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EFFECT OF MORPHINE ON CEREBRAL GLYCOLYTIC INTERMEDIATES AND ENZYMES OF RATS *IN VITRO**

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Abstract—Morphine caused a stimulation of glucose utilization in rat cerebral homogenates after a 30-min lag period. The fructose diphosphate concentration of the incubation medium in the presence of morphine *in vitro* was higher after 60 min of incubation, while the concentration of pyruvate was lower at both 30 and 60 min than those in the absence of morphine. Adenosine monophosphate (AMP) concentration was lower and the adenosine triphosphate (ATP) level was higher in the presence than in the absence of morphine after 30 and 60 min of incubation. The concentration of glyceraldehyde-3-P was decreased while the level of 3-P-glycerate was increased by morphine, indicating a facilitation of the glyceraldehyde-3-P dehydrogenase step of glycolysis. AMP inhibited the activity of glyceraldehyde-3-P dehydrogenase appreciably in rat cerebral homogenates. The initial facilitation by morphine is thought to be at the glyceraldehyde-3-P dehydrogenase step due to the low AMP concentration and a secondary facilitation at the P-fructokinase step due to the accumulated concentrations of ATP and fructose diphosphate. Additionally, the time required to alter adenine nucleotide concentrations may explain the lag period that occurs before the stimulation of glucose utilization by morphine is manifested.

IT WAS previously shown that the aerobic and anaerobic glucose uptakes of cerebral cortical slices taken from morphinized rats were greater than those of slices taken from control rats.[1] Other central nervous system depressants such as pentobarbital and ethanol did not display this effect. The glucose uptake of slices also increased when morphine was added *in vitro*. In addition, morphine *in vitro* increased the glucose utilization of cerebral homogenates and there was a positive correlation between glucose utilized by cerebral homogenates and the concentration of morphine in the range of 1×10^{-3} to 1×10^{-6} M. Kinetic studies of this effect showed that during the initial 30 min of incubation, the rate of glucose utilization in the presence and absence of morphine was the same.[2] The increase in rate due to morphine was seen only during the subsequent 30 min of incubation.

The present investigation was performed in order to determine the mechanism by which morphine increased glucose utilization of cerebral homogenate of rats. Experiments were also performed to gain some insight into the reasons for the lag period before the effect of morphine is manifested.

METHODS

Preparation of cerebral homogenates. Male Holtzman rats, 150–250 g, were used in

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all experiments. The animals were decapitated and the cerebral hemispheres were rapidly removed and homogenized in 9 vol. of ice-cold 0·2 M Tris-HCl buffer, pH 7·4, containing 0·01 M nicotinamide.[2] Usually cerebral hemispheres from two rats were pooled to provide enough homogenate for one experiment. A small aliquot of the homogenate was always taken for the determination of protein content by the method of Lowry *et al.*[3] The protein content of the cerebral homogenates did not vary greatly from sample to sample and was between 12·5 and 13·5 mg/ml. The homogenates were always kept on ice and were used within 1 hour after preparation.

Measurement of glucose utilization by cerebral homogenates. The content of the usual reaction mixture was similar to that described by Takemori.[2] The reaction mixture contained: 12 mM glucose; 4 mM $MgCl_2$; 5 mM ATP; 1 mM NAD^+; 20 mM inorganic phosphate as K_2HPO_4-KH_2PO_4 buffer, pH 7·4; 16 mM Tris-HCl buffer, pH 7·4; and 0·75 ml of cerebral homogenate in a total of 2·5 ml. The mixture, contained in a 25-ml Erlenmeyer flask, was incubated in a Dubnoff metabolic shaker at 30° with air as the gas phase. The reaction was stopped after various times of incubation by the addition of 0·5 ml of 18% perchloric acid and the incubation flask was immediately placed on ice. After the precipitated protein was removed by centrifugation, the supernatant was neutralized by the addition of 0·6 ml of 1·25 M K_2CO_3. The neutralized extract was then used for determining the concentration of glucose enzymically by coupling the hexokinase and glucose-6-P dehydrogenase reactions.[4] Glucose utilization was taken as the difference in glucose content of the medium between zero and various times of incubation.

