Role of Adenosine Monophosphate in Regulation of Metabolic Pathways of Perfused Rat Liver

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1. By perfusion of rat livers with 3 mM-AMP in the perfusion medium we obtain increased intracellular concentrations of AMP. 2. These high intracellular concentrations of AMP lead to an increased output of glucose and urea into the perfusion medium. 3. The increased output of glucose in livers from fed rats is brought about primarily by an AMP-stimulated breakdown of liver glycogen. In livers from starved rats the increase in glucose output is not as great, reflecting the low contents of glycogen in livers from starved rats. 4. AMP inhibits gluconeogenesis from lactate in perfused livers. In the presence of high concentrations of lactate, however, the counteracting effects of AMP to increase glycogenolysis and to inhibit gluconeogenesis result in little change in the net glucose output. 5. The increased urea output is brought about by increased breakdown of amino acids that are present in the perfusion medium. In livers from starved rats the overall urea production is much higher, indicating increased catabolism of amino acids and other nitrogenous substrates in the absence of carbohydrate substrates. 6. AMP causes an inhibition of incorporation of labelled precursors into protein and nucleic acid. This may result from increased catabolism of precursors of proteins and nucleic acids as reflected by the more rapid breakdown of nitrogenous compounds. In support of this hypothesis, cell-free systems for amino acid incorporation isolated from livers perfused with and without AMP are equally capable of supporting protein synthesis. 7. The labelling pattern of RNA in perfused livers corresponds very closely to those found by pulse-labelling *in vivo*. AMP in no way alters the qualitative nature of the labelling patterns. 8. We consider these results as supporting evidence for the role of the concentration ratio of AMP to ATP in controlling the metabolic pathways that lead to the formation of ATP.

Atkinson & Walton (1967), Stadtman (1966), Krebs (1964), and Newsholme & Gevers (1967) proposed that the concentration ratio of AMP to ATP in the cell may be an important control mechanism for carbohydrate metabolism and more especially for those processes that lead to the formation of ATP. Since the total concentration of adenine nucleotides in the liver cell remains reasonably constant under a variety of conditions (Bucher & Swaffield, 1966), any decrease in the concentration of ATP necessarily leads to a rise in the concentration of ADP, and also to a rise in the concentration of AMP, which is in equilibrium with ADP and ATP through the enzyme adenylate kinase. Because the concentration of ATP of the cell is always main-

tained at a value that is at least 20 times that of AMP, a very small rise in AMP concentration leads to a significant change in the AMP/ATP ratio. A control system based on the maintaining of this ratio at a constant value would be a very sensitive method of keeping the energy level of the cell at a particular value. AMP is known to be an allosteric effector for a number of enzymes that are in key positions for controlling energy-producing metabolic pathways (see Stadtman, 1966). There is therefore a considerable body of evidence to support an adenylate-control hypothesis.

It is, however, very difficult to obtain direct evidence of this *in vivo*. We decided that one possible approach was the perfusion of rat liver with AMP. By perfusing rat livers with AMP we were able to observe a number of metabolic changes that are consistent with AMP's stimulating an increased catabolic activity in the liver.

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MATERIALS AND METHODS

Animals. Female Wistar rats weighing 130–160 g. from a strain bred in this Laboratory were used. They were fed *ad libitum* on a diet of Purina laboratory chow. When rats were starved they were deprived of food for 12–18 hr. before the experiment.

Perfusion of livers. Rats were anaesthetized with sodium pentobarbital (50 mg./kg. body wt.) and the livers were perfused *in situ* by the technique of Mortimore (1961). The perfusion medium was Krebs bicarbonate buffer (Krebs & Henseleit, 1932) containing bovine plasma albumin powder (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) (3%, w/v), EDTA (0.05 mM), and sufficient saline-buffer-washed rat erythrocytes (Exton & Park, 1967) to give a haematocrit of 20%, and, unless otherwise noted, 10 mM-glucose and an amino acid mixture at three times the normal rat plasma concentration (Jefferson & Korner, 1967a). The labelled amino acid or nucleic acid precursor was added as described in the legend of each Table. The initial recirculating volume of medium was 50 ml., and samples (2 ml.) were taken when indicated and analysed for glucose content by the method of Huggett & Nixon (1957) and for urea content by the method of Archibald (1945). The flow rate was 7.5 ml./min. At the end of the perfusion the liver was rapidly excised into ice-cold 0.25 M-sucrose medium containing 100 mM-KCl, 40 mM-NaCl, 10 mM-magnesium acetate and 20 mM-tris-HCl buffer, pH 7.6. The liver was then weighed by displacement and homogenized in 3 vol. of the sucrose medium with five strokes of a Dounce glass glass homogenizer fitted with a Perspex pestle.

