ripple marks and the inclination of the floor near the sidewall lead one to speculate that a current of higher velocity than elsewhere on the canyon floor flows intermittently at the right (west) side of the canyon.

In summary, the fragmentary evidence can be taken to indicate mass transport down the canyon axis, a previous episode of canyon filling followed by partial erosion of the fill, and perhaps intermittent flow of strong water currents on the canyon floor.

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Adenosine 3′,5′-Cyclic Phosphate: Stimulation of Steroidogenesis in Sonically Disrupted Adrenal Mitochondria

Abstract. *Adenosine 3′5′-cyclic phosphate stimulated the conversion of added cholesterol to pregnenolone in "coupled" rat adrenal mitochondria provided with succinate, and in "leaky" mitochondria fortified with reduced nicotinamide adenine dinucleotide phosphate. Adenine nucleotides other than adenosine 3′,5′-cyclic phosphate did not duplicate these actions. The cyclic nucleotide was also effective in supernatants from sonically disrupted mitochondria. The minimum effective concentration was 50 micromoles per liter or less. The results suggest that adenosine 3′,5′-cyclic phosphate stimulates corticosteroidogenesis by activating the mitochondrial enzymes which are rate-limiting in the utilization of cholesterol.*

On the pathway to corticosteroids in the adrenal cortex the initial conversions of cholesterol are carried out by mitochondrial steroid hydroxylases which require reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (*1*). Formation of pregnenolone from cholesterol involves as intermediates 20α-hydroxycholesterol and 20α,22-dihydroxycholesterol (*2*). Side-chain cleavage of the latter steroid completes this early sequence. Several experimental observations (*3*) are consistent with the concept that one or both of these hydroxylation steps is rate-limiting in corticosteroidogenesis and may be activated acutely by adrenocorticotropin (ACTH). Mechanisms proposed for this action of ACTH include enhanced passage of cholesterol or pregnenolone through the adrenal mitochondrial membranes, activation or induction of certain catalytic proteins in the adrenal cortex, and increased provision of cytoplasmic NADPH to adrenal steroid hydroxylase systems (*4*). Adenosine 3′,5′-cyclic phosphate (3′,5′-AMP), which is formed in the adrenal cortex in response to ACTH, has been implicated as a possible intermediate in the action of this hormone (*5*). This cyclic nucleotide stimulates certain steroid transformations in surviving adrenal tissue (*6*), adrenal homogenates (*7*), and isolated adrenal mitochondria (*8*). Our experiments reveal that relatively low concentrations of 3′,5′-AMP activate the conversion of cholesterol to pregnenolone in sonically treated suspensions of rat adrenal mitochondria as well as in intact mitochondria in various functional states (*8*).

Adrenal glands were obtained from male Sprague-Dawley rats (6 weeks old) that were lightly anesthetized with sodium pentobarbital (Nembutal). We prepared the mitochondria by a method (*8*) which involved repeated washing in a medium composed of 0.25*M* sucrose and 0.8 m*M* tris-HCl at *p*H 7.1. This procedure resulted in cholesterol-depleted mitochondria which contained about 3 μg of free cholesterol per milligram of mitochondrial protein. When these mitochondria were incubated at 37°C in buffer composed of 24 m*M* $NaHCO_3$ (*p*H 7.5) and 130 m*M* KCl, they swelled rapidly and required exogenous NADPH to support the utilization of added cholesterol-4-^{14}C ("leaky" mitochondria). In contrast, adrenal mitochondria did not swell at 37°C in a medium containing 20 m*M* tris-HCl (*p*H 7.4) and 70 m*M* sucrose, they were dependent upon exogenous substrates of the citric acid cycle to generate NADPH intramitochondrially, and they gave other evidence of being tightly "coupled," including ADP acceptor control ratios greater than 3. For sonic disruption, we suspended washed mitochondria in distilled water at 0°C and treated them for varying periods at 20 kc/sec with a Branson Sonifier, model S-75, equipped with a microtip. The sonically treated suspension was then centrifuged in a Sorvall SM-24 rotor at 0°C and 15,000 rev/min for 15 minutes. This procedure removed from suspensions similar to those subjected to sonic disruption essentially all structures recognizable under the electron microscope as mitochondria. However, mitochondrial membrane fragments undoubtedly remained. The supernatant was incubated in the tris-sucrose medium. In all experiments, the *p*H of 3′,5′-AMP was adjusted to that of the incubation medium at 37°C. After incubation, the media were extracted with methylene chloride. Steroid products were analyzed by thin-layer chromatography on silica gel GF in an isooctane,*tert*-butanol solvent system (5 : 1). Once-repeated development of the chromatograms in this system afforded good separation of pregnenolone from progesterone as well as from the large amount of radioactive cholesterol remaining. Other solvent systems used for identification included benzene, hexane, and ethyl acetate (4 : 15 : 8); methylene chloride and methanol (94 : 6); benzene and ethyl acetate (3 : 1); hexane and ethyl acetate (1 : 1); and systems A, D, H, and L of Lisboa (*9*). Radioactivity in the separated steroids was measured with a Packard radiochromatogram scanner equipped with a disc integrator. The overall efficiency of counting was about 10 percent (that is, 1 μc was equivalent to approximately 2.2 $\times$ 10^5 count/min). The reproducibility and accuracy of quantitation were established by mea-

