# Inhibition of Hepatic Lipogenesis by Adenine Nucleotides

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## ABSTRACT

Incubation of liver slices and isolated liver cells with adenosine cyclic-3′,5′-monophosphate at concentrations which inhibit lipogenesis was found to expand the pool size of the noncyclic adenine nucleotides in the intact cells of the preparations. This observation led to studies which demonstrated that adenosine and adenosine-5′-monophosphate also inhibited lipogenesis and expanded the adenine nucleotide pool size. It is proposed but not proven that the increase in intracellular nucleotides produced by adenosine-5′-monophosphate, adenosine cyclic-3′,5′-monophosphate, and adenosine may have an adverse effect upon the synthesis of fatty acids. Because of the expansion of the adenine nucleotide pool size, high concentrations of adenosine cyclic-3′,5′-monophosphate should not be used to investigate the mechanism responsible for hormonal regulation of lipogenesis. As an added complication, exogenous adenosine-5′-monophosphate was found to produce a small but significant increase in the intracellular concentration of adenosine cyclic-3′,5′-monophosphate of isolated liver cells. This effect also may be a factor in the inhibition of lipogenesis by adenosine-5′-monophosphate. Low concentrations of $N^6$,$O^{2'}$-dibutyryl adenosine cyclic-3′,5′-monophosphate were found to inhibit lipogenesis without increasing the intracellular adenine nucleotide content of either liver slices or isolated liver cells. It is concluded that studies on the mechanism of glucagon regulation of lipogenesis should be carried out with glucagon or low concentrations of $N^6$,$O^{2'}$-dibutyryl adenosine cyclic-3′,5′-monophosphate.

## INTRODUCTION

Adenosine cyclic-3′,5′-monophosphate (cyclic AMP) has been reported in a number of studies (1-4) to inhibit lipogenesis in liver tissue preparations. An inhibitory action of the cyclic nucleotide on acetyl CoA carboxylase (E.C. 6.4.1.2) has been suggested to account for the inhibition of lipogenesis (5,6). In addition, an inhibition of aerobic glycolytic activity has been shown to be responsible in part for $N^6$,$O^{2'}$-dibutyryl adenosine-3′,5′-monophosphate (dibutyryl cyclic AMP) and glucagon inhibition of lipogenesis (7). On the other hand, Raskin, et al., (8) have seriously questioned the physiological significance of this response to glucagon and cyclic AMP. We demonstrate here that high concentrations of cyclic AMP expand the intracellular nucleotide pool size, and, therefore, should not be used in studies on the mechanism responsible for glucagon inhibition of lipogenesis.

## METHODS AND MATERIALS

Male Wistar rats weighing ca. 200 g were starved for 48 hr and refed for 48 hr on a high sucrose diet ("Fat-Free" test diet, Nutritional Biochemicals Corp., Cleveland, OH) (9). Liver slices were prepared with a Harvard Tissue slicer. Isolated liver cells were prepared by the method of Berry and Friend (10) with modifications described previously (7). Incubations were carried out in Krebs-Henseleit saline equilibrated with 95% oxygen:5% carbon dioxide in a Dubnoff metabolic shaking incubator at 37.5 C. In experiments conducted with isolated cells, the medium was supplemented with 2.5% albumin which had been charcoal treated to remove the fatty acids (11).

Incubations conducted for determining the rate of fatty acid synthesis were carried out in 4 ml of medium containing 100-200 mg of slices or isolated cells wet wt and 1 mCi of $^3$HOH. Incubations were terminated by the addition of 0.4 ml of 50% (w/v) perchloric acid. The precipitate was extracted twice for total lipids with methanol:chloroform (2:1) as described by Kates (12). The total lipids were saponified at 73 C with a solution which was 0.3 M in NaOH and contained 90% (v/v) methanol (12). The nonsaponifiable fraction was extracted and discarded. Fatty acids were extracted and counted for radioactivity with a scintillation counter. Benzene was added to the total lipid extract and to the final fatty acid extract before complete evaporation of the solvent under nitrogen. Addition of benzene reduced zero time controls to background levels of radioactivity as traces of water were removed more effectively. Calculations of the rate of fatty acid synthesis were based on the assumption (13) that 1.0 μmole of $^3$HOH incorporated

corresponded to 1.15 μmole of acetyl groups incorporated.