Measurement of uptake of radioactive precursors. The supernatant from the initial acid precipitation of the liver homogenate with 5 ml. of 10% (w/v) trichloroacetic acid/ml. of homogenate was extracted with two 5 ml. washes of ether to remove trichloroacetic acid, which otherwise would heavily quench radioactive scintillation. A portion (0.2 ml.) of this supernatant was diluted with 0.2 ml. of water and transferred to 4.0 ml. of Bray's (1960) aqueous scintillation fluid, and the radioactivity was determined in a Nuclear-Chicago scintillation counter. On occasions when double labelling was used, the counter was set up specifically to count two radioactive isotopes. Results were expressed as counts/min. taken up/ml. of acid-soluble supernatant. Great care was taken to ensure that each homogenate had the same protein concentration.

Measurement of glycogen. For the measurement of changes in glycogen content of perfused livers it was insufficient to determine the content of glycogen after perfusion, because of the inherent variability in the glycogen content of livers from fed rats. This difficulty was overcome by tying off a small lobe of the liver before the perfusion was started and determining its glycogen content by the method of Walaas & Walaas (1950). The alteration in glycogen content for each liver during the perfusion was determined by assaying the glycogen content after perfusion and subtracting this value from the initial content.

Measurement of [^{14}C]glucose. The amount of labelled substrate converted into glucose was determined exactly as described by Exton & Park (1967). The amount of label in liver glucose and in liver glycogen was taken into account in the calculation of [^{14}C]glucose produced by gluconeogenesis.

Measurement of $^{14}\text{CO}_2$ production. Perfused livers were

circulated with medium that was continuously gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and contained 10^5 counts/min. of a uniformly ^{14}C -labelled amino acid mixture/ml. The effluent gas was passed through three successive traps each containing 30 ml. of aq. 2 M- NH_3 solution. A sample from each was counted in Bray's (1960) aqueous scintillation fluid. The efficiency of this method and further details were discussed by Exton & Park (1967).

Determination of the radioactivity incorporated into protein. Samples (1 ml.) of the homogenized liver were treated with 5 ml. of 10% (w/v) trichloroacetic acid. The procedure of Munro, Jackson & Korner (1964) was used to free the resulting precipitate of amino acids, RNA and lipid and to measure the radioactivity in the sample. Corrections were made for background counts and self-absorption. The efficiency of counting was about 20%.

Determination of the radioactivity incorporated into nucleic acid. The procedure for isolating nucleic acids was essentially that described by Wool & Moyer (1964). Samples (1 ml.) of the homogenate were treated with 5 ml. of ice-cold 20% (w/v) trichloroacetic acid. The precipitate was washed twice with 20% (w/v) trichloroacetic acid and once with ethanol-chloroform-diethyl ether (2:2:1, by vol.), all in the cold, before being extracted with 2 ml. of 10% (w/v) NaCl at 100° for 60 min. The extract was cooled and centrifuged at 1000 g for 10 min., and the supernatant was removed. A portion (0.1 ml.) of this supernatant was diluted to 3 ml. and E_{260} was read to measure the quantity of nucleic acids present. A further 0.1 ml. of this supernatant was diluted to 0.5 ml. with water and transferred to 4.0 ml. of Bray's (1960) aqueous scintillation fluid, and the radioactivity was measured in a Nuclear-Chicago scintillation counter. Corrections were made for background counts. Quenching was assessed and found to be reasonably uniform throughout each series of samples, and lowered the counting efficiency from about 40% to about 20% for ^3H . No correction was made for quenching. Counting efficiency for ^{14}C was about 50%. An extinction coefficient of 32.2 units/mg./ml./cm. for RNA was used, and the results were expressed as counts/min. incorporated into nucleic acid/mg. of nucleic acid.

When it was desired to determine the radioactivity in both protein and nucleic acid from the same homogenate, the procedure for nucleic acid extraction was followed to the final centrifugation and the residual precipitate was then treated by the method of Munro *et al.* (1964) for the extraction of protein.

Cell-free incorporation of amino acids. The liver homogenate was centrifuged for 10 min. at 10000 g and at 0° to remove mitochondria, nuclei and debris. The postmitochondrial supernatant, treated with Triton X-100 to a final concentration of 1% (Hunter & Korner, 1966), was used for the preparation of polysomes and ribosomes. Polysomes were prepared essentially as described by Munro *et al.* (1964) with the slight modification of a 1.0 M-sucrose step replacing the 0.5 M-sucrose step. Ribosomes were prepared as the total ribonucleoprotein particles as described by Munro *et al.* (1964). After aspiration of the supernatant fluid and rinsing of the inside of the tubes and the surface of the pellets, the polysomes and ribosomes were stored at -20° . No loss of activity was noted on storage for up to 1 month.

Cell sap was prepared by centrifuging the postmitochondrial supernatant for 1.5 hr. at 165000 g in the Spinco model L2-50 ultracentrifuge and passing the supernatant

through a column of Sephadex G-25 to remove inhibitors of protein synthesis (Munro *et al.* 1964). Unless otherwise noted, cell sap was prepared from the postmitochondrial supernatant obtained from non-perfused livers of normal female rats in the manner described above.

