE-Sephadex failed to reveal more than the three UV-absorbing components. Furthermore, the inorganic phosphate content of the fractions accounted for nearly all the phosphate released from ATP by

TABLE 5

Requirements for activation of liver fluke phosphofructokinase

The complete system for enzyme activation contained potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; AMP, 2.1 mM; ADP, 3.5 mM; ATP, 3.6 mM; KH_2PO_4, 6.9 mM; cyclic 3′,5′-AMP, 10^{-4} M; and 0.02 ml 150-sediment in a final volume of 0.10 ml. After incubation for 12 min at 30°, the activation mixture was diluted 200-fold and assayed by assay procedure B as described under Materials and Methods. Prior to activation the enzyme had an activity of 0.31 unit per milliliter of 150-sediment.

Component(s) omitted	Phosphofructokinase activity (units/ml 150-sediment)
None	2.57
Cyclic 3′,5′-AMP	0.31
$MgCl_2$	0.26
AMP, ADP, ATP	0.43
KH_2PO_4	1.21
AMP	2.48
ADP	2.38
ATP	2.07
AMP, ADP	0.82
AMP, ATP	2.53
ADP, ATP	0.49
All except buffer	0.23

the formation of ADP and AMP (Table 4).

A mixture containing ATP, ADP, AMP, inorganic phosphate and Mg^{++} in the same proportions found in the thermostable fraction was tested for its effect in activating phosphofructokinase in the presence of cyclic 3′,5′-AMP in step II (see above). The nucleotide-inorganic phosphate-Mg^{++} mixture was found to activate phosphofructokinase to the same extent as the thermostable fractions when combined with cyclic 3′,5′-AMP (Table 5).

Requirements for Activation of Liver Fluke Phosphofructokinase

The above results indicated that a mixture of ATP, ADP, AMP, inorganic phosphate, and Mg^{++} can replace the thermostable fraction in activating phosphofructokinase. The effect of omission of different components of this mixture on activation of phosphofructokinase was tested. The data are summarized in Table 5. Prior to activation the enzyme had an activity of 0.31 unit/ml 150-sediment. Incubation with the nucleotide-phosphate-Mg^{++} mixture and cyclic 3′,5′-AMP according to the procedure in step II (see above) resulted in an 8-fold increase in enzyme activity. Omission of cyclic 3′,5′-AMP, $MgCl_2$, or all three nucleotides (AMP, ADP, ATP) resulted in no activation. Activity was reduced by about half in the absence of inorganic phosphate. When the three nucleotides were omitted one at a time, little reduction in activity occurred. However, when the nucleotides were omitted two at a time, significant activation was obtained only when ADP remained (AMP and ATP omitted). Under these conditions activation was equivalent to that obtained with the full system. This indicates that maximal activation requires Mg^{++}, ADP, inorganic phosphate, and cyclic 3′,5′-AMP. However, ADP can be replaced with a combination of AMP and ATP with little loss in activity.

The nucleotide specificity for phosphofructokinase activation in the presence of cyclic 3′,5′-AMP (step II) was studied in a system containing glycylglycine buffer, $MgCl_2$, KH_2PO_4, and cyclic 3′,5′-AMP. Nucleotides were added to give a final concentration of 3.5 mM in the activation mixture. The experimental conditions were similar to those given in Table 5. Of the compounds tested, only ADP gave consistently full activation of the enzyme. The presence of ATP alone gave a variable effect which ranged from nearly no activation to full activation. Four other nucleoside triphosphates (ITP, UTP, CTP, GTP) were approximately one-half as active as ADP in activating phosphofructokinase. Other nucleotides which were tested and found to show no effect on the activation system included AMP, adenosine, IDP, IMP, inosine, GDP, UDP, and CDP.

TABLE 6

Effect of hexose phosphates on activation of phosphofructokinase

Activation was carried out in a final volume of 0.10 ml containing potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; cyclic 3′,5′-AMP, 10^{-3} M; caffeine, 13 mM; 0.02 ml 150-sediment; and the indicated additions. After incubation for 12 min at 30°, the activation mixture was diluted 200-fold and assayed by assay procedure B as described under Materials and Methods.