In studies involving the determination of tissue adenine nucleotide and metabolite concentrations of tissue slices, incubations were conducted in triplicate and terminated after 30 min by rapid filtration of the slices from the incubation medium with a tea strainer. The slices were tapped cleanly onto a liquid nitrogen cooled freeze clamp and frozen with a second clamp. In this manner, the slices were isolated from the medium and frozen solid in less than 3 sec. The slices were homogenized in 6% perchloric acid with a Polytron PT-10 homogenizer operated at full speed for 20 sec. Residues obtained after centrifugation were re-extracted with 6% perchloric acid. The combined extracts were adjusted rapidly to pH 5-6 with 20% KOH and centrifuged to remove potassium perchlorate. In studies involving the determination of the adenine nucleotide content of isolated liver cells, incubations were terminated by rapid separation of the cells from the medium by centrifugation into 6% perchloric acid with the hepatocyte separation tubes described in detail previously (14). Perchloric acid extracts of the cells were treated as described above for slices. Inorganic phosphate was determined by the method of Martin and Doty as described by Lindberg and Ernster (15). Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and α-ketoglutarate were determined by enzymatic analysis using methods described by Williamson and Corkey (16); lactate, malate, and pyruvate were determined as described by Hohorst, et al., (17); and citrate was determined as described by Möllering and Gruber (18). Cytoplasmic-free nicotinamide adenine dinucleotide ($NAD^+$) to NADH ratios were calculated from the concentrations of lactate and pyruvate and the equilibrium constant of lactate dyhydrogenase (19).

For the determination of cyclic AMP, isolated liver cells were incubated and then centrifuged into 5% trichloroacetic acid via the hepatocyte separation tubes. Tritiated, general labeled, cyclic AMP in tracer quantities was added to the extract to determine percent recoveries. Purification of the extracts was carried out by column chromatography on AG 1-X8 formate as described by Kneer, et al., (20). Cyclic AMP content was determined by the protein kinase binding assay of Gilman (21,22). The quantity of other adenine nucleotides in the purified extracts was established by enzymatic analysis to be too low to interfere with the determination of cyclic AMP.

The oxidation of succinate to malate was used to estimate the viability of liver slice preparations (14). The slices, 100 mg wet wt in a volume of 8 ml of Krebs-Henseleit saline, were preincubated for 5 min at 37 C. Succinate was added to the flasks at an initial concentration of 20 mM and the incubation terminated after 2 and 5 min with perchloric acid. The rates of malate accumulation by slices were compared with the rates by homogenates prepared with a Potter-Elvehjem homogenizer in which all cells were established to be broken by failure to exclude trypan blue.

Radioactive 5′-AMP was prepared enzymatically from [$^{14}C_8$] adenosine triphosphate by the combined action of purified mitochondrial ATPase ($F_1$) and adenylate kinase (E.C. 2.7.4.3). Purification was carried out by ascending paper chromatography with the solvent system of n-butanol:acetone:acetic acid: aq. 5% $NH_4OH$:$H_2O$: 0.1 M ethylene diamine tetracetic acid (EDTA), (45:15:10:10:19:1, v/v) (23). [$^{14}C_8$] Adenosine cyclic 3′,5′-monophosphate was purified prior to use by ascending paper chromatography with the solvent system of isopropanol:$NH_4OH$:$H_2O$ (7:1:2, v/v). Radioactive adenine nucleotides of perchloric acid extracts of tissue slices were separated by column chromatography on AG 1-X8 formate by the method described by Groot and Van den Bergh (24). All radioactive isotopes were obtained from New England Nuclear (Boston, MA). All enzymes, except the purified ATPase, and most chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

## RESULTS

### Studies with Liver Slices

In an attempt to gain insight into the mechanism responsible for cyclic AMP inhibition of lipogenesis, freeze stop studies were conducted with liver slices incubated with and without cyclic AMP. As shown in Table I, cyclic AMP was found to produce a substantial increase in the ATP, ADP, AMP, and inorganic phosphate content of freeze clamped liver slices. In contrast, significant decreases were observed in the concentrations of lactate, pyruvate, malate, citrate, and α-ketoglutarate. The cytoplasmic-free $NAD^+$ to NADH ratio also was decreased significantly by the cyclic nucleotide.

Adenosine-5′-monophosphate (5′-AMP) also was found to inhibit lipogenesis (Table II) and to increase the adenine nucleotide content of liver slices (Table I). As shown in Table II, both cyclic AMP and 5′-AMP brought about an inhibition of lipogenesis at similar concentrations with a trend noted for cyclic AMP to be more effective. The changes in metabolite levels

