The Effect of Cyclic Adenosine 3', 5'-Monophosphate on Carbohydrate Metabolism of Rats[†]

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The effects of cyclic adenosine 3', 5'-monophosphate (c-AMP) on carbohydrate metabolism, especially gluconeogenesis, glycolysis, glycogenesis, and glycogenolysis in rats fed a stock diet (commercial pellets) were investigated. Increases in blood glucose levels in rats injected with two different doses of c-AMP intraperitoneally were observed. The rates of increase in blood glucose levels of rats administered 20 mg of c-AMP per 100 g of body weight were higher than those of rats administered 2 mg of c-AMP per 100 g of body weight. *In vivo* gluconeogenic capacity was increased by the injection of either dose of c-AMP. *In vivo* glucose oxidation was depressed and liver glycogen levels were decreased in rats administered 20 mg of c-AMP per 100 g of body weight but these changes were not recognized in rats administered 2 mg of c-AMP per 100 g of body weight. Renal gluconeogenesis was merely stimulated by the injection of the lower dose of c-AMP. Glucose formation from FDP and/or F6P in both liver and kidney cortex homogenates of rats was not stimulated by the injection of c-AMP.

Since c-AMP was discovered by Rall *et al.*¹⁾ and Cook *et al.*²⁾ in 1957, its physiological function has been investigated by many researchers. *In vitro*, c-AMP accelerated gluconeogenesis

in perfused rat livers,³⁻⁶⁾ rat kidney tubules,^{7,8)} kidney cortex slices,^{9,10)} and perfused kidneys.¹¹⁾ *In vivo*, c-AMP caused hyperglycemia of human beings,¹²⁾ dogs,^{13,14)} cats,¹⁵⁾ and rats,¹⁶⁻¹⁸⁾

† The abbreviations used are: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; c-AMP, cyclic adenosine 3', 5'-monophosphate; FDP, D-fructose 1, 6-diphosphate; F6P, D-fructose 6-phosphate; GTP, guanosine 5'-triphosphate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase [GTP-oxaloacetate carboxylase (transphosphorylating), EC 4.1.1.32]; PC, pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), EC 6.4.1.1.]; PK, pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40).

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however, the effect of c-AMP on *in vivo* gluconeogenesis has received little attention.

During the course of our study on the relationship between dietary composition and carbohydrate metabolism, especially gluconeogenesis,¹⁹⁻²¹⁾ it is tempting to suppose that c-AMP may be a mediator of major nutrients which exert control on hepatic and renal gluconeogenesis. In order to test this hypothesis, we have to show the stimulation of *in vivo* gluconeogenesis by c-AMP as the first step. In addition, the results of Northrop and Parks^{16,17)} reminded us of being necessary to check the effects of different doses of c-AMP on carbohydrate metabolism of rats.

This paper deals with the stimulation of *in vivo* gluconeogenesis by c-AMP injected into rats and the effects of two different doses (2 and 20 mg per 100 g of body weight) of c-AMP on carbohydrate metabolism of rats.

MATERIALS AND METHODS

Animals and diets. Female Donryu strain rats were purchased from Nippon Rat Co., Urawa. The rats were placed in individual cages at about 25°C and 12 hr per day of darkness and light. The animals were allowed to have a stock diet (pellets purchased from Central Laboratories for Experimental Animals, Tokyo) and water *ad libitum* until about 180 g in body weight. Experiments were started thereafter.

Materials. c-AMP-H₂O, ITP-Na₃, FDP-Na₃, F6P-Na₂, PEP-Na-H₂O, and oxaloacetic acid were purchased

from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Sodium pyruvate-3-¹⁴C (specific activity, 5~20 mCi/mmol) was obtained from Radiochemical Centre, Amersham, Buckinghamshire, England. Glucose-U-¹⁴C (specific activity, 4~10 mCi/mmol) was purchased from Daiichi Pure Chemicals Co., Tokyo. Chemicals were used without further purification.