Additions	Phosphofructokinase activity (units/ml 150-sediment)
None	0.13
ADP, 3.5 mM	0.98
KH_2PO_4, 6.9 mM	0.30
ADP + KH_2PO_4	1.57
Fructose-1,6-di-P, 1 mM	0.31
ADP + fructose-1,6-di-P	1.49
Fructose-6-P, 1 mM	0.31
ADP + fructose-6-P	1.66
Glucose-6-P, 1 mM	0.21
ADP + glucose-6-P	1.36
Glucose-1-P, 1 mM	0.31
ADP + glucose-1-P	1.44

The specificity of the inorganic phosphate requirement for phosphofructokinase activation was studied. It was found that sodium sulfate in concentrations of approximately 10 mM could substitute for KH_2PO_4. Several organic phosphate compounds were also found to affect the activation of phosphofructokinase. When

tested at a concentration of 1 mM, fructose-1,6-di-P, fructose-6-P, glucose-6-P, and glucose-1-P all had approximately the same activity as 6.9 mM inorganic phosphate (Table 6). None of the hexose phosphates tested had any activity in the absence of ADP.

Effect of Sulfate Ions on Assay Levels of Phosphofructokinase

Results summarized above (see Table 2) as well as the work of others (14, 15) indicated that $(NH_4)_2SO_4$ can stimulate phosphofructokinase activity when added directly to the assay reaction mixture in high concentrations. That the stimulatory effect was due to the sulfate anion rather than to the ammonium cation was indicated by the fact that sodium sulfate was just as effective as ammonium sulfate. On the other hand both NH_4Cl and NaCl were inactive (Table 7). In other experiments it was found that phosphate anion in the form of potassium phosphate had an effect similar to the sulfate salts when added directly to the assay mixture in a concentration of 0.1 M. These experiments indicate that a polyvalent anion could increase enzyme activity when added to the phosphofructokinase reaction mixture.

TABLE 7

Effect of several salts on phosphofructokinase activity

The 150-sediment was diluted 1000-fold and assayed for phosphofructokinase activity by assay procedure B (see Materials and Methods). Salts were added where indicated to the assay reaction mixture in a final concentration of 0.1 M. Under these conditions maximal enzyme activity was reached approximately 5 min after addition of the enzyme to the reaction mixture. Enzyme activity was calculated from the change in optical density occurring between 5 and 10 min.

Additions	Phosphofructokinase activity (units/ml 150-sediment)
None	0.20
Na_2SO_4	2.13
$(NH_4)_2SO_4$	2.53
NH_4Cl	0.43
NaCl	0.28

Effect of Serotonin on the Thermostable Fraction

The effect of serotonin on the different steps of enzyme activation and assay was tested. Addition of serotonin during activation (step II) or during spectrophotometric assay (step III) had no effect on the enzyme activity. This was true both in the presence and absence of cyclic 3′,5′-AMP in the activation mixture. Since earlier work demonstrated that serotonin increases the production of cyclic 3′,5′-AMP from ATP and Mg^{++} in particulate fractions of the fluke (5), it seemed probable that addition of serotonin to step I which included incubation of the heavy particles with ATP and Mg^{++} would lead to the formation of cyclic 3′,5′-AMP in the thermostable fraction. Under these conditions, some activation should occur in the absence of added cyclic 3′,5′-AMP in the step II incubation. Table 8 demonstrates that when serotonin in the presence of caffeine was added at step I, the thermostable fraction prepared after such incubation was found to activate phosphofructokinase in the absence of added cyclic 3′,5′-AMP. The presence of caffeine was found to be essential for the activating effect of serotonin. Caffeine alone had no effect. The activation produced by thermostable fractions prepared in the presence of 10^{-4} M serotonin and 13 mM caffeine was equivalent to that of control thermostable fractions combined with 10^{-5} M cyclic 3′,5′-AMP. Significant phosphofructokinase activation was observed with thermostable fractions which had been perpared in the presence of 13 mM caffeine and as little as 10^{-6} M serotonin.

