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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References and Notes

1. I. D. K. Halkerston, J. Eichhorn, O. Hechter, *Arch. Biochem. Biophys.* **85**, 287 (1959); *J. Biol. Chem.* **236**, 374 (1961).
2. G. Constantopoulos, P. S. Satoh, T. T. Tchen, *Biochem. Biophys. Res. Commun.* **8**, 50 (1962); G. Constantopoulos and T. T. Tchen, *ibid.* **4**, 460 (1961); K. Shimizu, R. I. Dorfman, M. Gut, *J. Biol. Chem.* **235**, PC 25 (1960); K. Shimizu, M. Gut, R. I. Dorfman, *ibid.* **237**, 699 (1962); K. Shimizu, M. Hayano, M. Gut, R. I. Dorfman, *ibid.* **236**, 695 (1961); S. Solomon, P. Levitan, S. Lieberman, *Rev. Can. Biol.* **15**, 282 (1956).
3. M. Hayano, N. Saba, R. I. Dorfman, O. Hechter, *Recent Progr. Hormone Res.* **12**, 79 (1956); G. C. Karaboyas and S. B. Koritz, *Biochemistry* **4**, 462 (1965); S. B. Koritz, *Biochim. Biophys. Acta* **56**, 63 (1962); N. Saba and O. Hechter, *Fed. Proc.* **14**, 775 (1955); D. Stone and O. Hechter, *Arch. Biochem. Biophys.* **51**, 457 (1954).
4. R. V. Farese, *Biochim. Biophys. Acta* **87**, 701 (1964); J. J. Ferguson, Jr., *ibid.* **57**, 616 (1962); *J. Biol. Chem.* **238**, 2754 (1963); L. D. Garren, R. L. Ney, W. W. Davis, *Proc. Nat. Acad. Sci. U.S.* **53**, 1443 (1965); R. C. Haynes, Jr., and L. Berthet, *J. Biol. Chem.* **225**, 115 (1957); R. C. Haynes, Jr., E. W. Sutherland, T. W. Rall, *Recent Progr. Hormone Res.* **16**, 121 (1960); O. Hechter, *Vitamins Hormones* **13**, 293 (1955); O. Hechter and I. D. K. Halkerston, in *The Hormones* **5**, 697 (1964); I. N. Hirshfield and S. B. Koritz, *Biochemistry* **3**, 1994 (1964); K. W. McKerns, *Biochim. Biophys. Acta* **90**, 357 (1964); S. Roberts and J. E. Creange, in *Functions of the Adrenal Cortex*, K. W. McKerns, Ed. (Appleton-Century-Crofts, New York, 1967), in press.
5. R. C. Haynes, Jr., *J. Biol. Chem.* **233**, 1220 (1958).
6. R. C. Haynes, Jr., S. B. Koritz, F. G. Péron, *ibid.* **234**, 1421 (1959); G. C. Karaboyas and S. B. Koritz, *Biochemistry* **4**, 462 (1965).
7. J. E. Creange and S. Roberts, *Steroids, Supplement II*, 13 (1965); R. Makoff, S. Roberts, P. L. Young, D. D. Fowler, *J. Biol. Chem.* **239**, 4124 (1964).
8. J. E. Creange, S. Roberts, P. L. Young, *Fed. Proc.* **25**, 221 (1966); S. Roberts, J. E. Creange, P. L. Young, *Biochem. Biophys. Res. Commun.* **20**, 446 (1965); S. Roberts and J. E. Creange, in *Functions of the Adrenal Cortex*, K. W. McKerns, Ed. (Appleton-Century-Crofts, New York, 1967), in press.
9. J. Avigan, D. S. Goodman, D. Steinberg, *J. Lipid Res.* **4**, 100 (1963); B. P. Lisboa, *Acta Endocrinol.* **43**, 47 (1963); Y. Nakamura and B.-I. Tamaoki, *Biochim. Biophys. Acta* **85**, 350 (1964); L. I. Smith and T. Foell, *J. Chromatogr.* **9**, 339 (1962).
10. K. F. Beyer and L. T. Samuels, *J. Biol. Chem.* **219**, 69 (1956); M. Hayano, N. Saba, R. I. Dorfman, O. Hechter, *Recent Progr. Hormone Res.* **12**, 79 (1956); N. Saba and O. Hechter, *Fed. Proc.* **14**, 775 (1955).
11. P. Satoh, G. Constantopoulos, T. T. Tchen, *Biochemistry* **5**, 1646 (1966).
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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.

Table 1. Reduced glutathione levels (GSH), packed cell volumes (PCV), and hemoglobins (Hb) of normal and glutathione-deficient sheep. The GSH was determined by DTNB method (*4*), PCV by microhematocrit, and the hemoglobin by the cyanmethemoglobin method. Values are means and, for normal sheep, ± standard deviation.

No. of sheep	GSH (milligrams per 100 ml of RBC)	PCV (%)	Hb (grams per 100 ml)
	Normal sheep		
101	104.0 ± 27.2	34.7 ± 5.8	12.6 ± 2.2
	Glutathione-deficient sheep		
	10.5	32.3	10.9
	19.3	34.2	11.8
	4.5	30.6	10.5

During investigations of the concentration of GSH in sheep blood, we recently found three ewes with a marked decrease in erythrocyte GSH (Table 1). The lowered GSH levels were determined by both the 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (*4*) and "Alloxan 305" (*5*) methods. This decrease has been seen for over 2 months and is unaccompanied by a readily apparent hemolytic disorder. The blood copper, glucose-6-phosphate dehydrogenase, glutathione reductase, serum glutamic pyruvic transaminase, blood urea nitrogen, sulfobromophthalein clearance, GSH stability, and bilirubin are all within the normal range for this species. Two animals have hemoglobin type A and one has type AB, and all have a low concentration of red cell potassium. The three ewes have delivered six lambs, one of which died before the GSH determination could be performed. Of the five remaining lambs, one has a GSH value (28.2 mg/100 ml of erythrocytes) that is approximately the same as his dam (19.3 mg), while his twin has a normal level. The other lambs have concentrations that are in the normal range for their age.