surements of radioactivity eluted from the thin-layer plates, as well as by repeated scanning of different portions derived from mixtures of known radioactive steroids and representative incubation samples. The material from the radioactive peaks designated pregnenolone and progesterone migrated with the same mobility as authentic samples of these steroids in each of the nine solvent systems. The areas corresponding to these and other peaks were recovered from a number of thin-layer plates and were subjected to additional identification procedures (*7*). These included, where applicable, analyses of ultraviolet and sulfuric acid spectra, as well as chromatography of mixtures of the original steroid or its acetylation and oxidation products with authentic samples of each suspected structure. Recoveries of radioactive steroids carried through the incubation and extraction procedures averaged 95 percent.

Under appropriate conditions, incubation of "leaky" or "coupled" rat adrenal mitochondria with cholesterol-4-^{14}C resulted in the formation of radioactive pregnenolone and progesterone, and other more polar products such as 11β-hydroxyprogesterone—which traveled in a peak about 3 cm from the origin in the chromatography system routinely used. The presence of the steroid isomerase and dehydrogenase involved in the conversion of pregnenolone to progesterone did not appear to be due to microsomal contamination, but rather to the firm association of these enzymes with isolated adrenal mitochondria (*8, 10*). Basal utilization of cholesterol was linear between 20 and 100 μmole of steroid per liter and 15 and 60 minutes of incubation. Cyclic 3′,5′-AMP stimulated the utilization of exogeneous cholesterol by "leaky" adrenal mitochondria in the presence of optimum concentrations of NADPH (Table 1) and by "coupled" mitochondria incubated with succinate (Table 2). Phosphate buffer (*p*H 6.0 to 7.6) could be substituted for bicarbonate in the experiments with "leaky" mitochondria. The minimum effective concentration of the cyclic nucleotide for stimulation of overall cholesterol utilization was 50 μmole/liter or less in "leaky" mitochondria. In "coupled" mitochondria, the effective concentration of 3′,5′-AMP was considerably greater (about 1 mmole/liter), probably as a consequence of the relative impermeability of intact membranes to this compound (see *6*). The most striking action of the cyclic nucleotide in both "leaky" and "coupled" mitochondria was on the accumulation of pregnenolone. Enhanced formation of progesterone and of the more polar products also occurred in the "leaky" preparation (Table 1). However, formation of progesterone and of the polar products was often inhibited by 3′,5′-AMP in "coupled" mitochondria (Table 2). We were unable to detect radioactive peaks other than cholesterol when cholesterol-26-^{14}C was used as the radioactive substrate in the presence or absence of 3′,5′-AMP. The actions of 3′,5′-AMP could not be duplicated by adenosine 5′-phosphate, adenosine 5′-diphosphate, or adenosine 5′-triphosphate in concentrations varying from 0.05 to 2 mmole/liter.

The supernatants prepared from sonically disrupted rat adrenal mitochondria formed pregnenolone, progesterone, and other more polar products from cholesterol in the presence of NADPH (Table 3). In common with undisrupted mitochondria, these sonicates responded to low concentrations of 3′,5′-AMP with enhanced pregnenolone formation and cholesterol utilization. The minimum effective concentration of the cyclic adenylate was 50 μmole/liter or less. This amount of 3′-5′-AMP augmented pregnenolone production 35 to 40 percent in each of three experiments, without significantly altering the other radioactive

Table 1. Stimulation of cholesterol utilization in "leaky" rat adrenal mitochondria by 3′,5′-AMP. The incubation medium (1 ml) contained cholesterol-^{14}C (1 μc per 0.1 μmole) in 0.01 ml of absolute ethanol, 0.5 m*M* NADPH, 24 m*M* $NaHCO_3$, 130 m*M* KCl, and 0.3 mg of mitochondrial protein. Incubation was carried out for 15 to 30 minutes at 37°C and *p*H 7.5 under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide. Each value represents the average ± standard error of the mean for four incubation samples. Total products include all radioactive peaks other than cholesterol.