Several compounds related to serotonin were studied for their effect on the step I incubation. Thermostable fractions prepared in the presence of epinephrine or norepinephrine at concentrations of 10^{-4} M (and 13 mM caffeine) were ineffective in activating the 150-sediment phosphofructokinase (Table 8). Other compounds which were studied and found to be ineffective in concentrations of 10^{-4} M included L-5-hydroxytryptophan, 5-hydroxyindole acetic

TABLE 8

Effect of serotonin and related compounds on the thermostable fraction

Thermostable fractions were prepared by incubation of the 80-sediment with ATP and Mg^{++} as described under Materials and Methods. Where indicated, 13 mM caffeine and 10^{-4} M serotonin or other test compounds were added to the step I incubation (see text). Thermostable fractions were used for enzyme activation in a system containing 0.02 ml 150-sediment and 0.065 ml thermostable fraction in a final volume of 0.10 ml. Cyclic 3′,5′-AMP, in a final concentration of 10^{-4} M, was included in the activation mixture (step II) where indicated. After incubation for 12 min at 30°, the activation mixture was diluted 200-fold and assayed for phosphofructokinase activity by assay procedure B as described under Materials and Methods. Results are expressed as units of phosphofructokinase activity/ml of 150-sediment.

Expt. No.	Additions to step I		Additions to step II	
	Test compound, 10^{-4} M	Caffeine, 13 mM	None	C-AMP, 10^{-4} M
I	None	−	0.34	2.94
	None	+	0.20	2.82
	Serotonin	−	0.44	2.85
	Serotonin	+	1.21	3.25
II	None	+	0.54	3.23
	Serotonin	+	1.61	2.76
	Epinephrine	+	0.39	2.62
	Norepinephrine	+	0.43	2.94

acid, *d*-lysergic acid diethylamine, and *d*-amphetamine.

Nature of Phosphofructokinase Activation

From the above results it appeared that activation by the thermostable fraction and cyclic 3′,5′-AMP was the only possible physiological mechanism for increasing the activity of the 150-sediment phosphofructokinase. Several experiments were carried out in an attempt to understand the nature of this activation process. Initial experiments demonstrated that the activation process was both time and temperature dependent. Incubation of the 150-sediment with the thermostable fraction and cyclic 3′,5′-AMP for 12 min at 0° resulted in only a 2-fold increase in phosphofructokinase activity (Fig. 2). With increasing temperature of incubation, the rate of activation increased. At 30°, the incubation temperature routinely used in the experiments reported in this paper, the activation process was nearly complete after 4 min of incubation. A 12-min incubation time was routinely employed to assure complete activation of the 150-sediment enzyme.

Since the activation process was found to be time dependent, an attempt was made to determine the order of the reaction from the rate of activation data. In order to slow the rate of activation, incubation was carried out at 10° in these experiments. For a first-order reaction a plot of log of the concentration of the inactive enzyme against time should be linear. This could be expressed in the form of the following integrated equation:

$$\log (V_{max} - V_t) = -\frac{k}{2.303} t + \text{constant}$$

where the concentration of the inactive enzyme is the maximum velocity (V_{max}) minus the velocity at time t (V_t). If the reaction is second order a plot of $1/(V_{max} - V_t)$ against time should be linear according to the following equation:

$$\frac{1}{V_{max} - V_t} = kt + \text{constant}$$

A typical plot of phosphofructokinase activity *vs.* time of activation at 10° is given in Fig. 3a. In Fig. 3b, the same data are plotted according to the rate equations for first (left ordinate) and second (right ordinate) order reactions. Only the first-order plot yielded a straight line indicating that the rate of the activation reac-