TABLE I

Effect of 3′,5′-cyclic AMP and 5′-AMP on the Adenine Nucleotide and Metabolite Levels of Liver Slices[a]

| Metabolite or metabolite ratio[b] | Experiment I | | Experiment II | |
|---|---|---|---|---|
| | No addition (μmol/g) | 3′,5′-cyclic AMP (μmol/g) | No addition (μmol/g) | 5′-AMP (μmol/g) |
| ATP | 0.67 ± 0.07 | 0.88 ± 0.08[c] | 0.61 ± 0.02 | 0.81 ± 0.03[c] |
| ADP | 0.24 ± 0.01 | 0.39 ± 0.02[c] | 0.23 ± 0.02 | 0.28 ± 0.01[c] |
| AMP | 0.09 ± 0.01 | 0.14 ± 0.02[c] | 0.07 ± 0.01 | 0.05 ± 0.01 |
| Σ ATP + ADP + AMP | 1.00 ± 0.10 | 1.51 ± 0.10[c] | 0.91 ± 0.02 | 1.17 ± 0.04[c] |
| Inorganic phosphate | 3.2 ± 0.3 | 3.9 ± 0.3[c] | 3.6 ± 0.3 | 5.6 ± 0.3[c] |
| Lactate | 2.6 ± 0.4 | 1.4 ± 0.2[c] | 2.3 ± 0.2 | 2.0 ± 0.2 |
| Pyruvate | 0.16 ± 0.01 | 0.04 ± 0.01[c] | 0.14 ± 0.01 | 0.07 ± 0.01[c] |
| Malate | 0.61 ± 0.06 | 0.30 ± 0.05[c] | 0.58 ± 0.04 | 0.45 ± 0.09 |
| Citrate | 0.19 ± 0.03 | 0.12 ± 0.02[c] | 0.14 ± 0.03 | 0.08 ± 0.01[c] |
| α-ketoglutarate | 0.12 ± 0.01 | 0.05 ± 0.01[c] | 0.09 ± 0.02 | 0.05 ± 0.01[c] |
| Free [NAD]/[NADH]cyto | 603 ± 63 | 278 ± 64[c] | 540 ± 72 | 341 ± 33[c] |

[a]The initial concentrations were 5 mM for 3′,5′-cyclic adenosine monophosphate (AMP) in Experiment I and 5 mM for 5′-AMP in Experiment II. Concentrations of metabolites are expressed as μmol/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 6 animals in Experiment I and 5 animals in Experiment II. Values which are significantly ($P < 0.05$) different from the no addition controls by the Student's t test for paired data.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; NAD = nicotinamide adenine dinucleotide.

[c]Values which are significantly ($P<0.05$) different from the no additions controls by the student's t test for paired data.

TABLE II

Effect of 5′-AMP, 3′, 5′-cyclic AMP, and Dibutyryl Cyclic AMP on Fatty Acid Synthesis by Liver Slices[a]

| Addition (mM) | Rate of fatty acid synthesis (nmoles/min/g wet wt) |
|---|---|
| None | 52 ± 12 |
| 5′-AMP (0.5) | 32 ± 13 |
| 5′-AMP (2.5) | 7 ± 3 |
| 5′-AMP (5.0) | 8 ± 2 |
| 3′, 5′-cAMP (0.5) | 18 ± 5 |
| 3′, 5′-cAMP (2.5) | 4 ± 1 |
| 3′, 5′-cAMP (5.0) | 2 ± 1 |
| Dibutyryl cyclic AMP (0.05) | 3 ± 1 |

[a]AMP = adenosine monophosphate; rates of lipogenesis are expressed as nmoles of "$C_2$" units incorporated into fatty acids/min/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 3 animals. Rates were determined for the last 30 min of 60 min incubations with the additions listed. All values are significantly ($P < 0.05$) different from the no addition control by the Student's t test for paired data.

caused by 5′-AMP (Table I) mimicked in part those produced by cyclic AMP. Significant increases were found in ATP, ADP, and inorganic phosphate levels, whereas, significant decreases were found in pyruvate, citrate, α-ketoglutarate, and the cytoplasmic-free $NAD^+$ to NADH ratio. However, significant changes were not induced by 5′-AMP in the liver slice content of lactate, malate, and 5′-AMP. The lack of effect on the 5′-AMP content was surprising in view of the fact that the slices were not washed free of the external medium. However, much of the exogenous 5′-AMP had been lost from the medium during the incubation.