Analysis of substrates and cofactors. The analyses of glucose-6-P, fructose-6-P, fructose diphosphate, dihydroxyacetone-P, pyruvate, lactate, glyceraldehyde-3-P, 3-P-glycerate, ATP, ADP, and AMP were performed fluorometrically on the neutralized extracts described above, by slight modifications of methods described by Lowry *et al.*[4] All fluorometric measurements were made in a volume of 3 ml in 3·5-ml round Pyrex glass cuvettes on a Turner fluorometer, model 111. The primary filter was a Turner No. 7-60 which passes wavelengths peaking at 360 mμ, and the secondary filters were a combination of Turner No. 3 and Turner No. 48 which passes wavelengths peaking at 460 mμ.

Assay of P-fructokinase activity. Cerebral hemispheres from rats were homogenized in 9 vol. of 0·25 M sucrose. The homogenate was placed in a SS-34 rotor and centrifuged in a Servall RC-2 centrifuge at 3000 rpm for 10 min at 0°–2°. The supernatant was removed and centrifuged for a second time. The supernatant was then placed in a No. 40 rotor and centrifuged in a Spinco model L ultracentrifuge at 40,000 rpm for 60 min at 0°–2°. The resulting soluble fraction was used to assay for enzymic activity.

Enzymic activity was determined by a modification of the method of Shonk and Boxer.[5] The assay mixture consisted of 20 mM imidazole-HCl buffer, pH 7·0, 5 mM Na_2H_2EDTA, 10 mM $MgCl_2$, 2·8 mM ATP, 2·8 mM fructose-6-P, 0·2 mM NADH, 1 μg per ml aldolase, 0·4 μg per ml triose-P isomerase, 6·7 μg per ml glycero-P dehydrogenase and 0·1 ml of soluble fraction in a total volume of 3 ml. Blank rates were negligible and the reaction was initiated by the addition of fructose-6-P. The oxidation of NADH was followed at 340 mμ for 10 min on a Gilford model 220 absorbance indicator with an attached recorder.

Assay of glyceraldehyde-3-P dehydrogenase activity. Both soluble fractions and whole homogenates were used to assay for enzymic activity. The soluble fraction

employed in this assay was prepared as described for the P-fructokinase assay. Cerebral homogenates were prepared in 9 vol. of an aqueous solution containing 0·15 M KCl, 0·05 M $KHCO_3$ and 0·006 M Na_2H_2EDTA as described by Shonk and Boxer.[5] The assay system consisted of 0·05 M triethanolamine-HCl buffer, pH 7·6, 6 mM Na_2H_2EDTA, 5 mM arsenate, 0·4 mM NAD^+, 0·5 mM glyceraldehyde-3-P and 20 μl of soluble fraction or homogenate in a total volume of 3 ml. A 2-min period was allowed for blank stabilization and the reaction was initiated by the addition of glyceraldehyde-3-P. The reaction was followed at 340 mμ on a Gilford model 220 absorbance indicator with an attached recorder.

Substrates and enzymes. All of the substrates and enzymes used in these studies were obtained from Boehringer Mannheim Corp.

Statistics. The data were analyzed by the paired Student's *t*-test.

RESULTS AND DISCUSSION

It is well established that hexokinase and P-fructokinase occupy special control points in aerobic glycolysis.[6–9] Therefore the steps catalyzed by these enzymes were examined as possible sites of facilitation to explain the increased glucose utilization of rat cerebral homogenates produced by morphine.

Effect of morphine on glycolytic substrate and cofactor levels. Initially, the effect of 1×10^{-3} M morphine was determined on a number of intermediates of glycolysis and