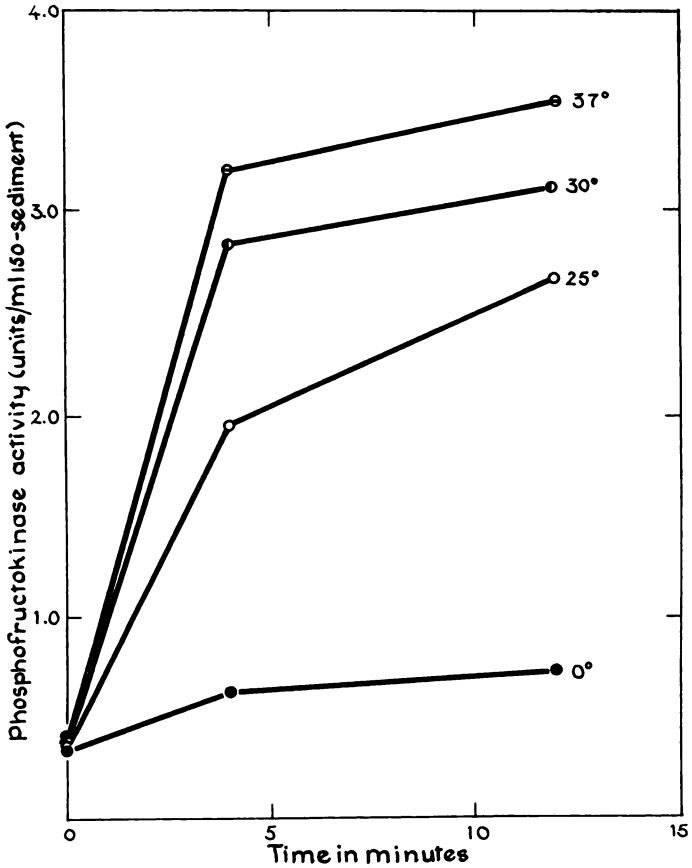

FIG. 2. *The effect of temperature on phosphofructokinase activation*

The activation mixtures contained 0.02 ml 150-sediment, 0.065 ml thermostable fraction, and 10^{-4} M cyclic 3',5'-AMP in a final volume of 0.10 ml. Activation was carried out for periods of 4 and 12 min at temperatures of 0°, 25°, 30°, and 37°. After incubation, the activation mixtures were diluted 200-fold and assayed for phosphofructokinase activity by assay procedure B (see Materials and Methods).

tion is directly proportional to the first power of the concentration of the inactive enzyme. A first-order rate constant of 0.18 min^{-1} was calculated from the slope of the first-order rate curve.

Since activation of phosphofructokinase from other tissues appears to involve a monomer–polymer type conversion (14), experiments were carried out to determine the sedimentation behavior of the liver fluke enzyme before and after activation. The sucrose gradient centrifugation method of Martin and Ames (13) was used in these experiments. Inactive, as well as activated, enzyme was detected in the gradient samples by assaying in the presence of high concentrations of fructose-6-P (20 mM). Under these assay conditions the inactive enzyme had approximately one-third of the activity of the enzyme after full activation. A total of 147 milliunits of inactive enzyme and 417 milliunits of activated enzyme were applied to separate gradients. Following centrifugation for 4.5 hr at 39,000 rpm the two forms of the enzyme had markedly different sedimentation patterns (Fig. 4). The inactive enzyme had an $S_{20,w}$ of 5.5 S while the peak for the activated enzyme occurred at 12.8 S. Recoveries for the inactive and activated forms of the enzyme were 52% and 114%, respectively.