Experiments conducted with radioactive cyclic AMP and 5′-AMP demonstrated that uptake and phosphorylation of these nucleotides accounted for the observed increase in the intracellular content of ATP (Table III). Based on the specific activity of the exogenous nucleotides and the total radioactivity of the isolated ATP, ca. 75% of the total ATP of the slices was derived from the exogenous nucleotides. No attempt was made to determine whether the nucleotides were dephosphorylated prior to penetration into the cells (see Discus-

TABLE III

Incorporation of [$^{14}C_8$]-AMP and [$^{14}C_8$]-cyclic AMP into the ATP of Liver Slices[a]

| Addition | Total ATP (μmol/g wet wt) | ATP derived from exogenous nucleotide (μmol/g wet wt) | ATP derived from exogenous nucleotide (%) |
|---|---|---|---|
| None | 0.54 | ------- | ------- |
| [$^{14}$C]AMP (5 mM) | 0.88 | 0.76 | 77 |
| [$^{14}$C]cyclic AMP (5 mM) | 0.93 | 0.68 | 73 |

[a]AMP = adenosine monophosphate; ATP = adenosine triphosphate; incubations were conducted for 60 min in duplicate with 500 mg wet wt liver slices per flask. Incubations were terminated by freeze clamping. Perchloric acid extracts were analyzed for the quantity and radioactivity of ATP.

TABLE IV

Effect of Dibutyryl Cyclic AMP on the Adenine Nucleotide and Metabolite Levels of Liver Slices[a]

| Metabolite or metabolite ratio[b] | No addition (μmol/g) | Dibutyryl cyclic AMP (μmol/g) |
|---|---|---|
| ATP | 0.41 ± 0.06 | 0.37 ± 0.02 |
| ADP | 0.22 ± 0.02 | 0.22 ± 0.04 |
| AMP | 0.09 ± 0.02 | 0.12 ± 0.02 |
| Σ ATP + ADP + AMP | 0.72 ± 0.07 | 0.71 ± 0.06 |
| Inorganic phosphate | 2.16 ± 0.54 | 2.64 ± 0.44 |
| Lactate | 1.4 ± 0.3 | 0.66 ± 0.20[c] |
| Pyruvate | 0.12 ± 0.01 | 0.05 ± 0.06[c] |
| Malate | 0.34 ± 0.07 | 0.25 ± 0.05[c] |
| Citrate | 0.10 ± 0.02 | 0.08 ± 0.01 |
| α-ketoglutarate | 0.06 ± 0.01 | 0.04 ± 0.01 |
| free [NAD]/[NADH]cyto | 878 ± 120 | 969 ± 303 |

[a]The initial concentration of dibutyryl cyclic adenosine monophosphate (AMP) was 0.05 mM. Concentrations of metabolites are expressed as μmol/g wet wt of slices, as means ± S.E.M. for liver slices prepared from 4 animals incubated for 30 min.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate; NAD = nicotinamide adenine diphosphate.

[c]Values which are significantly ($P < 0.05$) different from controls by the Student's t test for paired data.

TABLE V

Effect of 5′-AMP, Cyclic AMP, ADP, ATP, Adenosine and Dibutyryl Cyclic AMP on Fatty Acid Synthesis by Isolated Cells[a]

| Addition (mM) | Rate of fatty acid synthesis nmoles/min/gm wet wt |
|---|---|
| None | 260 ± 40 |
| 5′-AMP (0.1) | 170 ± 30 |
| 5′-AMP (0.5) | 20 ± 10 |
| Cyclic AMP (0.1) | 100 ± 10 |
| Cyclic AMP (0.5) | 60 ± 10 |
| ADP (0.1) | 140 ± 10 |
| ADP (0.5) | 20 ± 10 |
| ATP (0.1) | 100 ± 50 |
| ATP (0.5) | 10 ± 5 |
| Adenosine (0.1) | 100 ± 20 |
| Adenosine (0.5) | 20 ± 10 |
| Dibutyryl cyclic AMP (0.05) | 50 ± 10 |

[a]AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate. Rates of lipogenesis are expressed as nmoles of "$C_2$" units incorporated into fatty acids/min/g wet wt of cells, as means ± S.E.M. for isolated cells prepared from 3 animals. Rates were determined for the last 30 min of 60 min incubations with the additions listed. All values are statistically ($P < 0.05$) different from the no addition control by the Student's t test for paired data.

TABLE VI

Effect of Cyclic AMP, 5′-AMP, Adenosine, and Dibutyryl Cyclic AMP on the Adenine Nucleotide Content of Isolated Liver Cells[a]

| Addition (mM) | ATP[b] (μmol/g) | ADP[b] (μmol/g) | AMP (μmol/g) | ΣATP + ADP + AMP (μmol/g) |
|---|---|---|---|---|
| None | 2.46 ± 0.11 | 1.09 ± 0.07 | 0.38 ± 0.18 | 3.93 ± 0.21 |
| Cyclic AMP (0.5) | 3.76 ± 0.37[c] | 1.57 ± 0.08[c] | 0.50 ± 0.04 | 5.83 ± 0.42[c] |
| 5′-AMP (0.5) | 4.37 ± 0.49[c] | 1.54 ± 0.19 | 0.48 ± 0.03 | 6.39 ± 0.69[c] |
| Adenosine (0.5) | 3.96 ± 0.39[c] | 1.67 ± 0.05[c] | 0.50 ± 0.03 | 6.13 ± 0.41[c] |
| Dibutyryl cyclic AMP (0.05) | 2.33 ± 0.03 | 1.17 ± 0.04 | 0.45 ± 0.04 | 3.95 ± 0.07 |

[a]AMP = adenosine monophosphate; values are expressed as μmol/g wet wt of liver cells, as means ± S.E.M. for liver cells prepared from 3 animals and incubated for 45 min.