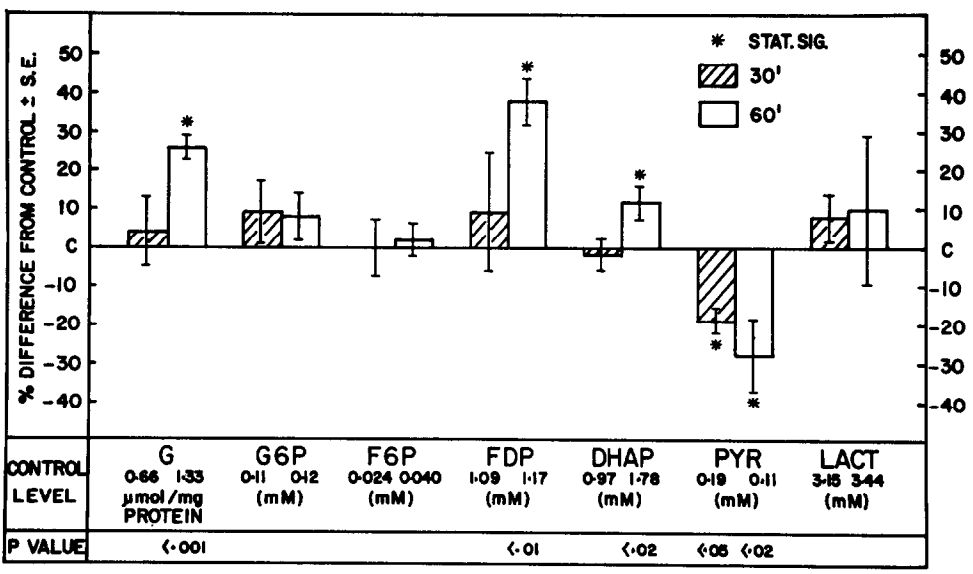

FIG. 1. Effect of morphine on a number of glycolytic intermediates. The system described under Methods to study glucose utilization by cerebral homogenates was used. The concentration of morphine *in vitro* was 1×10^{-3} M. After precipitation of homogenate, the concentrations of the various glycolytic intermediates were determined in the neutralized supernatant. The figures for glucose represent utilization rate rather than concentration. Each histogram represents the average value of six experiments (twelve rats). The abbreviations for the intermediates are G (glucose), G6P (glucose-6-P), F6P (fructose-6-P), FDP (fructose diphosphate), DHAP (dihydroxyacetone-P, PYR (pyruvate) and LACT (lactate).

* A P value of $<0·05$ was considered necessary to show significant changes.

is illustrated in Fig. 1. The characteristic 30-min lag period is seen, during which time the rate of glucose utilization in the presence of morphine is the same as that seen in the absence of morphine. This is followed by a significant increase in the glucose metabolism rate in the presence of morphine during the next 30 min. Both fructose diphosphate and dihydroxyacetone-P are significantly increased at 60 min but not at 30 min, while the pyruvate concentration is decreased at both 30 and 60 min.

A facilitation of the P-fructokinase step should lead to an increase in the concentration of fructose diphosphate and a fall in the concentrations of the hexose monophosphates. In Fig. 1, the increase in fructose diphosphate is seen, but there is no concomitant fall in either fructose-6-P or glucose-6-P. This discrepancy might be explained by the fact that in these experiments a constant source of glucose in high concentration is available for phosphorylation and thus the hexose monophosphate levels are kept high.

Time course of changes in adenine nucleotide concentration. The increase in fructose diphosphate concentration at 60 min seen in Fig. 1 was of great interest, since it can reflect a stimulation of the P-fructokinase step of glycolysis. Since there was a 30-min lag period prior to the increase in fructose diphosphate concentration, the effect of morphine on phosphofructokinase is probably indirect and may possibly be mediated through changes in adenine nucleotide concentrations. It has been shown by several