The activity of ribosome preparations as an amino acid-incorporating system was tested in cell-free incubation. Samples of ribosome preparations (0.15 mg. of RNA) were incubated at 37° in a total volume of 0.5 ml. containing 20 mM-tris-HCl buffer, pH 7.6, 10 mM-magnesium acetate, 40 mM-NaCl, 100 mM-KCl, 6 mM-mercaptoethanol, 5 mM-ATP, 0.1 mM-GTP, 0.5 μ C of [14 C]phenylalanine, the L-isomer of each of the other amino acids (each 10 μ M) and cell sap (5 mg. of protein). Incubation was stopped by the addition of 5 ml. of 0.5 M-HClO₄ containing unlabelled L-phenylalanine. The resulting precipitate was freed of amino acids, RNA and lipid, and the radioactivity in protein was determined by the procedure of Munro *et al.* (1964). Corrections were made for the radioactivity of the zero-time controls, background counts and self-absorption. Results are reported as counts/min. incorporated into protein/mg. of particle RNA incubated.

Polysome aggregation was analysed by sucrose-density-gradient centrifugation. Polysome pellets were suspended in 20 mM-tris-HCl buffer, pH 7.6, containing 5 mM-magnesium acetate, 40 mM-NaCl and 100 mM-HCl to give a concentration of approx. 200 E_{260} units/ml. It was found that a less aggregated suspension could be obtained by incubating the polysomes for 30 min. at 37° (A. R. Hunter & L. S. Jefferson, unpublished work). Portions of suspended polysomes (50 μ l.) were layered on 5 ml. of 15–30% (w/v) sucrose density gradients in the suspension medium and centrifuged at 204 000 g for 30 min. in the SW50 rotor of the Spinco model L2-50 ultracentrifuge. Gradients were prepared and centrifuged at 0°. After centrifugation the pattern of E_{260} on the gradient was recorded automatically with a ratio-recording spectrophotometer (Beckman model DK-2A) by using the flow-cell attachment.

Extraction of RNA. RNA was extracted from the post-mitochondrial supernatant as described by Munro *et al.* (1964) with the omission of the sodium dodecyl sulphate from the phenol. RNA was extracted from the combined nuclear and mitochondrial pellet with phenol at 65°, a treatment that is supposed to extract most of the nuclear RNA (Georgiev, 1961). Linear 5–20% (w/v) sucrose gradients containing 0.1 M-NaCl and 0.01 M-sodium acetate buffer, pH 5.2, were used for analysis of RNA.

Determination of RNA and protein. RNA was determined by the method of Fleck & Munro (1962) as described by Munro *et al.* (1964). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with standards of bovine serum albumin.

Statistical treatment of results. The experimental and control values of parameters measured in perfused livers were analysed and expressed as their means and S.E.M. The significance between means was tested by Student's *t* test and expressed as a *P* value. We have only stated the significant difference when the mean values of the control and experimental groups showed such a difference that the *P* value was less than 0.05.

Materials. DL-[1- 14 C]Phenylalanine (44.2 mc/m-mole), DL-[14 C]leucine (37 mc/m-mole), [3 H]adenine (600 mc/m-mole), [3 H]uridine (13 000 mc/m-mole) and [14 C]AMP (21.4 mc/m-mole) were purchased from The Radiochemical

Centre (Amersham, Bucks.). Sodium DL-[2- 14 C]lactate (6 mc/m-mole) was obtained from Nuclear-Chicago Corp. (Des Plaines, Ill., U.S.A.). The uniformly 14 C-labelled amino acid mixture (approx. 150 mc/m-mole) was obtained from the New England Nuclear Corp. (Boston, Mass., U.S.A.). Adenosine, AMP, ADP and ATP were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). They were added to the perfusion medium to a final concentration of 3 mM.

RESULTS

Uptake of [14 C]AMP. [8- 14 C]AMP was added to the perfusion medium to ascertain whether AMP could be taken up by perfused rat liver. The experiment was done in the presence of 3 mM-AMP to get some idea of how much AMP was taken up by the liver under these conditions. The acid-soluble supernatant derived from the precipitation of 1.0 ml. of the liver homogenate with 5.0 ml. of 10% (w/v) trichloroacetic acid contained $35\,660 \pm 6060$ counts/min./ml. when livers were perfused for 1 hr. with a medium containing 0.16 μ C of [14 C]AMP/ml. On the basis of assumptions about the efficiency of counting and the ratio of extracellular to intracellular water volume, there is an uptake of 5–10 μ moles of the adenine moiety of AMP/g. wet wt. of liver. This value is very high, but it is likely that there is a large extracellular contamination because AMP is a charged molecule. The specific radioactivity of nucleic acids isolated from livers under these conditions was 801 ± 73 counts/min./mg. In case considerable breakdown of the adenine nucleus occurred in the liver during perfusion, the incorporation of 14 C into liver protein was assayed under these conditions. Over 1 hr. less than 5 counts/min./mg. was incorporated into protein.