[b]ATP = adenosine triphosphate; ADP = adenosine diphosphate.

[c]Values which are significantly ($P < 0.05$) different from no addition controls by the Student's t-test for paired data.

sion). Nevertheless, it can be concluded that the cells of liver slices take up at least the adenine moiety of 5′-AMP and cyclic AMP and that this uptake leads to an increase in the pool size of adenine nucleotides within liver slices.

As reported by other investigators (3) and previously (7), dibutyryl cyclic AMP inhibited lipogenesis at much lower concentrations than cyclic AMP (Table II). At such low concentrations, dibutyryl cyclic AMP was not found to affect the adenine nucleotide content of liver slices (Table IV). However, significant decreases in the concentrations of lactate, pyruvate, and malate were produced by dibutyryl cyclic AMP.

#### Studies with Isolated Liver Cells

Concern as to whether the above studies represented an artifact of the liver slice preparation prompted an investigation of the effect of adenine nucleotides on isolated hepatocytes. As shown in Table V, fatty acid synthesis was effectively inhibited with this preparation by comparable concentrations of 5′-AMP, 3′,5′-cyclic AMP, ADP, ATP, and adenosine. The isolated cells, however, were considerably more sensitive to these inhibitors than the liver slice preparation. For example, 0.5 mM 5′-AMP produced only 38% inhibition with liver slices, but produced 92% inhibition with isolated liver cells. The observation that ADP, ATP, and adenosine have the same action as 5′-AMP makes it unlikely that the inhibitory effect is caused by some contaminant of the 5′-AMP used in these studies. As reported previously (7), dibutyryl cyclic AMP was found to be a very effective inhibitor of fatty acid synthesis by isolated liver cells (Table V) and was considerably more effective than the other nucleotides.

As reported above for slices, 3′,5′-cyclic AMP and 5′-AMP increased dramatically the adenine nucleotide content of the isolated liver cells (Table VI). Because adenosine also was found to inhibit lipogenesis (Table V), the study was extended to include this nucleoside which was found to greatly expand the adenine nucleotide pool size (Table VI). Dibutyryl cyclic AMP, however, did not have this effect (Table VI) at a concentration which produced a striking inhibition of lipogenesis (Table V). Hence, the results obtained with liver cells are qualitatively similar to those reported with liver slices. The rates of lipogenesis were greater with liver cells as was the sensitivity of this process to adenine nucleotides. In addition, the expansion produced in the intracellular nucleotide pool size by the various adenine derivatives was more pronounced with liver cells than with liver slices.

The possibility that exogenous 5′-AMP affected an increase in the 3′,5′-cyclic AMP content of the cells also was investigated. As shown in Figure 1, glucagon at $10^{-6}$ M produced a dramatic but transient increase in 3′,5′-cyclic AMP. A much smaller but nevertheless significant increase in 3′,5′-cyclic AMP was observed with exogenous 5′-AMP. The increase was significant ($P < 0.05$) at 15 min, but not at 30 and 45 min of incubation.

## DISCUSSION

This study developed during an investigation of the mechanism responsible for cyclic AMP inhibition of lipogenesis. Incubation of liver slices with this nucleotide was found to increase the noncyclic adenine nucleotide content of the slices. This observation suggested that the adenine nucleotide derived by the hydrolysis of cyclic AMP might be involved in the inhibition of lipogenesis. Therefore, studies were conducted with 5′-AMP which also was found to inhibit lipogenesis and to increase the adenine