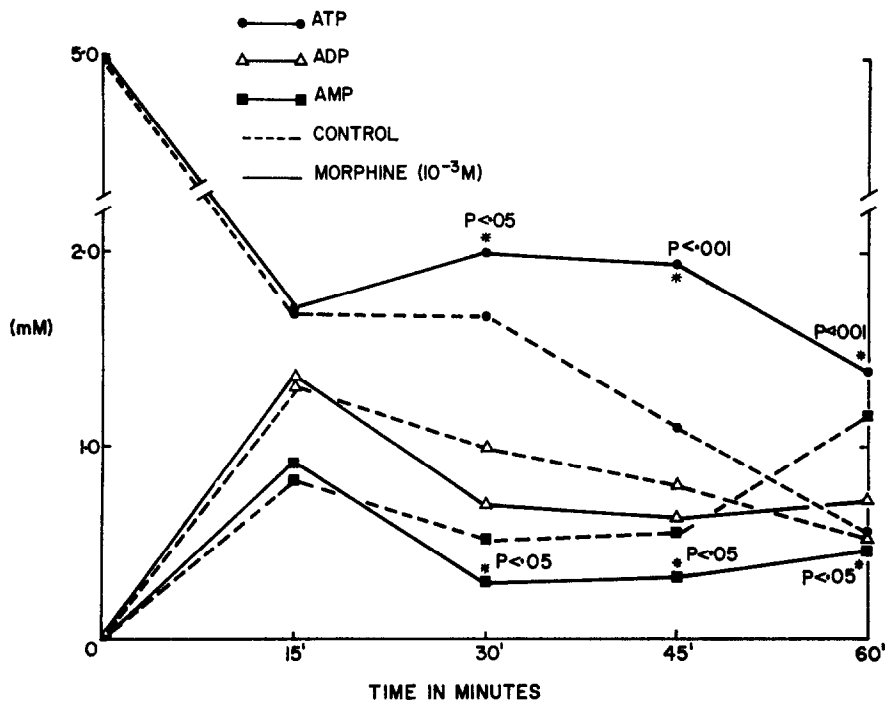

FIG. 2. Time course of the effect of morphine on adenine nucleotide concentration. The system described under Methods to study glucose utilization by cerebral homogenates was used. The concentration of morphine *in vitro* was 1×10^{-3} M. After precipitation of the homogenate at various times of incubation, the concentrations of the adenine nucleotides were determined in the neutralized supernatant. The initial concentration of ATP in the assay system was 5 mM. Each point on the curves represents the average value of four experiments (eight rats).

authors that changes in activity of the P-fructokinase step can be initiated by changes in the concentrations of effectors or inhibitors such as the adenine nucleotides.[10–12] Thus the adenine nucleotide concentrations were examined in the glucose utilization experiments and the results are presented in Fig. 2.

The ATP level decreases quickly in the first 15 min of incubation, partly due to the ATPase activity found in brain homogenates. Morphine does not alter the ATP level at 15 min, but it progressively maintains ATP levels above the level in the control flask during the remainder of the incubation period. The ATP does not appear to be kept high by an inhibition of ATPase activity, since Takemori[12] has shown that cerebral ATPases are not affected by morphine.

The AMP concentration follows an inverse pattern compared to ATP. There is no difference at 15 min, but throughout the rest of the incubation period the AMP concentration in the flask containing morphine is lower than that in the control flask. Morphine does not alter ADP concentrations at any of the time periods.

Since ATP can act as an inhibitor of P-fructokinase, it would be expected that the elevated ATP concentration in the presence of morphine would lead to a decrease in the glycolytic rate rather than the increase that was seen. Passonneau and Lowry[12] proposed two ATP sites on P-fructokinase, a primary active site and a secondary inhibitory site. They also pointed out that inorganic phosphate can compete with ATP for this inhibitory site and that it is not inhibitory itself. Inorganic phosphate can thereby deinhibit the inhibitory influence of ATP. The inorganic phosphate concentration in the incubation mixture (20 mM) probably explains the lack of ATP inhibition in the present study. ATP in the presence of inorganic phosphate occupies the primary stimulatory site and morphine increases the glycolytic rate by providing more ATP for phosphorylation of fructose-6-P.

Effect of morphine on utilization of certain glycolytic intermediates. Selected glycolytic intermediates were used as substrates instead of glucose to by-pass certain enzymic steps of glycolysis and further delineate the locus of facilitation produced by morphine.

When glucose is replaced by 12 mM pyruvate in the reaction mixture, morphine at 1×10^{-3} does not stimulate pyruvate disappearance after either 30 or 60 min of incubation (Table 1). Additionally, 1×10^{-3} M morphine does not alter the P:O ratio of pyruvate oxidation by rat cerebral mitochondria. Since morphine does not alter oxidation of pyruvate, the effect of morphine on glucose utilization probably is entirely on glycolysis.

When glucose is replaced by 12 mM glucose-6-P as the substrate, morphine at 1×10^{-3} M causes a stimulation of glucose-6-P utilization at 60 min of incubation similar in magnitude to that of glucose utilization. Thus the hexokinase step can be eliminated as the site of morphine stimulation.

In further experiments, the P-fructokinase step was by-passed by using 12 mM fructose diphosphate as the substrate instead of glucose. The utilization of fructose diphosphate by cerebral homogenates is not influenced by 1×10^{-3} M morphine at the initial 30 min of incubation, but is significantly increased during the subsequent 30 min (Table 1). The lag period is very similar to that associated with the effect of morphine on glucose or glucose-6-P utilization; however, the enhanced fructose diphosphate utilization is not as pronounced as the increase in glucose utilization. Thus morphine may stimulate another site of glycolysis below the P-fructokinase step.