Effect of AMP on glucose production. The output of glucose into the perfusion medium was measured at 30 min. and 60 min. and the results were expressed as changes in net glucose output as depicted in Table 1. With livers from fed rats there was a dramatic stimulation of glucose output both at 30 min. (111%) and at 60 min. (132%) in those livers perfused with AMP. On the other hand in livers from starved rats the stimulation of glucose output by AMP was not so marked, there being a 31% increase at 30 min. and a 36% increase at 60 min. The higher glucose output in livers from fed rats can be attributed to their initially high content of glycogen.

Changes in glycogen contents were determined to ascertain whether the increased glucose output with AMP was due to increased glycogenolysis. We were only able to carry out this experiment with livers from fed rats, because the content of glycogen in livers from starved rats is virtually nil. Because of the natural variance from rat to rat in glycogen contents in fed rats, the net change in glycogen

Table 1. *Effect of AMP on the production of glucose by perfused livers from fed and starved rats*

Livers were perfused under normal conditions except that the perfusion medium contained 3mm-glucose initially. AMP was present in the perfusion medium at an initial concentration of 3mm. In Expt. 2 a small lobe of the liver was taken at the start of perfusion for determination of the initial glycogen content. The values for glycogen are in μ moles of glucose equivalent. The numbers in parentheses are the numbers of observations. Means $\pm$ s.e.m. are given. N.S., Not significant. For other details, see the text.

		Net glucose output (μ moles/100 g. body wt.)				Net loss of glycogen (μ moles/g. of liver/hr.) Fed rats
		Fed rats		Starved rats		
Time (min.)	...	30	60	30	60	
Expt. 1						
Control (4)		91.2 $\pm$ 4.1	146.2 $\pm$ 7.0	65.7 $\pm$ 10.1	99.0 $\pm$ 17.3	
With AMP (4)		196.5 $\pm$ 16.4 <i>P</i> < 0.001	340.0 $\pm$ 16.7 <i>P</i> < 0.001	86.3 $\pm$ 18.3 N.S.	135.7 $\pm$ 15.9 <i>P</i> < 0.05	
Expt. 2						
Control (4)		124.4 $\pm$ 6.7	148.3 $\pm$ 7.7			15.6 $\pm$ 2.3
With AMP (4)		160.6 $\pm$ 7.7 <i>P</i> < 0.02	244.3 $\pm$ 8.8 <i>P</i> < 0.001			27.2 $\pm$ 2.6 <i>P</i> < 0.02

Table 2. *Effect of AMP on gluconeogenesis from lactate in perfused livers from fed and starved rats*

Livers were perfused for 1 hr. with medium containing initially 3mm-glucose, 20mm-L-lactate and 18×10^3 counts/min. of DL-[2- 14 C]lactate/ml. The numbers in parentheses are the numbers of observations. [14 C]Glucose synthesis was measured exactly as described by Exton & Park (1967) and represents [14 C]glucose output plus [14 C]glucose converted into glycogen. Means $\pm$ s.e.m. are given. AMP was present in the perfusion medium at 3mm. N.S., Not significant. For other details, see the text.

Time (min.) ...	Net glucose output (μ moles/100g. body wt.)		$10^{-3} \times$ [14 C]Glucose synthesis (counts/min./100g. body wt.)	
	30	60	30	60
Fed rats				
Control (4)	201.3 $\pm$ 5.6	305.8 $\pm$ 17.4	56.2 $\pm$ 5.3	122.9 $\pm$ 2.6
With AMP (4)	203.3 $\pm$ 17.6 N.S.	374.6 $\pm$ 13.8 <i>P</i> < 0.05	22.4 $\pm$ 3.3 <i>P</i> < 0.01	72.1 $\pm$ 5.0 <i>P</i> < 0.001
Starved rats				
Control (4)	153.3 $\pm$ 6.9	246.1 $\pm$ 13.0	86.4 $\pm$ 4.6	183.1 $\pm$ 7.3
With AMP (4)	142.2 $\pm$ 5.4 N.S.	232.2 $\pm$ 6.0 N.S.	67.8 $\pm$ 4.8 <i>P</i> < 0.05	144.8 $\pm$ 7.6 <i>P</i> < 0.02

content of each liver was measured as the difference in glycogen content between a lobe of liver taken before the start of the perfusion and the rest of the liver at the end of the perfusion. These results are shown in Table 1, Expt. 2. Assays of net glucose production performed at the same time again showed a very high stimulation of glucose output associated with a 77% increase in the breakdown of glycogen in the presence of AMP.