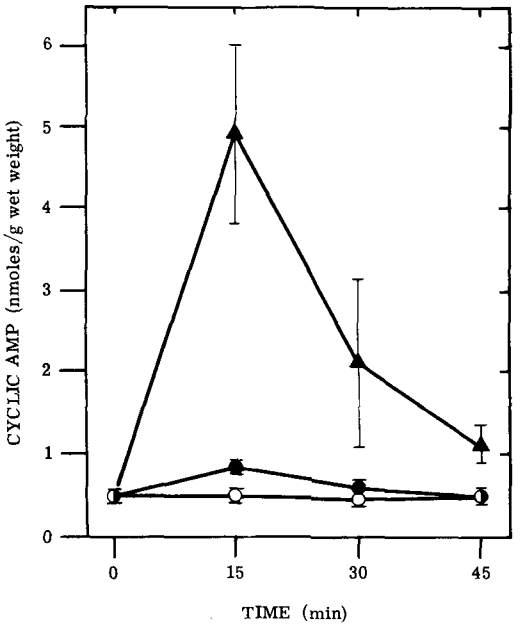


FIG. 1. Effect of 5'-AMP (adenosine monophosphate) and glucagon on the cyclic AMP content of isolated liver cells. ○ = no additions; ● = 5'-AMP at 0.5 mM; and ▲ = glucagon at $10^{-6}$ M. The results are given as means ± S.E.M. for 5-7 liver cell preparations. The increase caused by glucagon was statistically ($P < 0.05$) significant at all time points; 5'-AMP only at 15 min.

nucleotides of liver slices. These observations would suggest that one mechanism by which cyclic AMP inhibits lipogenesis is by the generation of 5'-AMP or adenosine, which in turn increases the adenine nucleotide content of the cell to bring about an inhibition of lipogenesis. The mere slicing of liver is well established to cause a dramatic decrease in the adenine nucleotide content of this tissue (25). Hence, at first inspection, it might seem paradoxical to suggest that an expansion of the pool size of the adenine nucleotides of the cells of liver slices would lead to an inhibition of a metabolic process such as lipogenesis. Indeed, exogenous 5'-AMP and cyclic AMP failed to increase the apparent intracellular content of adenine nucleotides back to the level of the intact liver. Hence, it might be argued that exogenous adenine nucleotides should tend to restore normal metabolic activity. However, this requires the assumption that all cells of liver slices are deficient in adenine nucleotides. From the results reported above with isolated liver cells and below with succinate oxidation to malate by liver slices, it can be suggested that this assumption is not valid. A more likely explanation is that part of the cells are damaged and completely void of adenine nucleotides, whereas, other cells are intact and contain a complete complement of adenine nucleotides. Thus, the only cells which would be competent to respond to exogenous adenine nucleotides would be the intact cells which already contain a full complement of adenine nucleotides. By this analysis, the increase in adenine nucleotides of liver slices caused by exogenous 5'-AMP or cyclic AMP would represent a considerable expansion of the pool size of the intact cells of the tissue. This interpretation is supported by the results obtained with isolated liver cells. Most of the cells of this preparation are intact, i.e., >95% exclude trypan blue, and contain a full complement of adenine nucleotides (26) (Table VI). Exogenous cyclic AMP, 5'-AMP, and adenosine were observed to expand greatly the adenine nucleotide pool size of these cells and to inhibit concurrently fatty acid synthesis. Although the percentage is difficult to estimate, it is recognized that liver slices contain a great number of damaged cells. With the proposal stated above that only intact cells of liver slices contain significant quantities of adenine nucleotides, it can be calculated from the adenine nucleotide content of intact liver, 4 μmoles/g wet wt (26), that only ca. 25% of the cells of liver slices used in this study and that of Krebs (25) would meet this criterion of intactness. The notion discussed by Krebs (25), that the adenine nucleotides of liver slices might be confined to the mitochondrial compartment, is untenable. Liver cells which lacked cytoplasmic adenine nucleotides would be unable to maintain transmembrane ion gradients. The resulting influx of $Ca^{++}$ would lead to swelling and disruption of the mitochondria with a resulting loss of intramitochondrial adenine nucleotides. The viability of isolated liver cells can be estimated by either trypan blue exclusion or succinate oxidation to malate (14). Although the former method cannot be applied to liver slices, the latter method can be used to give a minimum estimate of the number of cells damaged. Succinate is oxidized to malate only by damaged cells of a liver preparation because intact cells are nearly impermeable to succinate and malate (14). It was determined that the liver slices used in this study produced malate from succinate at a rate of 7.1 ± 0.3 μmoles/min/g wet wt (mean ± S.E.M. for liver slices prepared from 4 animals and incubated under the conditions described above). Homogenates of these livers produced malate from succinate at a rate of 19.5 ± 0.5 μmoles/min/g wet wt. Hence, by this analysis, at least 36% of the cells of the slices were not intact. In contrast to the estimate based on

ATP content, malate production probably gave a minimal estimate of the percentage of damaged cells. This is because cells which are damaged, yet not accessible to exogenous succinate and/or oxygen, would not convert succinate to malate. A considerably number of such cells would be expected to exist within the interior of liver slices. Hence, the suggestion from this analysis is that liver slices consist of a mixed population of extensively damaged cells and relatively intact cells, and that only the latter cells could be expected to respond like isolated cells to exogenous adenine nucleotides. Although it is clear that isolated liver cells should be used for metabolic studies in preference to liver slices, there is an important point to be made here. Slices represent the only system for studying human liver in which there is maintenance of structural integrity of the cells. The isolation of intact cells from human liver appears impossible. Therefore, as long as it is appreciated that the slice is composed of a mixed population of damaged and intact cells, meaningful studies can be carried out with slices of human liver. Such studies are currently in progress in this laboratory.