These limited genetic studies do not offer any definite evidence of the genetic transmission of this disorder. It is possible that all three ewes are homozygous and that the heterozygotes do not have a lowered erythrocyte GSH, as is the case in man (*4*). On the other hand, the three animals could be heterozygotes, and the lambs could be either homozygous or heterozygous for the normal GSH level. These animals should be useful in delineating the role of reduced glutathione in erythrocyte metabolism and drug sensitivity.

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References and Notes

1. E. Beutler, *Blood* **14**, 103 (1959).
2. N. A. Holtzman, D. A. Elliott, R. H. Heller, *New Engl. J. Med.* **275**, 347 (1966); J. Todd and R. Thompson, *Brit. Vet. J.* **119**, 161 (1963).
3. M. Oort, J. A. Loos, H. K. Prins, *Vox Sang.* **6**, 370 (1961).
4. E. Beutler, O. Duron, B. H. Kelly, *J. Lab. Clin. Med.* **61**, 882 (1963).
5. J. W. Patterson and A. Lazarow, in *Methods of Biochemical Analysis*, D. Glick, Ed. (Interscience, New York, 1959), vol. 2, p. 259.
6. C. Bincoe, and W. B. Dye, *J. Animal Sci.* **17**, 224 (1958); C. E. Cornelius, *Clinical Biochemistry of Domestic Animals* (Academic Press, New York, 1963), p. 230.
7. We are grateful to A. Tillman and T. Nelson for the determination of plasma copper levels. Investigation supported, in part, by NIH grant HE 10934 and the Oklahoma Agricultural Experiment Station.

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Potamotrygon spp.: Elasmobranchs with Low Urea Content

Abstract. *All previously reported species of Chondrichthyes, from both marine and fresh water, have contained urea at concentrations ranging from about 300 to 1300 milligrams of urea nitrogen per 100 milliliters of fluid. Body fluids from two species of* Potamotrygon, *permanent residents of the Amazon basin, contained only 2 to 3 milligrams of urea nitrogen per 100 milliliters. Although they have abandoned the retention of urea exhibited by other chondrichthyans, the extent to which they have lost the mechanisms of retaining and tolerating urea in a hypertonic medium has not been determined.*

Staedeler and Frerichs (*1*) first demonstrated that the blood and other body fluids of chondrichthyans contain a concentration of urea far above that retained by most other vertebrates. Unusually high concentrations of urea have also been shown in a few other vertebrate species, notably the aestivating African lungfish, *Protopterus aethiopicus* (*2*), the crab-eating frog, *Rana cancrivora* (*3*), and the coelacanth, *Latimeria chalumnae* (*4*). However, the class Chondrichthyes is the only large vertebrate taxon in which retention of urea has, until now, appeared to be universal. All marine species studied to date, both elasmobranchs and chimaeroids, have contained urea nitrogen in the range of approximately 750 to 1300 mg/100 ml (*5*). It has also been shown (*6*) that trimethylamine oxide (TMAO) is present in unusually high concentration in the blood and tissues of elasmobranchs (about 80 to 120 mmole/liter).

Urea concentrations have been reported for elasmobranchs occurring in freshwater by Smith (*7*), Urist (*8*), and Thorson (*9*). In general, all three papers reported a reduction to approximately 25 to 35 percent of the urea concentrations in marine elasmobranchs. In all cases, the freshwater animals studied were probably of marine or brackish-water origin, or, in any case, had access to the sea. Smith's work dealt with the sawfish *Pristis microdon*; the shark *Carcharhinus melanopterus*; and two rays, *Dasyatis warnak* and *Hypolophus sephen*, about 40 miles up the Perak River in the Federated Malay States (now Malaysia). Urist studied the shark *Carcharhinus leucas* of Lake Nicaragua, and Thorson studied *C. leucas* and the sawfish *Pristis perotteti*, both from Lake Nicaragua. This lake is drained into the Caribbean Sea by a large, broad river, the Río San Juan, whose rapids probably do not, as often claimed, restrict the passage of sharks and sawfish (*10*). These elasmobranchs have probably come from the sea, or at least are of very recent marine ancestry. The fate of the urea following return of the freshwater animals to the sea has not yet been determined, but it is reasonable to assume that it would return to the marine concentration, probably quite rapidly.

Since the only study of osmoregulation in freshwater elasmobranchs has involved species whose length of residence in freshwater was unknown, but probably brief, it appeared pertinent to study elasmobranch species known to live permanently in freshwater a long distance from the sea. These species would, therefore, have experienced a protracted absence from the hypertonic medium which apparently elicited the retention of urea in the Chondrichthyes. Such animals are the sting rays of the family Potamotrygonidae (the genera *Potamotrygon, Elipesurus*, and *Disceus*), which occur commonly in South America, particularly in the Orinoco and Amazon drainage systems, as much as 4000 or 4500 km from the sea. They have most likely been there for at least many