The effect of AMP in increasing glucose output by the perfused liver might also reflect changes in the rate of synthesis of glucose. To study the effects of perfusion with AMP on gluconeogenesis, the conversion of [14 C]lactate into glucose was assayed (Exton & Park, 1967). In the same experiment the total production of glucose into the perfusion

medium was measured. Experiments with livers from both fed and starved rats (Table 2) show that the inclusion of 20mm-lactate in the perfusion medium counteracted the effect of AMP on glucose production to some extent. In livers from fed rats the stimulation is decreased to 22% after 60min., and in livers from starved rats it is totally abolished. Table 2 also shows that AMP has a significantly inhibitory effect on gluconeogenesis from lactate in livers from both fed and starved rats: at 60min. the inhibition was 44% in livers from fed rats and 21% in livers from starved rats.

Urea production from perfused livers. The urea output into the perfusion medium, measured after 60min., of fed rats was stimulated by 77% in the presence of AMP (control, 1.27 ± 0.07 ; with AMP,

2.25 ± 0.15 mg./100 g. body wt.; $P < 0.001$), but in starved rats the increase was only 23% (control, 4.60 ± 0.08; with AMP, 5.65 ± 0.13 mg./100 g. body wt.; $P < 0.001$). The output of urea in livers from starved rats was thus two to three times that from livers from fed rats.

Production of $^{14}\text{CO}_2$ and gluconeogenesis from ^{14}C -labelled amino acids in perfused livers. The production of $^{14}\text{CO}_2$ from uniformly labelled amino acids in the perfusion medium was assayed (Exton & Park, 1967), in conjunction with the net output of glucose and the synthesis of [^{14}C]glucose. The high concentration of glucose (9 mM) in the medium apparently inhibited the total output of glucose by the livers during 30 min. of perfusion (30–40% inhibition), possibly by a feedback mechanism (control, 66.9 ± 2.0; with AMP, 132.5 ± 15.0 $\mu\text{moles}/100\text{ g. body wt.}$; $P < 0.001$; cf. Table 1) (see Exton & Park, 1967). There was no difference in $^{14}\text{CO}_2$ production between control and AMP-perfused livers of fed rats (control, 427.5 ± 27.0 × 10³; with AMP, 437.5 ± 29.6 × 10³ counts/min./100 g. body wt.; not significant). The synthesis of glucose from the ^{14}C -labelled amino acids was also measured. Unexpectedly, the synthesis of labelled glucose was stimulated by AMP (51%) (control, 211.3 ± 11.1 × 10³; with AMP, 318.0 ± 12.9 × 10³ counts/min./100 g. body wt.; $P < 0.001$). This result apparently contradicts the earlier finding that AMP inhibits gluconeogenesis from lactate. This point is raised in the Discussion section.

Uptake and incorporation of amino acids into perfused liver. Perfusion of livers from fed or starved rats for 60 min. with medium containing [^{14}C]phenylalanine leads to the incorporation of the labelled amino acid into liver protein (Jefferson & Korner, 1967a). The inclusion of AMP at an initial concentration of 3 mM in the perfusion medium of the livers from starved rats caused a fall of 36% in the incorporation of [^{14}C]phenylalanine into protein

(control, 135 ± 13; with AMP, 87 ± 6 counts/min./mg. of liver protein; $P < 0.01$). With livers from fed rats the fall in incorporation was 24% (control 256 ± 14; with AMP, 196 ± 4 counts/min./mg. of liver protein; $P < 0.01$). With leucine as the ^{14}C -labelled amino acid the decrease in incorporation into protein was 37% in livers from starved rats (control, 128 ± 19; with AMP, 80 ± 8 counts/min./mg. of liver protein; $P < 0.01$). The uptake of amino acids into liver was measured by the amount of radioactivity occurring in acid-soluble form in the liver homogenate. The value derived in this way is high owing to contamination both from trapped perfusion medium and from material non-specifically absorbed on to the outside of liver cells. In livers from starved rats there was no significant difference in the uptake of phenylalanine by livers perfused with or without AMP (control, 3220 ± 255; with AMP, 3650 ± 70 counts/min./ml.).

Uptake and incorporation of nucleic acid precursors into nucleic acids. [^3H]Orotic acid, which is rapidly incorporated into RNA in rat liver (Munro & Korner, 1964), was the chief nucleic acid precursor used. In livers from starved rats perfusion with AMP caused a decrease in incorporation of 77% (Table 3). The uptake of orotic acid into livers from starved rats was inhibited in the presence of AMP by 52% (Table 3). Since we could not be certain that the large decrease in incorporation of labelled precursors into nucleic acids was not just due to the inhibition of uptake, we tested a number of other nucleic acid precursors in our system. There was an inhibition of incorporation of [^3H]adenine into nucleic acids by AMP of 75%, but the uptake of adenine was apparently not affected (Table 3). On the other hand the uptake of [^3H]uridine was inhibited to the extent of 30% by AMP, though the incorporation of uridine into nucleic acids was not affected (Table 3). We used [^3H]orotic acid-labelled RNA extracted from perfused livers to ascertain whether AMP

Table 3. *Effect of AMP on the uptake and incorporation of [^3H]orotic acid, [^3H]adenine and [^3H]uridine into nucleic acids of perfused livers of starved rats*

Livers were perfused for 60 min. under normal conditions with a medium containing either 2.5 μC of [^3H]orotic acid/ml. or 5.0 μC of either [^3H]adenine or [^3H]uridine/ml. The acid-soluble supernatant was derived from the precipitation of 1.0 ml. of liver homogenate with 5.0 ml. of 10% (w/v) trichloroacetic acid. The numbers in parentheses are the numbers of observations. Means ± S.E.M. are given. The concentration of AMP in the perfusion medium was 3 mM. N.S., Not significant. For other details, see the text.