Cyclic AMP may inhibit lipogenesis by limiting the activity of acetyl CoA carboxylase (5,6), either by phosphorylation of the enzyme into an inactive form (27) or by induced changes in established effectors of the enzyme (28-30). Another attractive possibility is that cyclic AMP limits substrate supply for lipogenesis by suppressing glycolytic activity (7) and/or pyruvate dehydrogenase activity. However, no attempt is made here to define the mechanism responsible for cyclic AMP inhibition of lipogenesis nor to explain why Raskin, et al., (8) failed to observe inhibition of lipogenesis by cyclic AMP in the perfused liver. Instead, this report makes it clear that studies attempting to deal with the mechanism of action of cyclic AMP should be conducted under experimental conditions that preclude increases in intracellular adenine nucleotides. This could be accomplished most readily by the generation of intracellular cyclic AMP by hormonal activation of adenylate cyclase or by the judicious use of the dibutyryl derivative.

This study should not be construed to support the work of Raskin, et al., (8) in suggesting that cyclic AMP inhibition of lipogenesis is an artifact of the liver slice preparation. Glucagon and dibutyryl cyclic AMP now have been shown to inhibit lipogenesis by liver slices and isolated liver cells without affecting the adenine nucleotide content of the cells. Indeed, studies in this laboratory now have demonstrated that dibutyryl cyclic AMP and glucagon effectively inhibit lipogenesis in the perfused liver as well (R.A. Harris, J.P. Mapes, and C.S. Stewart, unpublished data).

The inhibition of lipogenesis by adenine nucleotides reported in this study suggests that some step in fatty acid synthesis per se or in substrate supply may be affected by changes in the balance of adenine nucleotides and/or inorganic phosphate within the liver slices. Indeed, adenine nucleotides are recognized as important regulators of metabolic processes (31). On the other hand, exogenous 5′-AMP was found to lead to a significant increase in the cyclic AMP content of isolated liver cells. Although this increase was small compared to the increased caused by glucagon, it nevertheless could be a factor in the inhibition of lipogenesis caused by 5′-AMP. Exogenous adenine nucleotides have been shown in other studies to increase the cyclic AMP content of slices of guinea pig cerebral cortex (32) and cultured astrocytoma cells (33). Whether this response with liver cells was due to a surface receptor which activates adenylate cyclase or a secondary effect caused by the increase in intracellular adenine nucleotides is not known.

In retrospect, it is not surprising that exogenous 5′-AMP and cyclic AMP increase the adenine nucleotide content of liver cells. Adenosine has been shown to increase the adenine nucleotide content of liver in vivo (34) and liver cells in vitro (P. Lund, N. Cornell, and H.A. Krebs, personal communication). In addition, 5′-AMP at concentrations similar to those used in this study have been shown to increase the adenine nucleotide and inorganic phosphate content of perfused and sliced kidney (35) and to exert strong metabolic effects in both kidney (35) and liver (36). Evidence has been presented in other studies that exogenous nucleotides such as 5′-AMP are degraded rapidly by enzymes located at the outer surface of the plasma membranes of liver cells (37) and granulocytes (38). Presumably cyclic AMP, 5′-AMP, ADP, and ATP are all degraded to the neutral molecule adenosine prior to penetration into liver cells in quantities sufficient to expand the adenine nucleotide pool size. The lack of an effect of dibutyryl cyclic AMP on the adenine nucleotide content of liver slices can be explained on the basis that this compound must be a poor substrate for the external enzymes, as well as the phosphodiesterases that hydrolyze cyclic AMP (39). Also, very low concentrations of dibutyryl cyclic AMP were used in these studies because of its greater effectiveness as an inhibitor of lipogenesis. The mechanism responsible for the action of adenosine, adenine nucleotides, dibutyryl cyclic AMP, and gluca-

gon on lipogenesis is being investigated further with the isolated cell and perfused liver preparations.