Plan of experiments. For the determination of blood glucose, liver glycogen, and *in vivo* glucose formation from pyruvate-3-¹⁴C, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Fifteen min after injection of the anesthetic the animals were injected intraperitoneally either 2 or 20 mg of c-AMP dissolved in 0.15 M potassium phosphate buffer, pH 7.3, per 100 g of body weight. In the case of isotope experiments, sodium pyruvate-3-¹⁴C (1.25 μ Ci/0.5 mmole pyruvate/100 g body weight) was injected intraperitoneally together with c-AMP and the animal was kept in a glass metabolic chamber for collection of the expired carbon dioxide. Thirty min after injection of c-AMP, blood samples were obtained by heart puncture using heparin to prevent coagulation. Liver samples were taken immediately after killing the animals.

For the other assays, namely, the determination of glucose formation by means of tissue slices and tissue homogenates, activity of PEPCK, and oxidation of glucose-U-¹⁴C, an anesthetized rats were killed 30 min after the injection of c-AMP. In the case of isotope experiments, glucose-U-¹⁴C (2.5 μ Ci/100 g body weight) was injected intraperitoneally together with c-AMP and the animal was kept in a glass metabolic chamber.

Assay for blood glucose, liver glycogen, and rates of incorporation of radioactivity from pyruvate-3-¹⁴C into blood glucose and liver glycogen. Blood was deproteinized by the addition of an aliquot of 0.3 N perchloric acid and blood glucose was determined by a modification¹⁹⁾ of a glucose-oxidase method.²²⁾ For radioactivity assay, blood glucose was isolated and purified by a modification of the method of Friedman *et al.*²³⁾ Blood samples were deproteinized by the Somogyi's method²⁴⁾ and mixtures were kept in a boiling water bath for 5 min. After centrifugation at

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2500 rpm for 10 min an aliquot of supernatant was passed through an Amberlite MB-2 column (0.8×15 cm). The column was eluted with deionized water and the first 15 ml of the eluates was collected. Aliquots of the purified eluate of the supernatant were added into 10 ml of Bray's solution²⁵⁾ in 20-ml counting vials. Blood glucose-¹⁴C was counted in a Packard Tri-Carb model 500 type liquid scintillation spectrophotometer. In preliminary experiments blood glucose was either precipitated as phenylhydrazine and crystalline glucosotriazoles prepared for counting²⁶⁾ or counted as described above. Similar results were obtained by either method. Total amount of blood glucose was calculated on the assumption that the glucose is essentially that of the extracellular fluid, taken to be 30 % of the body weight.^{23,27)} Liver glycogen was isolated according to Hassid *et al.*²⁸⁾ For radioactivity assay, crude glycogen was purified by a modification³⁰⁾ of the method of Stetten and Boxer.³¹⁾ An aliquot of purified glycogen was added in 3 N sulfuric acid and hydrolyzed in a boiling water bath for 2 hr.³²⁾ After being neutralized, glucose-¹⁴C was purified by passing through an Amberlite MB-2 column and counted as described for blood glucose-¹⁴C.

Assay for respiratory ¹⁴CO₂. The respiratory carbon dioxide was collected for 30 min after the injection of pyruvate-3-¹⁴C or glucose-U-¹⁴C by trapping into a mixture of ethanolamine and ethylene glycol monomethyl ether (1:2 v/v).³³⁾ Aliquots of the mixture were transferred to 20-ml counting vials. Ten ml of Bray's solution were added and ¹⁴C was determined with the liquid scintillation spectrophotometer.