Precursor		Uptake (counts/min./ml. of acid-soluble supernatant)		Incorporation (counts/min./mg. of nucleic acid)	
[^3H]Orotic acid	Control (8)	142000 ± 6000	$P < 0.001$	7445 ± 377	$P < 0.001$
	With AMP (8)	68000 ± 5000		1706 ± 122	
[^3H]Adenine	Control (5)	8930 ± 1155	N.S.	2039 ± 331	$P < 0.05$
	With AMP (5)	8715 ± 195		529 ± 77	
[^3H]Uridine	Control (3)	96.8 ± 7.4	$P < 0.05$	955 ± 105	N.S.
	With AMP (3)	66.8 ± 7.5		834 ± 96	

Table 4. *Effects of ATP, ADP and adenosine on a number of parameters measured in perfused livers*

The results are expressed as a percentage of control values at 60 min. No attempts were made to assess the significance of the results. AMP, adenosine, ADP and ATP were present in the perfusion medium at 3 mM.

	Effect of addition (% of control value)					
	Glucose output	Urea output	[¹⁴ C]Phenylalanine uptake	[¹⁴ C]Phenylalanine incorporation	[³ H]Orotic acid uptake	[³ H]Orotic acid incorporation
AMP	211	177	100	76	48	37
Adenosine	100	115	100	90	50	30
ADP	100	106	100	35	40	10
ATP	—	—	—	—	25	15

qualitatively altered the RNA labelled during the course of the perfusion. RNA was extracted from the postmitochondrial supernatant of a liver homogenate and also from the combined mitochondrial and nuclear pellet. The RNA was analysed by sucrose-density-gradient centrifugation. The RNA from the nuclear fraction of the control liver showed a typical short-time pulse-labelling pattern (Georgiev, 1967; Penman, Smith & Holtzman, 1966), with RNA of heterogeneous molecular weight showing labelling. The RNA from liver perfused with AMP showed a very similar labelling pattern. The cytoplasmic RNA also showed the typical pattern obtained from pulse-labelling *in vivo* (Henshaw, Revel & Hiatt, 1965), there being considerable labelling of the soluble-RNA fraction and also some labelling of the 18s peak. The cytoplasmic RNA from liver perfused with AMP showed no qualitative difference in labelling. It is therefore concluded that AMP does not, in any significant way, alter the types of RNA synthesized in the liver. The uptake and incorporation of orotic acid were, however, both significantly inhibited.

Cell-free systems for amino acid incorporation derived from perfused livers. Ribosomes and polysomes were prepared from livers of starved rats that had been perfused with and without AMP for 1 hr. The resulting particle preparations were analysed on sucrose density gradients. To maintain the polysomes in perfused livers intact, the concentration of amino acids in the perfusion medium has to be very high (Jefferson & Korner, 1967b); the optimum may be as high as ten times the plasma concentration of amino acids. Differences in polysome profiles are therefore very hard to interpret, as they may be due to minor variations in energy levels and other factors. There was not, however, any demonstrable difference between the profiles obtained.

The ability of these preparations of ribosomes to support protein synthesis in the presence and absence of polyuridylic acid was tested. There was no difference in the ability of ribosomes from livers

perfused with and without AMP to incorporate phenylalanine (control, 9505; with AMP, 9039 counts/min./mg. of ribosomal RNA). Addition of polyuridylic acid caused a sixfold stimulation in each case (control, 58279; with AMP, 53522 counts/min./mg. of ribosomal RNA). We conclude that AMP had neither altered the ability of isolated ribosomes to incorporate amino acids into protein nor impaired their ability to synthesize protein on exogenous RNA.

Perfusion with adenosine, ATP and ADP. To check whether the effects we were observing with AMP were specific for that nucleotide, livers were perfused with perfusion medium containing 3 mM-adenosine, -ADP or -ATP. Table 4 shows the qualitative effects of these substances. All the adenine nucleotides and the nucleoside are very potent inhibitors of orotic acid uptake by perfused rat liver. Possibly as a consequence, they all inhibit the incorporation of orotic acid into nucleic acids. ADP has a marked inhibitory effect on the incorporation of amino acids into protein. AMP is the only one of these compounds that has significant effects on the output of glucose and urea by perfused liver. The slight effect of ADP and adenosine in increasing urea output may reflect the destruction of these substances and their subsequent detoxication.