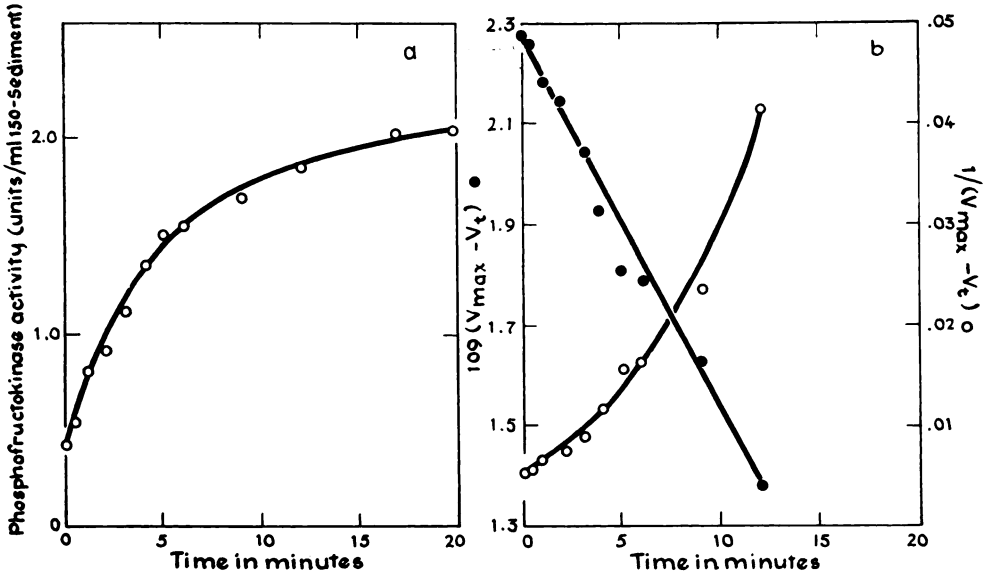

FIG. 3. *Phosphofructokinase activation as a function of time*

Activation mixtures containing 0.02 ml 150-sediment, 0.065 ml thermostable fraction, 10^{-3} M cyclic 3',5'-AMP, and 13 mM caffeine in a final volume of 0.10 ml were incubated at 10°. At various times of incubation aliquots of the activation mixture were diluted 100-fold and assayed by assay procedure B as described under Materials and Methods. Phosphofructokinase activity is plotted as a function of time of incubation in (a). Functions of the concentration of inactive enzyme (V_{max}-V_t) are plotted against the time of incubation in (b). Functions plotted are log (V_{max}-V_t) for a first-order reaction (*left ordinate*, ●) and $1/(V_{max}$-$V_t)$ for a second-order reaction (*right ordinate*, ○).

TABLE 9

Reversible inactivation of liver fluke phosphofructokinase by dialysis

Phosphofructokinase activation was carried out in a final volume of 0.60 ml containing potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; ADP, 3.5 mM; KH_2PO_4, 6.9 mM; cyclic 3',5'-AMP, 10^{-4} M; caffeine, 13 mM; and 0.24 ml 150-sediment. After incubation at 30° for 12 min, the mixture was centrifuged in the cold for 10 min at 4000 *g* to remove a slight precipitate which formed during activation. This precipitate had no enzyme activity. Of the activated enzyme, 0.5 ml was dialyzed for 2 hr against 50 ml of a solution containing potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; and caffeine, 13 mM. The dialyzed enzyme was reactivated by incubation with the original activation components for 12 min at 30°. The complete reactivation system contained potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; ADP, 3.5 mM; KH_2PO_4, 6.9 mM; cyclic 3',5'-AMP, 10^{-4} M; caffeine, 13 mM; and 0.05 ml dialyzed enzyme in a final volume of 0.10 ml. Aliquots of the enzyme at each stage were diluted and assayed by assay procedure B as described under Materials and Methods.

Condition	Milliunits per ml	Protein (mg/ml)	Specific activity (units/g protein)
1. Before activation	180	27.8	6
2. After activation (supernatant)	872	9.85	89
3. After dialysis for 2 hr	125	9.35	13
4. Reactivation with complete system	282	4.58	62
5. Reactivation without cyclic 3',5'-AMP	10	4.58	2
6. Reactivation without KH_2PO_4	72	4.58	16
7. Reactivation without ADP	13	4.58	3

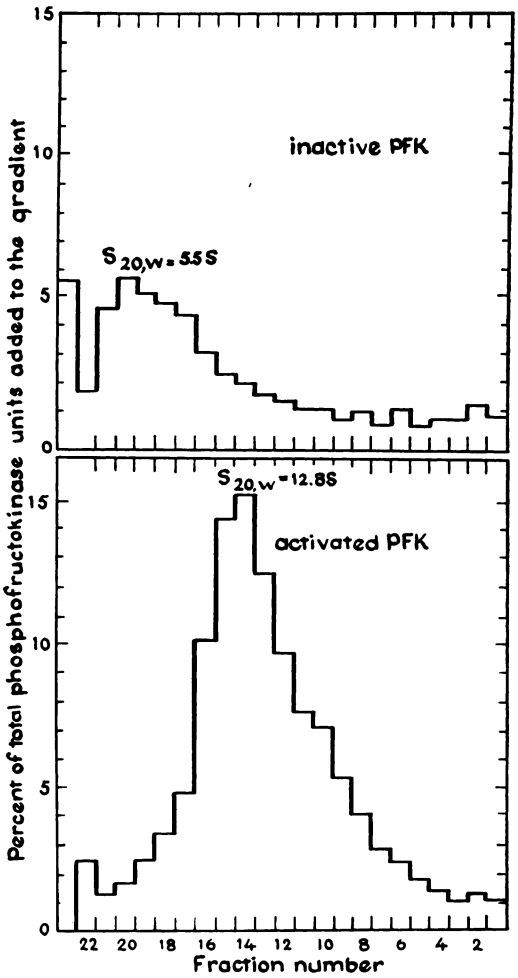

FIG. 4. *Distribution of inactive and activated phosphofructokinase (PFK) after ultracentrifugation in sucrose gradients*