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Glucose formation in liver and kidney cortex slices. Preparation of slices, incubation procedures and the determination of glucose were described in the previous papers.^{19,21)}

Glucose formation in liver and kidney cortex homogenates. Preparation of homogenates and incubation procedures were described in the previous paper.²⁰⁾

Measurement of PEPCK activity. Preparation of enzyme extracts and assay of enzyme activity were described in the previous paper.²¹⁾

RESULTS

Levels of blood glucose and liver glycogen in rats injected with 2 mg and 20 mg of c-AMP per 100 g of body weight are summarized in Tables I and II, respectively, together with rates of incorporation of radioactivity from pyruvate-¹⁴C into blood glucose and liver glycogen. Levels of blood glucose in rats injected with either dose of c-AMP were significantly higher than those of controls ($p < 0.001$). The higher the injected doses of c-AMP, the more increase in levels of blood glucose were observed. Rats injected with 2 mg of c-AMP per 100 g of body weight maintained normal levels of liver glycogen but rats injected with 20 mg of c-AMP per 100 g of body weight showed a significant depression in levels of glycogen ($p < 0.001$). The rates of incorporation of radioactivity into blood glucose from pyruvate-3-¹⁴C increased in rats injected with either dose of c-AMP than those of controls ($p < 0.01$). The rates of incorporation into liver glycogen, however, significantly decreased only in rats injected with 20 mg of c-AMP per 100 g of body weight as compared with those of controls ($p < 0.001$).

Table III shows the effect of c-AMP on *in vivo* oxidation of pyruvate-3-¹⁴C and glucose-U-¹⁴C. The rates of incorporation of radioactivity from pyruvate-3-¹⁴C into respiratory carbon dioxide of rats injected with either dose of c-AMP were similar to those of

TABLE I. EFFECT OF CYCLIC 3', 5'-AMP INJECTION (2 mg/100 g B. W.) ON LEVELS OF BLOOD GLUCOSE AND LIVER GLYCOGEN AND RATES OF INCORPORATION OF RADIOACTIVITY FROM PYRUVATE-3-¹⁴C INTO BLOOD GLUCOSE AND LIVER GLYCOGEN OF RATS

	without c-AMP (controls)	with c-AMP (2 mg/100 g B. W.)	<i>p</i> ^{b)}
Concentration of:	mg per 100 ml blood		
Blood glucose	85.9±8.02 ^{a)} (5)	115±7.36(5)	<0.001
	mg per g wet liver		
Liver glycogen	42.7±4.42(4)	39.9±3.37(4)	NS
Incorporation of radioactivity into:	% recovery of ¹⁴ C administered		
Blood glucose	4.38±0.727(4)	7.15±1.42(5)	<0.01
Liver glycogen	0.121±0.0682(4)	0.0885±0.0356(5)	NS

^{a)} Mean±standard deviation for number of rats indicated in parentheses.^{b)} Probability of difference between controls and c-AMP administered rats being significant by *t* test. NS; not significant.TABLE II. EFFECT OF CYCLIC 3', 5'-AMP INJECTION (20 mg/100 g B. W.) ON LEVELS OF BLOOD GLUCOSE AND LIVER GLYCOGEN AND RATES OF INCORPORATION OF RADIOACTIVITY FROM PYRUVATE-3-¹⁴C INTO BLOOD GLUCOSE AND LIVER GLYCOGEN OF RATS

	without c-AMP (controls)	with c-AMP (20 mg/100 g B. W.)	<i>p</i> ^{b)}
Concentration of:	mg per 100 ml blood		
Blood glucose	74.9±11.5 ^{a)} (4)	203±7.53(5)	<0.001
	mg per g wet liver		
Liver glycogen	49.6±1.86(7)	38.1±2.03(6)	<0.001
Incorporation of radioactivity into:	% recovery of ¹⁴ C administered		
Blood glucose	3.80±1.19(7)	6.85±2.03(6)	<0.01
Liver glycogen	0.191±0.0518(4)	0.0731±0.0275(4)	<0.001