DISCUSSION

Stadtman (1966), in his review on the allosteric regulation of enzyme activity, listed a number of enzymes that show a response to AMP as an allosteric effector. Examples of enzymes whose activity is stimulated in the presence of AMP are glycogen phosphorylase (Sutherland & Wosilait, 1956; Krebs & Fischer, 1962; Bishop & Larnier, 1967), phosphofructokinase (Underwood & Newsholme, 1965b) and isocitrate dehydrogenase (Atkinson, Hathaway & Smith, 1965). Examples of enzymes whose activity is inhibited in the presence of AMP are fructose 1,6-diphosphatase (Underwood &

Newsholme, 1965a), UDP-glucose synthetase (Kornfeld, 1965), glycerol kinase and β -hydroxymethylglutaryl-CoA synthetase (Stadtman, 1966). AMP also inhibits a number of enzyme steps in the biosynthesis of purines and pyrimidines (Stadtman, 1966). It can be seen that the general pattern of allosteric interaction by AMP is to inhibit a number of different types of synthetic process and at the same time to stimulate the pathways of carbohydrate catabolism.

Previous studies on the effects of AMP on partially intact metabolic processes were carried out by Krebs (1964) and Gevers & Krebs (1966), who studied the effect of AMP on carbohydrate metabolism in a pigeon liver homogenate in the presence and absence of lactate. They found that the control of glycolysis was mediated by the concentration of AMP, which affected the relative activities of phosphofructokinase and fructose 1,6-diphosphatase. AMP caused a speed-up of glycolysis in the homogenate and at the same time slowed gluconeogenesis from lactate. A further study was made by Beloff-Chain *et al.* (1965), who added AMP to intact diaphragm; this caused an increased rate of glycolysis in the diaphragm. Adenosine, on the other hand, was virtually without effect on glycolysis.

It is uncertain whether the perfusion of rat liver with 3mm-AMP leads to uptake of AMP by the liver cells. It is recognized that the uptake of nucleotides by cells is in general not easily accomplished (Prescott, 1964). Leibman & Heidelberger (1955) tested the uptake of ^{32}P -labelled AMP into liver slices and came to the conclusion that only 1–2% of the added AMP was taken up as such. There was, however, a significant rise in the intracellular AMP concentration, which on the basis of specific-radioactivity measurements they attributed to the resynthesis of AMP intracellularly, after previous dephosphorylation of the AMP extracellularly and passage of the adenosine and phosphate across the membrane. It may be the ionic character of the nucleotides that prevents their free passage across the cell membrane. Our own investigations with ^{14}C -labelled AMP demonstrated that the adenine moiety of AMP can be taken up by perfused liver and subsequently be incorporated into nucleic acids. We could not, however, show that the AMP molecule had been taken up intact, although the metabolic changes observed are consistent with increased intracellular concentrations of AMP.

When we consider the results obtained from perfusing rat liver with AMP, we are confronted by two different situations, that in fed and that in starved rats. In fed rats there is a considerable glycogen store, which is used for the secretion of glucose into the bloodstream. In the presence of

AMP there is a marked stimulation of glucose output, which is coupled to a significant decrease in the glycogen content of the perfused livers. A rough calculation shows that over 60% of the glucose secreted comes from the breakdown of glycogen. The increased breakdown of glycogen is due to activation of glycogen phosphorylase by AMP (Krebs & Fischer, 1962; Sutherland & Wosilait, 1956; Bishop & Larnier, 1967). The remainder of the glucose output under these conditions presumably comes from the gluconeogenic pathway, for which the substrate is the amino acids in the perfusion medium. That there is an increased breakdown of amino acids in the presence of AMP is borne out by the increased production of urea into the perfusion medium.

There is no doubt that gluconeogenesis plays a large part in the production of glucose by the liver (Exton & Park, 1967). Exton & Park (1967) also showed that the rate of utilization of glucose by glycolysis in the liver is low compared with the rate of gluconeogenesis. In other words an increased glycolytic activity mediated through an AMP stimulation of phosphofructokinase is unlikely to have a great effect on the rate of glucose production. By using labelled lactate we have shown that AMP inhibits gluconeogenesis by about 30% in livers from both fed and starved rats, presumably through inhibition of fructose 1,6-diphosphatase (Underwood & Newsholme, 1965a). A number of glucogenic amino acids, however, feed into the gluconeogenic pathway at the oxaloacetate stage via the citric acid cycle. Since AMP may increase the turnover of the citric acid cycle this will effectively increase the production of oxaloacetate from amino acids. Hence by a mass-action effect the AMP inhibition of gluconeogenesis may be overcome in the presence of AMP without affecting the rate of utilization of lactate. We attempted to show that AMP increased the turnover of the citric acid cycle by perfusing livers with labelled amino acids and were able to show an increase in the synthesis of labelled glucose, which is in accordance with AMP increasing the turnover of the citric acid cycle and increasing the availability of labelled oxaloacetate for gluconeogenesis. We could show no increase of output of $^{14}\text{CO}_2$ from ^{14}C -labelled amino acids, but it is possible that the $^{14}\text{CO}_2$ is 'fixed' by the liver before it can be released. A possible site of fixation is urea synthesis, which is stimulated by AMP in perfused liver.