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REFERENCES

1. Akhtar, M., and D.P. Bloxham, Biochem J. 120:11P (1970).
2. Bricker, L.A., and G.S. Levey, J. Biol. Chem. 247:4914 (1972).
3. Tepperman, H.M., and J. Tepperman, in "Insulin Action," Edited by J.B. Fritz, Academic Press, New York, NY, 1972, p. 543.
4. Allred, J.B., and K.L. Roehrig, Biochem. Biophys. Res. Commun. 46:1135 (1972).
5. Allred, J.B., and K.L. Roehrig, J. Biol. Chem. 248:4131 (1973).
6. Kain, G.J., and P.L. Weiser, Biochem. Biophys. Res. Communs. 55:76 (1973).
7. Harris, R.A., Arch. Biochem. Biophys. 169:168 (1975).
8. Raskin, P., J.D. McGarry, and D.W. Foster, J. Biol. Chem. 249:6029 (1974).
9. Woolley, J.G., and W.H. Sebrell, J. Nutr. 29:191 (1945).
10. Berry, M.N., and D.S. Friend, J. Cell Biol. 43:506 (1969).
11. Chen, R.F., J. Biol. Chem. 242:173 (1967).
12. Kates, M., "Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids," North Holland Publishing Co., Amsterdam, The Netherlands, 1972, pp. 349 and 363.
13. Jungas, R.L., Biochemistry 7:3708 (1968).
14. Mapes, J.P., and R.A. Harris, F.E.B.S. Lett. 51:80 (1975).
15. Lindberg. O., and L. Ernster, Methods Biochem. Anal. 3:1 (1956).
16. Williamson, J.R., and B.E. Corkey , in "Methods of Enzymology," Vol. XIII, Edited by J.M. Lowenstein, Academic Press, New York, NY, 1969, p. 434.
17. Hohorst, H.J., F.H. Kreutz, and T. Bucher, Biochem. Z. 332:18 (1959).
18. Möllering, H., and W. Gruber, Anal. Biochem. 17:369 (1966).
19. Williamson, D.H., P. Lund, and H.A. Krebs, Biochem. J. 103:514 (1967).
20. Kneer, N.M., A.C. Bosch, M.G. Clark, and H.H. Lardy, Proc. Natl. Acad. Sci., U.S.A. 71:4523 (1974).
21. Gilman, A.G., Ibid. 67:305 (1970).
22. Michal, G., and P. Wunderwald, in "Methods of Enzymatic Analysis," 2nd Edition, Edited by H.U. Bergmeyer, Academic Press, New York, NY, 1974, p. 2136.
23. Randerath, K., and H. Struck, J. Chromatog. 6:365 (1961).
24. Groot, G.S.P., and S.G. Van den Bergh, Biochem. Biophys. Acta 153:22-31 (1968).
25. Krebs, H.A., in "Advances in Enzyme Regulation," Vol. 8, Edited by G. Weber, Pergamon Press, New York, NY, 1969, p. 335.
26. Krebs, H.A., N.W. Cornell, P. Lund, and R. Hems, in "Regulation of Hepatic Metabolism," Edited by F. Lundquist and N. Tygstrup, Alfred Benzon Symposium 6, Mlinksgaard, Copenhagen, 1973, p. 718.
27. Carlson, C.A., and K.H. Kim, J. Biol. Chem. 248:378 (1973).
28. Goodridge, A.G., Ibid. 248:4318 (1973).
29. Gregolin, C., E. Ryder, and M.D. Lane, Ibid. 243:4227 (1968).
30. Lynen, F., in "Control Processes in Multicellular Organisms," Edited by G.E.W. Wolstenholme and J. Knight; J. and A. Churchill, London, England, 1970, p. 23.
31. Newsholme, E.A., and C. Start, "Regulation in Metabolism," John Wiley and Sons, London and New York, 1973.
32. Sattin, A., and T.W. Rall, Mol. Pharmacol. 6:13 (1970).
33. Clark, R.B., K. Gross, Y.F. Su, and J.P. Perkins, J. Biol. Chem. 249:5296 (1974).
34. de Sanchez, C.V., A. Brunner, and E. Pina, Biochem. Biophys. Res. Commun. 46:1441 (1972).
35. Weidemann, M.J., D.H. Hems, and H.A. Krebs, Biochem. J. 115:1 (1969).
36. Hunter, A.R., and L.S. Jefferson, Ibid. 111:537 (1969).
37. Decker, K., and M. Liersch, in "Isolated Liver Perfusion and its Application," Edited by I. Bartosek, A. Guaitani, and L.L. Miller, Raven Press, New York, NY, 1973, p. 241.
38. De Pierre, J.W., and M.C. Kanovsky, Science 183:1096 (1974).
39. Simon, L.E., D.A. Shuman, and R.K. Robins, Adv. Cyclic Nucleotide Res. 3:225 (1973).

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