^{a), b)} See Table I, footnotes 1 and 2.TABLE III. EFFECT OF CYCLIC 3', 5'-AMP INJECTION ON OXIDATION OF PYRUVATE-3-¹⁴C AND GLUCOSE-U-¹⁴C IN RATS

	without c-AMP (controls)	with c-AMP (2 mg/100 g B. W.)	without c-AMP (controls)	with c-AMP (20 mg/100 g B. W.)
Incorporation of radioactivity into ¹⁴ CO ₂ from:	% recovery of ¹⁴ C administered			
Pyruvate-3- ¹⁴ C	5.38±0.93 ^{a)} (4)	4.54±0.44 (5)	5.06±0.72 (7)	4.69±0.96 (5)
Glucose-U- ¹⁴ C	8.78±1.57 (5)	8.53±2.34 (5)	6.30±1.06 (4)	3.57±1.48 ^{b)} (5)

^{a)} See Table I, footnote 1.^{b)} Differs significantly from corresponding values of controls at the level of 2 %, by *t* test.

TABLE IV. EFFECT OF CYCLIC 3', 5'-AMP INJECTION (2 mg/100 g B. M.) ON GLUCOSE FORMATION FROM PYRUVATE IN RAT LIVER SLICES

without c-AMP (controls)	with c-AMP (2 mg/100 g B. W.)	p^b
μ moles of extra glucose formed/hr/liver/ 100 g body weight		
12.8 ± 6.82^{a1} (5)	37.9 ± 16.6 (5)	< 0.02

$a1, b1$ See Table I, footnotes 1 and 2.

controls. Rats injected with 2 mg of c-AMP per 100 g of body weight maintained normal rates of incorporation from glucose- $U-^{14}C$ into respiratory carbon dioxide but rats injected with 20 mg of c-AMP per 100 g of body weight showed a significant depression in rates of incorporation from glucose- $U-^{14}C$ into

respiratory carbon dioxide ($p < 0.02$).

In liver slices of rats injected with 2 mg of c-AMP per 100 g of body weight, glucose formation from pyruvate was stimulated (Table IV), while net glucose formation from F6P and/or FDP in liver homogenates of rats injected with either dose of c-AMP was similar to controls. Activities of hepatic PEPCK of rats injected 20 mg of c-AMP per 100 g of body weight increased significantly ($p < 0.01$, Table V).

In kidney cortex slices of rats injected with 2 mg of c-AMP per 100 g of body weight, glucose formation from pyruvate increased significantly ($p < 0.01$), while those injected with 20 mg of c-AMP per 100 g of body weight showed similar capacity of glucose formation from pyruvate as compared with

TABLE V. EFFECT OF CYCLIC 3', 5'-AMP INJECTION ON NET GLUCOSE FORMATION FROM FRUCTOSE 6-PHOSPHATE AND FRUCTOSE 1, 6-DIPHOSPHATE IN LIVER HOMOGENATES AND HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY OF RATS

	without c-AMP (controls)	with c-AMP (2 mg/100 g B. W.)	without c-AMP (controls)	with c-AMP (20 mg/100 g B. W.)
μ moles of extra glucose formed/hr/liver/100 g body weight				
Glucose formed from:				
F6P	2860 ± 49.9^{a1} (4)	2830 ± 115 (3)	2860 ± 49.9 (4)	2830 ± 279 (4)
FDP	876 ± 64.8 (4)	911 ± 142 (4)	876 ± 64.8 (4)	822 ± 101 (4)
μ moles of PEP formed/min/liver/100 g body weight				
Activity of PEPCK	18.5 ± 1.22 (4)	19.9 ± 0.88 (4)	16.2 ± 5.30 (4)	26.4 ± 3.69^{b1} (3)

$a1$ See Table I, footnote 1.

$b1$ Differs significantly from corresponding values of controls at the level of 1 %, by t test.

TABLE VI. EFFECT OF CYCLIC 3', 5'-AMP INJECTION ON GLUCOSE FORMATION FROM PYRUVATE AND PYRUVATE CONSUMPTION IN KIDNEY CORTX SLICES OF RATS

	with c-AMP (2 mg/100 g B. W.)	without c-AMP (controls)	with c-AMP (20 mg/100 g B. W.)
μ moles of extra glucose formed from pyruvate/hr/g dry kidney cortex			
Glucose formation	269 ± 14.5^{a1} (5)	232 ± 17.0 (5)	242 ± 31.1 (4)
μ moles of pyruvate taken up/hr/g dry kidney cortex			
Pyruvate consumption	2300 ± 242 (5)	2330 ± 239 (5)	2170 ± 188^{b1} (4)

$a1$ See Table I, footnote 1.