Effect of morphine on glyceraldehyde-3-P and 3-P-glycerate. Balazs[8] suggested that

glycolysis of brain is inhibited under aerobic conditions at the glyceraldehyde-3-P dehydrogenase step in addition to the hexokinase and P-fructokinase steps. Rolleston and Newsholme[9] also pointed out that glyceraldehyde-3-P dehydrogenase may be part of a regulatory system of glycolysis in cerebral cortex slices. Thus, in addition to the substrates examined in Fig. 1, the effect of morphine on the concentration of glyceraldehyde-3-P and 3-P-glycerate in the reaction medium was examined (Fig. 3).

TABLE 1. EFFECT OF MORPHINE (10^{-3} M) ON THE UTILIZATION OF CERTAIN GLYCOLYTIC INTERMEDIATES BY CEREBRAL HOMOGENATES OF RATS

Substrate*	Time of incubation (min)	Substrate utilization mean % difference from control†
Glucose	30	+3
	60	+38‡
Pyruvate	30	+1
	60	0
Glucose-6-P	30	0
	60	+43‡
Fructose diphosphate	30	+10
	60	+28‡

* All substrates were employed at a concentration of 12 mM.
† The reaction mixture employed to study glucose utilization by cerebral homogenates was used and its contents are described under Methods. Substrate utilization was taken as the difference in substrate content of the medium between zero and 30 or 60 min of incubation. The effects of morphine are expressed as % difference from the control flask and were analyzed by the paired Student's *t*-test. The figures represent an average of three to six experiments.
‡ $P < 0.05$.

If glyceraldehyde-3-P dehydrogenase is stimulated by morphine, the concentration of glyceraldehyde-3-P would be expected to fall and that of the intermediate (1,3-diphosphoglyceric acid) immediately following this step to rise. It was not possible to measure 1,3-diphosphoglyceric acid due to the extremely small amount of this compound present. As an alternative, the substrate immediately following 1,3-diphosphoglyceric acid in the glycolytic sequence, 3-P-glycerate, was measured.

In conjunction with the usual increase in glucose metabolism between 30 and 60 min of incubation, morphine causes a significant fall in glyceraldehyde-3-P concentration at 30 and 60 min. At the same time there is a rise in 3-P-glycerate concentration, which is especially pronounced at 60 min. This provides evidence for the facilitation of either the 3-P-glycerate kinase or the glyceraldehyde-3-P dehydrogenase step of glycolysis by morphine. The glyceraldehyde-3-P dehydrogenase step would probably be the more likely step because of its special role in glycolysis.

Effect of morphine on the activity of P-fructokinase and glyceraldehyde-3-P dehydrogenase. Since the possibility existed that morphine may have a direct stimulatory effect on the enzymes which catalyze the steps facilitated by morphine, the enzymic activities were measured as described under Methods, with and without the addition

of 1×10^{-3} M morphine to the reaction mixture just prior to the assay. Neither the activity of P-fructokinase nor that of glyceraldehyde-3-P dehydrogenase is influenced by morphine (Table 2).

The enzymic activities were also measured after the enzymes (soluble fraction) had been preincubated with 1×10^{-3} M morphine for 30 and 60 min. Preincubation