Lactate is the major gluconeogenic source of the liver. In the presence of high concentrations of lactate the glucose output of the liver increases greatly. It is therefore not surprising that the effects of AMP on livers perfused with lactate are somewhat moderated. There is an increase in glucose output in the presence of AMP and lactate

over that with lactate alone, but it is not so great as the stimulation with AMP alone. Presumably AMP inhibits the production of glucose from lactate, at the same time stimulating the production of glucose from glycogen.

In starved rats the picture is somewhat different. There is little or no stored glycogen in livers from starved rats, so that glucose output from glycogen is low. Starvation results in increased rates of gluconeogenesis and increased amounts of several of the gluconeogenic enzymes. The AMP stimulation of glucose output comes to some extent from the breakdown of residual glycogen, but more importantly from an increase in gluconeogenesis brought about by an increased availability of oxaloacetate, as discussed above. This is borne out by the greatly increased urea output in starved rats. Perfusion with lactate leads to an increase in glucose output equal in magnitude to the increased output seen with lactate in livers from fed rats (Tables 1 and 2). This corresponds to the glucose synthesized from lactate. With AMP and lactate there is no increase in glucose production over that with lactate alone. The increased output with AMP must be almost exactly balanced by the decrease in gluconeogenesis from lactate also caused by AMP. In livers from starved rats there may be a net synthesis of glycogen (Exton & Park, 1967), but since there were no detectable reserves of glycogen at the end of the perfusion the amount synthesized is obviously very small.

The overall effects of AMP on carbohydrate metabolism can be summarized as follows. There is an AMP-stimulated breakdown of glycogen mediated through glycogen phosphorylase, and an inhibition of gluconeogenesis from lactate mediated through fructose 1,6-diphosphatase. Gluconeogenesis from amino acids may, however, be stimulated because they enter the pathway via the citric acid cycle, which is speeded up by AMP, in contrast with lactate, which enters the pathway by the dicarboxylic acid shunt. The turnover of the citric acid cycle is speeded up by AMP, possibly through isocitrate dehydrogenase or through increased availability of ADP. AMP may also stimulate glycolysis through phosphofructokinase, although we have no evidence for this. The conversion of AMP into ADP by adenylate kinase means that those processes generating high-energy phosphate bonds will be speeded up. The presence of ADP will tend to increase the rate of turnover of the citric acid cycle and the rate of glycolysis.

AMP stimulated the production of urea by perfused livers. Urea production was much greater in livers from starved rats, indicating the breakdown of nitrogenous compounds in the absence of carbohydrate substrates. It seems likely that the stimulation of urea production by AMP is a

secondary effect and it could, for example, be brought about by an increased turnover of the citric acid cycle allowing the breakdown of more amino acids by this route.

The decreased incorporations, none of which were very large, into proteins and nucleic acids may be explained by the rapid destruction of their labelled precursors in AMP-treated livers, rather than by a specific inhibition of protein and nucleic acid synthesis. The case of orotic acid appears to be special in that all the adenine nucleotides strongly inhibit the uptake of the acid by the liver. This may reflect a particular mechanism of uptake for orotic acid. The fact that neither the protein-synthesizing ability of cell-free systems nor the polysome profiles were altered by perfusion with AMP suggests that the breakdown of labelled precursors may be the cause of the inhibition.

Analysis of nuclear-RNA and cytoplasmic-RNA fractions from AMP-perfused liver showed no qualitative differences in labelling patterns from those found in the controls, although the specific radioactivity of the fractions from AMP-perfused livers was considerably smaller. The labelling patterns of RNA in the control livers corresponded very closely to those found by pulse-labelling RNA *in vivo* (Penman *et al.* 1966; Henshaw *et al.* 1965). The AMP-stimulated breakdown of nucleic acid precursors was dependent on the particular precursor. AMP therefore may only affect certain breakdown pathways. The fact that uridine incorporation into nucleic acids appeared to be hardly affected at all by AMP is good evidence that AMP does not affect the ability of the liver to synthesize RNA. It appears that the effects of AMP on the uptake of protein and nucleic acid precursors are probably secondary.

Perfusion with adenosine, ADP and ATP did not result in such gross metabolic changes, suggesting that the effects are specific for AMP. All the effects of AMP on perfused liver that we have observed were either stimulations of pathways of ATP production (e.g. glycogenolysis, glycolysis and breakdown of nitrogenous compounds) or inhibitions of synthetic pathways that use ATP (e.g. gluconeogenesis). In agreement with other workers (Newsholme & Gevers, 1967) our results suggest that the concentration ratio of AMP to ATP in the cell may play an important role in controlling metabolic pathways that lead to the formation or destruction of ATP.

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