$b1$ Differs significantly from corresponding values of controls at the level of 1 % by t test.

TABLE VII. EFFECT OF CYCLIC 3', 5'-AMP INJECTION ON NET GLUCOSE FORMATION FROM FRUCTOSE 6-PHOSPHATE AND FRUCTOSE 1, 6-DIPHOSPHATE IN KIDNEY CORTEX HOMOGENATES AND RENAL PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY OF RATS

	without c-AMP (controls)	with c-AMP (2 mg/100 g B. W.)	without c-AMP (controls)	with c-AMP (20 mg/100 g B. W.)
Glucose formed from:	μ moles of extra glucose formed/hr/g wet kidney cortex			
F6P	347 $\pm$ 50.8 ^{a)} (4)	317 $\pm$ 13.4 (3)	347 $\pm$ 50.8 (4)	327 $\pm$ 44.6 (4)
FDP	131 $\pm$ 12.7 (4)	139 $\pm$ 13.1 (4)	131 $\pm$ 12.7 (4)	136 $\pm$ 27.9 (4)
Activity of PEPCK	μ moles of PEP formed/min/g wet kidney cortex			
	9.58 $\pm$ 1.41 (4)	9.01 $\pm$ 0.27 (4)	8.81 $\pm$ 2.59 (3)	7.19 $\pm$ 0.72 (3)

^{a)} See Table I, footnote 1.

controls (Table VI), although consumption of pyruvate by means of slices were similar among groups with or without c-AMP injection. Net glucose formation from F6P and/or FDP in kidney cortex homogenates and activities of renal PEPCK were not different among groups with or without c-AMP injection (Table VII).

DISCUSSION

The data described in Tables I and II indicate that there was a significant increase of 34% and 171% in levels of blood glucose in rats injected with 2 mg and 20 mg of c-AMP per 100 g of body weight, respectively. A possible explanation for the difference in the rates of increase in levels of blood glucose is that different dose responses of c-AMP is effective for each pathway of carbohydrate metabolism of rats. Although the higher dose of c-AMP not only accelerates gluconeogenesis and hepatic glycogenolysis but also depresses the oxidation of glucose and hepatic glycogenesis, c-AMP just accelerates gluconeogenesis at the lower dose. These results do not support the suggestion of Northrop and Parks^{16,17)} and Levine¹⁸⁾ that the hyperglycemic effect of c-AMP injection into rats was a stimulation of hepatic glycogenolysis.

Tables I and II provide experimental

evidence that c-AMP accelerates *in vivo* gluconeogenesis. Tissue slice studies with pyruvate as substrate demonstrate that rats injected with 2 mg of c-AMP per 100 g of body weight have increased capacities of hepatic and renal gluconeogenesis (Tables IV and VI). Animal cells has been considered relatively impermeable to exogenous c-AMP. Levine *et al.*³⁴⁾ showed, however, that c-AMP-8-¹⁴C added to the perfusate appeared intact in bile and expired carbon dioxide was labeled with ¹⁴C in the perfused rat liver. Moreover, Schaeffer *et al.*³⁵⁾ isolated tritiated c-AMP injected intraarterially into rats from liver, skeletal muscle, and epididymal fat pad. These data support the hypothesis that some exogenously administered c-AMP can enter cells in sufficient quantity to cause biological effects. In the liver, there is some good evidence for a direct action of exogenous c-AMP on glycogenolysis^{18,35)} and gluconeogenesis.⁶⁾ The main sites of gluconeogenesis in mammals are the liver and kidney cortex.³⁶⁾ In the kidney, Bowman¹¹⁾ showed a direct action of exogenous c-AMP on gluconeogenesis. While kidney cortex slices

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FIG. 1. Dose Responses of Cyclic 3', 5'-AMP Added *in vitro* on Glucose Formation from Pyruvate and Pyruvate Consumption in Rat Kidney Cortex Slices.