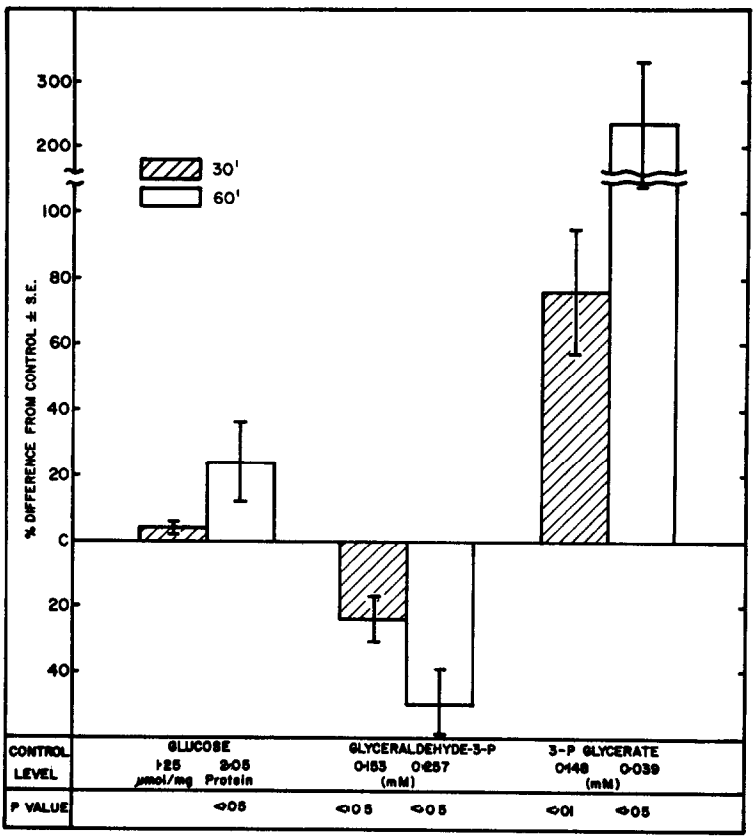

FIG. 3. Effect of morphine on glyceraldehyde-3-P and 3-P-glycerate concentrations in the assay medium. The contents of the reaction medium using cerebral homogenates and glucose as the substrate are described under Methods. Morphine was employed at a concentration of 1×10^{-3} M. The concentration of the triose phosphates were determined on neutralized acid extracts of the assay system. The values for glucose represent utilization rate rather than concentration. Each histogram represents the mean value for four experiments (eight rats).

procedures with glyceraldehyde-3-P dehydrogenase were performed at 0° due to the instability of this enzyme. In these experiments, no additional morphine was added to the assay system. Morphine does not appreciably alter the activities of either enzyme under these assay conditions (Table 2). Similar negative results are seen when purified P-fructokinase* from rabbit muscle is assayed under the above conditions.

* P-fructokinase was purified from rabbit muscle and supplied by Dr. Nelson Goldberg, Department of Pharmacology, University of Minnesota.

Effect of AMP and ATP on glyceraldehyde-3-P dehydrogenase activity. In further experiments, the direct effect of various substances on the activity of glyceraldehyde-3-P dehydrogenase was studied. The compounds which were tested were chosen because their concentrations were shown to be altered by morphine in the glucose utilization studies. It was seen that ADP, fructose diphosphate and ATP had no

TABLE 2. EFFECT OF MORPHINE *IN VITRO* ON THE ACTIVITY OF P-FRUCTOKINASE AND GLYCERALDEHYDE-3-P DEHYDROGENASE IN CERERBAL SOLUBLE FRACTIONS AND HOMOGENATES OF RATS

Enzyme	Experiment	Conditions prior to assay	(μmoles NADH oxidized/min) Control	Experiment
P-fructokinase (Soluble fraction)	a	1×10^{-3} M morphine added to cuvette just prior to assay	36·4	38·5
	b	1×10^{-3} M morphine incubated with soluble fraction for 30 min at room temperature	37·1	39·7
	c	1×10^{-3} M morphine incubated with soluble fraction for 60 min at room temperature	40·9	42·3
			(μmoles NADH formed/min) Control	Experiment
Glyceraldehyde-3-P dehydrogenase (Soluble fraction)	a	1×10^{-3} M morphine incubated with soluble fraction for 30 min at 0°	32·7	31·2
	b	1×10^{-3} M morphine incubated with soluble fraction for 60 min at 0°	31·0	30·6
(Homogenate)	c	1×10^{-3} M added to cuvette just prior to assay	36·1	33·8

effect on enzymic activity, while AMP appeared to lower it. In view of these results, it was decided to study the effect of AMP in greater detail.

The effect of various concentrations of ATP and AMP on glyceraldehyde-3-P dehydrogenase was studied with cerebral homogenates as the source of enzyme and is illustrated in Fig. 4. ATP, in concentrations of 0·5, 1·0 and 2·0 mM, has only a slight depressant effect on enzymic activity. AMP, in the same concentrations, produces a dramatic lowering of activity. AMP exhibits the same depressant effect on glyceraldehyde-3-P dehydrogenase even in the presence of ATP. Figure 5 shows the influence of a series of AMP concentrations with 1·5 mM ATP present under each assay condition. The concentrations of AMP and ATP are in the range of those found in the glucose utilization experiments at 30 and 60 min of incubation. These findings suggest that morphine produces at least a portion of its effect on glucose metabolism by lowering the AMP level, which in turn allows glyceraldehyde-3-P dehydrogenase to catalyze the reaction at a near optimal rate.