3′,5′-AMP (mmole/liter)	Steroids produced (mμmole per milligram of mitochondrial protein per hour)			
	3-cm Peak	Progesterone	Pregnenolone	Total products
		50 μM Cholesterol		
0	2.89 ± 0.35	4.94 ± 0.24	1.00 ± 0.08	10.78 ± 0.86
.05	5.63 ± .24*	6.95 ± .30*	1.51 ± .08*	17.23 ± .48*
1	6.06 ± .30*	5.64 ± .19	3.21 ± .22*	18.22 ± .46*
		100 μM Cholesterol		
0	3.89 ± 0.11	11.23 ± 0.54	3.13 ± 0.32	24.17 ± 0.97
.05	5.29 ± .11*	10.08 ± .43	5.51 ± .11*	25.06 ± .86†
1	5.40 ± .22*	12.10 ± .43	7.02 ± .54*	27.65 ±1.19*
2	5.72 ± .32*	11.88 ± .43	7.24 ± .54*	28.08 ± .86*

* $P < .01$ for the difference between this value and the corresponding control value. † $P = .03$ for the difference between this value and the corresponding control value.

Table 2. Stimulation of cholesterol utilization in "coupled" rat adrenal mitochondria by 3′,5′-AMP. The incubation medium (1 ml) contained cholesterol-4-^{14}C (1 μc per 0.1 μmole) in 0.01 ml of absolute ethanol, 20 m*M* tris-HCl buffer, 70 m*M* sucrose, 11.5 m*M* NaCl, 15.4 m*M* KCl, 10 m*M* sodium succinate, and 0.6 to 1.0 mg of mitochondrial protein. The final *p*H of the incubation medium was 7.4, except in the first experiment where the *p*H was 7.2. Incubation was carried out at 37°C for 30 to 60 minutes under 100 percent oxygen. Each value represents the average ± standard error of the mean for three to four incubation samples. Total products include all radioactive peaks other than cholesterol.

3′,5′-AMP (mmole/liter)	Steroids produced (mμmole per milligram of mitochondrial protein per hour)			
	3-cm Peak	Progesterone	Pregnenolone	Total products
		50 μM Cholesterol		
0	1.27 ± 0.14	0.52 ± 0.11	0.50 ± 0.13	2.72 ± 0.39
2	1.16 ± .14	.61 ± .05	1.59 ± .11*	3.90 ± .30†
0	1.49 ± .05	.70 ± .03	0.27 ± .06	3.04 ± .09
1	1.39.± .19	.73 ± .10	1.70 ± .10*	4.52 ± .26*
0	1.57 ± .15	.86 ± .04	0.39 ± .08	3.52 ± .20
1	1.13 ± .15	.55 ± .04*	2.24 ± .03*	4.55 ± .13*
		100 μM Cholesterol		
0	2.91 ± 0.27	1.62 ± 0.12	0.71 ± 0.12	6.29 ± 0.18
1	1.97 ± .07†	1.21 ± .22	3.31 ± .11*	7.42 ± .35†

* $P < .01$ for the difference between this value and the corresponding control value. † $P = .05$ for the difference between this value and the corresponding control value.

Table 3. Effects of 3′,5′-AMP on cholesterol and pregnenolone utilization in sonically treated rat adrenal mitochondria. The incubation medium (1 ml) contained 50 μM cholesterol-4-^{14}C (0.5 μc) or 10 μM pregnenolone-4-^{14}C (0.04 μc) in 0.01 ml of absolute ethanol, 20mM tris-HCl, 20 mM sucrose, 11.5 mM NaCl, 15.4 mM KCl, 0.5 mM NADPH, and 0.3 to 0.4 mg of supernatant protein from mitochondria sonically treated for 1 to 2 minutes. Incubation was carried out for 30 minutes at 37°C and pH 7.4 under 100 percent oxygen. Each value represents the average ± standard of the mean for four incubation samples, except where individual values are shown. Total products include all radioactive peaks other than cholesterol.