The blank circles indicate values of glucose formed and the solid circles those of pyruvate consumption. Values are expressed as % of controls. The vertical lines represent standard deviation of mean for number of rats in parentheses.

*,** Differs significantly from corresponding values of controls at the levels of 5 and 2%, respectively, by *t* test.

of rats injected with 2 mg of c-AMP per 100 g of body weight had an increased gluconeogenic capacity, slices of rats injected with 20 mg of c-AMP per 100 g of body weight did not change their gluconeogenic capacity as compared with controls (Table VI). As discussed later, concentration-dependent stimulatory and inhibitory effects of *in vivo* added c-AMP on renal gluconeogenesis from pyruvate may account for some parts of these results (Fig. 1).

The data shown in Tables V and VII suggest the sequence of reactions converting FDP or F6P to glucose is not likely to be important as the control site (or sites) at which c-AMP acts to stimulate hepatic and renal gluconeogenesis from pyruvate. From the measurement of levels of intermediary metabolites in rat liver perfused with or

without c-AMP, Exton and Park³⁷⁾ showed that the reaction limiting maximum rate of gluconeogenesis from lactate is not situated between triose phosphates and glucose but between pyruvate and PEP. They suggested PEPCK as a rate-limiting enzyme. On the contrary, Williamson *et al.*³⁷⁾ suggested PC as a rate-limiting enzyme and Rasmussen and Tenenhouse³⁸⁾ suggested a combined effect of PC stimulation and PK inhibition. Wicks *et al.*³⁹⁾ demonstrated that the injection of N⁶, O^{2'}-dibutyryl c-AMP stimulated activities of PEPCK and a few amino acid degrading enzymes of the rat liver. Tables V and VII show that c-AMP just stimulates the activity of hepatic PEPCK of rats administered the higher dose of c-AMP and exerts no effect on renal PEPCK, even though c-AMP increases *in vivo* gluconeogenic capacity from pyruvate of rats administered either the higher or lower dose of c-AMP and the renal gluconeogenic capacity increases in rats administered the lower dose. We can not, therefore, point out the exact enzyme reaction by which c-AMP exerts control on hepatic and renal gluconeogenesis from pyruvate.

Mallette *et al.*⁴⁰⁾ proposed that c-AMP acts to stimulate both the transport of amino acids into the hepatic cell and the conversion of pyruvate to PEP in the perfused rat liver. Consequently, the transport of pyruvate into the hepatic and renal cells should be considered as an alternative control point on gluconeogenesis from pyruvate. In kidney cortex slice studies, however, c-AMP does not exert any effect on the consumption of pyruvate from a medium in which slices are floated. This means c-AMP does not act to

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39) W. D. Wicks, E. T. Kenney and K. L. Lee, *J. Biol. Chem.*, **244**, 6008 (1969).

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stimulate or inhibit the transport of pyruvate in rat kidney cortex (Fig. 1).

The reason why the lower dose of c-AMP administered to rats stimulates renal gluconeogenesis from pyruvate and the higher dose exerts no effect can be explained as follows. Different effects of various concentrations of c-AMP added *in vitro* on gluconeogenesis from pyruvate in rat kidney cortex slices are shown in Fig. 1; namely, stimulation at 10^{-6} M,

inhibition at 10^{-3} M, and no effect at 10^{-8} and 10^{-5} M. In rats administered 2 mg of c-AMP per 100 g of body weight, the concentration of c-AMP in the kidney cortex cells may be similar to that in slices incubated with 10^{-6} M c-AMP and in rats administered 20 mg of c-AMP per 100 g of body weight, the concentration of c-AMP may be similar to that of slices incubated with 10^{-5} M c-AMP.