The *inactive* enzyme was prepared by diluting 0.15 ml of 150-sediment in a final volume of 0.30 ml containing potassium glycylglycine buffer, 50 mM, pH 7.5, and GSH, 10 mM. The *activated* enzyme was prepared by incubation (12 min at 30°) of 0.15 ml 150-sediment in a final volume of 0.30 ml containing potassium glycylglycine buffer, 50 mM, pH 7.5; $MgCl_2$, 8 mM; ADP, 3.5 mM; KH_2PO_4, 6.9 mM; cyclic 3',5'-AMP, 10^{-4} M; and GSH, 10 mM. Gradients were prepared from 10% and 3% sucrose solutions containing all components of the enzyme mixtures. Of the enzyme mixtures, 0.2 ml amounts (approximately 3 mg protein) were added to separate gradients. The gradients were spun in an SW 50L rotor of a Model L Spinco preparative ultracentrifuge for 4.5 hr at 39,000 rpm at a rotor temperature of approximately 0–2°. After centrifugation the contents of each tube were collected in 23 fractions of approximately 0.2 ml. Aliquots of the fractions were assayed for phosphofructokinase activity by assay procedure B as described under Materials and Methods. A fructose-6-P concentration of 20 mM was used in these assays. Under these assay conditions, the enzyme aliquots which were applied to the gradients contained 147 milliunits for the inactive enzyme and 417 milliunits for the activated enzyme. Recoveries for the inactive and activated forms of the enzyme were 52 and 114%, respectively. The enzyme activity recovered in each fraction is expressed as a percentage of the total units added to the respective gradients.

Finally, experiments were carried out to determine whether the activation process was reversible or irreversible. It was found that the activated enzyme could be readily inactivated by dialysis. The dialyzed enzyme could then be reactivated by incubation with the original activation components. The results of a typical experiment for inactivation and reactivation are given in Table 9. The specific activity of the enzyme increased from 6 to 89 units/g protein following activation. Dialysis for 2 hours in 100 volumes of solution reduced the activity to 13 units/g protein. Reactivation of the dialyzed enzyme by incubation with the activation components increased the activity to 62 units/g protein or to approximately 70% of the activity before dialysis. All three components not included in the dialysis fluid (ADP, KH_2PO_4, and cyclic 3',5'-AMP) were necessary for reactivation of the enzyme. This indicates that all three activation components could be reversibly dissociated from the enzyme.

DISCUSSION

Fluke phosphofructokinase appears to be invested with a complex system for activation which is dependent on many factors. The above results showed that conversion of the enzyme from the inactive to the active form is dependent on incubation at 30° with Mg^{++}, a nucleotide (ADP or a nucleoside triphosphate), a polyvalent anion such as inorganic phosphate or hexose phosphate,

and cyclic 3′,5′-AMP. An absolute requirement for the cyclic nucleotide was demonstrated since no other nucleotide could replace it. The specificity of cyclic 3′,5′-AMP in activating liver fluke phosphofructokinase is different from the action of the cyclic nucleotide in relieving ATP-inhibition of the enzyme. In the latter action 5-AMP as well as cyclic 3′,5′-AMP can activate the ATP-inhibited enzyme (17). The participation of another enzyme during the process of phosphofructokinase activation cannot be excluded since the enzyme isolated was not entirely pure. The multivalent nature of enzyme activation here indicates that phosphofructokinase activation in the liver fluke is dependent not only on an increase in the level of cyclic 3′,5′-AMP in the cell but also requires the participation of other adenylic nucleotides and a polyvalent anion. A multivalent system for activation appears also to be important in the case of the mammalian phosphofructokinase (14). It was reported before that the inactive mammalian enzyme is activated better by combinations of a nucleotide and a hexose phosphate than by either nucleotide or hexose phosphate alone. The physiological significance of such a multivalent system for activation of the fluke enzyme is as yet not determined. One can only speculate here that the process of activation of the enzyme is geared to changes in more than one substrate and therefore is influenced by changes in the activity of several other enzymes. Evidence was reported recently that activation of phosphorylase precedes that of phosphofructokinase in the frog skeletal muscle (18).