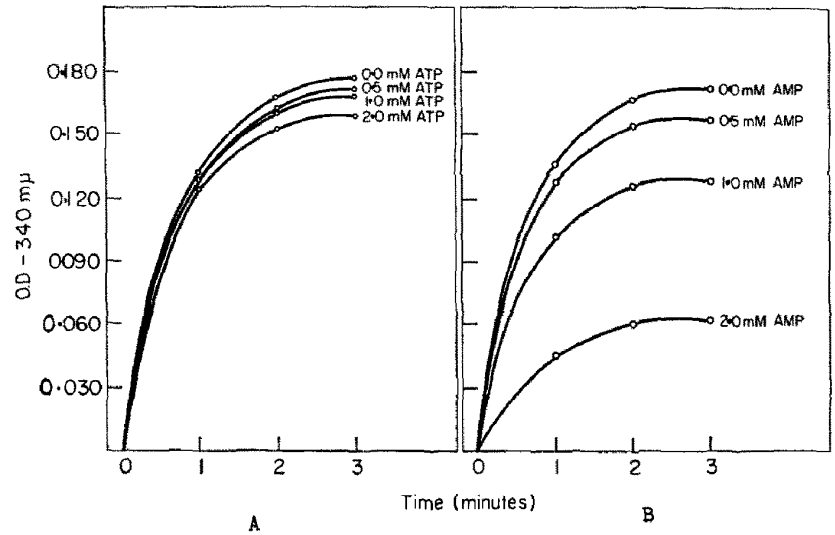

FIG. 4. Effect of various concentrations of ATP(A) and AMP(B) on the activity of glyceraldehyde-3-P dehydrogenase. Cerebral homogenates prepared as described under Methods were used as the enzymic source. These figures represent typical experiments which have been repeated and confirmed four to five times.

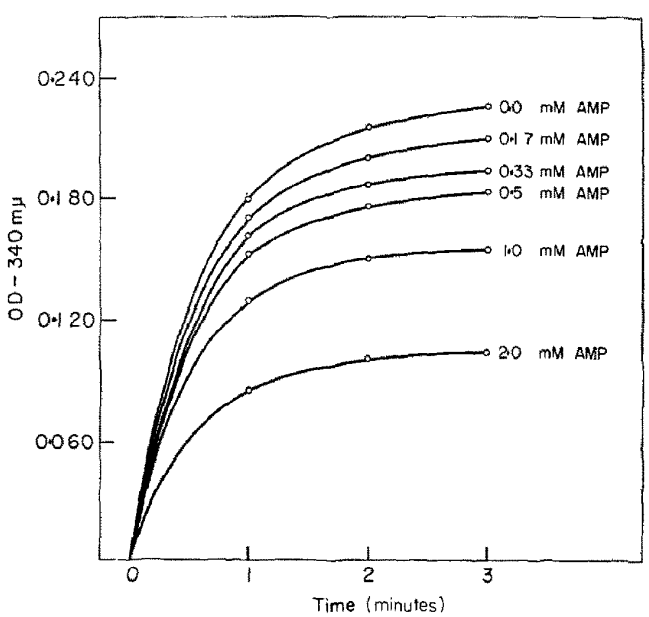

FIG. 5. Effect of various concentrations of AMP on the activity of glyceraldehyde-3-P dehydrogenase in the presence of ATP. Cerebral homogenates prepared as described under Methods were used as the enzymic source. These figures represent a typical experiment which has been repeated and confirmed four times.

Studies with other nucleoside monophosphates revealed that guanosine-5′-phosphate (GMP), cytidine-5′-phosphate (CMP) and uridine-5′-phosphate (UMP) have no appreciable effect on glyceraldehyde-3-P dehydrogenase activity. This indicates that the effect of AMP is quite specific.

The data above suggest that the following sequence of events may explain the stimulating effect of morphine on glucose metabolism *in vitro*. Since morphine decreases AMP concentration, the low AMP concentration may cause an apparent stimulation of glyceraldehyde-3-P dehydrogenase. This will stimulate glycolysis and lead to an increase in ATP level. The increase in ATP would provide more substrate for the P-fructokinase step and thereby stimulate this reaction and cause the increase in fructose diphosphate. Since fructose diphosphate is the most effective stimulator of P-fructokinase, its accumulation could then further stimulate P-fructokinase autocatalytically. Additionally, the time required to alter adenine nucleotide concentrations may explain the lag period that occurs before the stimulation of glucose utilization by morphine is manifested.

It would, of course, be extremely interesting to see if these changes occur *in vivo*. Similar changes in adenine nucleotides do appear to occur *in vivo* after a dose of morphine. These results will be reported soon in another communication.

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