3′,5′-AMP (mmole/liter)	Steroids produced (mμmole per milligram of mitochondrial protein per hour)			
	3 cm Peak	Progesterone	Pregnenolone	Total products
		Cholesterol		
0	1.00 ± 0.05	3.03 ± 0.27	1.93 ± 0.20	8.40 ± 0.45
0.1	1.25 ± .03	3.58 ± .20	2.68 ± .10*	10.35 ± .58†
.5	0.63 ± .13†	2.35 ± .08†	5.40 ± .25*	12.20 ± .53*
1.0	.31 ± .10*	1.15 ± .05*	6.13 ± .20*	9.25 ± .53
0	.95 ± .10	3.45 ± .18	2.50 ± .16	9.49 ± .29
.05	1.00 ± .05	3.62 ± .39	3.42 ± .13*	10.97 ± .80
.5	0.71 ± .16	2.45 ± .18*	5.44 ± .29*	11.15 ± .24*
1.0	.97 ± .11	1.92 ± .13*	6.44 ± .40*	11.65 ± .60*
		Pregnenolone		
0	9.1; 9.6	18.3; 18.3		37.8; 37.4
0.05	8.7; 9.6	17.0; 15.2		35.7; 36.1
.5	4.8; 4.8	10.4; 9.6		25.2; 26.5
1.0	2.2; 3.0	6.5; 6.1		18.7; 19.1

* P = .01 to .02 for the difference between this value and the corresponding control value. † P < .05 for the difference between this value and the corresponding control value.

products. We also observed an inhibition of pregnenolone utilization with concentrations of 3′,5′-AMP equal to or above 0.5 mmole/liter, when either cholesterol or pregnenolone was the added substrate. This inhibition sometimes obscured the stimulatory effect of these higher concentrations of 3′,5′-AMP on side-chain cleavage of cholesterol.

These experiments demonstrate a direct effect of 3′,5′-AMP on rat adrenal mitochondria and sonically disrupted mitochondrial suspensions, which resulted in enhanced utilization of cholesterol for corticosteroidogenesis. In low concentrations, the cyclic nucleotide stimulated the formation of pregnenolone from added cholesterol, but did not activate the conversion of pregnenolone to progesterone. Although mitochondrial integrity was not required, some degree of intact membrane structure may be necessary for the stimulatory effect of 3′,5′-AMP on cholesterol utilization, inasmuch as this action has not yet been demonstrated in completely soluble systems (*11*). Earlier investigations revealed that the cyclic nucleotide activated hydroxylations of progesterone and 11-deoxycorticosterone in "leaky" rat adrenal mitochondria (*8*). By analogy, it seems likely that the stimulation of cholesterol utilization may involve one or more of the complex mitochondrial multienzyme systems responsible for the hydroxylation of this steroid. The observed lack of accumulation of hydroxylated derivatives of cholesterol when cholesterol-26-^{14}C was the substrate may be explained by the relatively high activity of the side-chain cleavage enzyme (*2*). Our observations support the concept (*8*) that 3′,5′-AMP enhances corticosteroidogenesis by stimulating directly an early rate-limiting step in cholesterol utilization by adrenocortical mitochondria, and that this action may be of physiological significance in the control of adrenal steroid biosynthesis.

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Glutathione Deficiency in Sheep Erythrocytes

Abstract. *Three sheep have been found that have concentrations of erythrocyte glutathione less than 20 percent of the concentrations in normal sheep; they have no readily apparent hemolytic disorder.*

Reduced glutathione (GSH) is the major reducing substance in the erythrocyte and is apparently necessary for cellular integrity. When the red cell GSH decreases, as in subjects deficient in glucose-6-phosphate dehydrogenase who have been treated with 8-aminoquinoline antimalarials (*1*) or in sheep and humans with copper toxicity (*2*), there is a hemolytic crisis, with hemoglobinuria.

Oort *et al.* (*3*) have described an inherited absence of GSH with an associated hemolytic disorder in man. Patients with this defect have a borderline anemia, reticulocytosis, and a mild icterus. Administration of primaquine or the ingestion of fava beans has been shown to accelerate the hemolytic process. The biochemical defect is apparently a failure to synthesize the tripeptide from glycine, cysteine, and glutamic acid.