The above results demonstrate that serotonin by itself cannot activate phosphofructokinase in the absence of the particulate fraction. The same fraction was shown before to contain the system for cyclizing ATP to cyclic 3′,5′-AMP (5). Furthermore serotonin caused a marked increase in the synthesis of the nucleotide by the cyclase. The half-maximal concentration for serotonin was 10^{-6} M. In their survey on the relative activity of adenylcyclases in various animals and animal tissues Sutherland *et al.* (19) found that the liver fluke and the brain cortex had the highest activity. It appears then that the fluke contains an active system for the production of large amounts of the cyclic nucleotide. The fact reported above that in order to show an effect by serotonin it was found essential to inhibit the phosphodiesterase by caffeine adds more support to the idea that it is cyclic 3′,5′-AMP which mediates the action of serotonin. Since both serotonin and its synthesizing system have been shown to be present in the fluke (7, 8), it is tempting to speculate here that the effect of serotonin on fluke phosphofructokinase is part of hormonal control by the indolalkylamine on glycolysis. This would be similar to the action of epinephrine on glycogen phosphorylase in higher organisms.

It was previously reported that phosphofructokinase from guinea pig heart undergoes reversible dissociation from an active to an inactive form (14). Experiments reported above suggest that activation of liver fluke phosphofructokinase may involve a similar mechanism. Sucrose gradient ultracentrifugal analysis of the enzyme before and after activation gave an $S_{20,w}$ of 5.5 S for the inactive enzyme and 12.8 S for the activated enzyme. These results are consistent with a monomer–polymer system involving 3 or 4 subunits. These findings when added to the data presented in the next paper (17), where the kinetics of the activated enzyme are described, fulfills one of the main properties for an allosteric enzyme as reported by Monod *et al.* (20).

ACKNOWLEDGMENTS

This work was supported by Research Grant AI04214 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service; and a Research Career Development Award GM-K3-3848 from the Division of General Medical Sciences, U. S. Public Health Service.

REFERENCES

1. C. F. Cori, *in* "A Symposium on Respiratory Enzymes," p. 175. Univ. of Wisconsin Press, Madison, Wisconsin, 1942.

2. T. E. Mansour, *J. Pharmacol. Exptl. Therap.* **126**, 212 (1959).
3. T. E. Mansour, *J. Pharmacol. Exptl. Therap.* **135**, 94 (1962).
4. T. E. Mansour and J. M. Mansour, *J. Biol. Chem.* **237**, 629 (1962).
5. T. E. Mansour, E. W. Sutherland, T. W. Rall and E. Bueding, *J. Biol. Chem.* **235**, 466 (1960).
6. T. W. Rall and E. W. Sutherland, *J. Biol. Chem.* **232**, 1065 (1958).
7. T. E. Mansour, A. D. Lago and J. L. Hawkins, *Federation Proc.* **16**, 319 (1957).
8. T. E. Mansour, *Advan. Pharmacol.* **3**, 129 (1966).
9. D. B. Stone and T. E. Mansour, *Federation Proc.* **25**, 219 (1966).
10. H. V. Bergmeyer, ed., "Methods of Enzymatic Analysis." Academic Press, New York, 1963.
11. C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.* **66**, 375 (1925).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
13. R. G. Martin and B. N. Ames, *J. Biol. Chem.* **236**, 1372 (1961).
14. T. E. Mansour, *J. Biol. Chem.* **240**, 2165 (1965).
15. O. H. Lowry and J. V. Passonneau, *J. Biol. Chem.* **241**, 2268 (1966).
16. R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).
17. D. B. Stone and T. E. Mansour, *Mol. Pharmacol.* **3**, 177 (1967).
18. E. Helmreich and C. F. Cori, *Pharmacol. Rev.* **18**, 189 (1966).
19. E. W. Sutherland, T. W. Rall and T. Menon, *J. Biol. Chem.* **237**, 1220 (1962).
20. J. Monod, J. Wyman and J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).