Reports and Letters

Lowered P/O Ratios with Mitochondria Isolated from Livers Showing Cloudy Swelling

Morphological changes in the mitochondria of cells with "cloudy swelling" (the earliest histological evidence of cellular degeneration) have been demonstrated repeatedly. A reciprocal relationship between the shape of mitochondria and the rate of oxidative phosphorylation has been shown recently (*1*). Mitochondria isolated from normal cells were used to establish this interdependence; swelling was induced either by osmotic means or by incubation under conditions in which the oxidative phosphorylation was uncoupled.

It was of interest, therefore, to study the formation of high-energy phosphate bonds in cells affected by a typical cloudy swelling such as can be produced with certain bacterial toxins. Evidence has been presented in a previous paper (*2*) that, in cloudy swelling induced by diphtheria toxin, the easily hydrolyzable phosphate is decreased. Further experiments have shown that repeated injections of 2,4-dinitrophenol, a compound well known to uncouple oxidative phosphorylation *in vitro* (*3*), produced cloudy swelling of liver and kidneys in the rat (*4*). Analogous results were obtained by injecting rats with thyroxine (*5*), which also uncouples oxidative phosphorylation (*6*). This report is concerned with the phosphorylation quotients observed with mitochondria isolated from livers that showed cloudy swelling (*7*).

In order to produce cloudy swelling of the liver, rats were injected intraperitoneally with *S. typhi murium* toxin (the smallest dose that would kill such animals in 4 days) and guinea pigs were injected subcutaneously with diphtheria toxin (1 MLD/250 g of body weight). Animals were used 24 hours after the toxin injection. In all cases, the livers were examined histologically to confirm the occurrence of cloudy swelling in the treated animals. Liver mitochondria were prepared in 0.25*M* sucrose-0.005*M* versene (ethylenediaminetetraacetic acid, adjusted to *p*H 7.4 with NaOH), essentially by the procedure of Schneider (*8*).

The results of the phosphorylation experiments are shown in Table 1. They are expressed as P/O ratios (moles of inorganic orthophosphate which disappear per atom of oxygen consumed).

The complete reaction system contained the following: mitochondria derived from 500 mg of fresh tissue; 250 μmoles of sucrose (contributed from the added mitochondria); 15 μmoles of versene (including the amount of the mitochondrial suspension); 60 μmoles of potassium phosphate buffer at *p*H 7.4; 75 μmoles of KCl; 20 μmoles of $MgSO_4$; 40 μmoles of KF; 90 μmoles of succinate or 30 μmoles of α-ketoglutarate; 3×10^{-2} μmoles of cytochrome *c*; 3 μmoles of adenosine-5′-phosphate; 78 μmoles of glucose; and 20 mg of a hexokinase preparation (*9*) in a final volume of 3 ml. A Warburg bath was used for incubation at 25°C for 20 minutes, with air as the gas phase; CO_2 was absorbed with KOH. Inorganic orthophosphate in the trichloroacetic filtrates was estimated according to Fiske and Subbarow (*10*).

It can be seen that with both succinate and α-ketoglutarate, the phosphorylation quotient was lowered when mitochondria from livers showing cloudy swelling were used. The lowering of the P/O ratios has been observed with mitochondria prepared from livers of rats treated with *S. typhi murium* toxin and also from livers of guinea pigs injected with diphtheria toxin. In the latter system, the inhibition of phosphorylation is more marked with α-ketoglutarate as a substrate than it is with succinate as a substrate.

Table 1. Phosphorylation quotients with mitochondria from livers showing cloudy swelling as compared with the controls. The figures represent the mean ± standard error and those in parentheses give the number of observations.

Animal	Substrate	P/O	
		Controls	Treated animals
Rat	Succinate	1.8 ± 0.07 (5)	1.3 ± 0.09 (5)
Rat	α-Ketoglutarate	3.2 ± 0.07 (10)	2.5 ± 0.16 (10)
Guinea pig	Succinate	2.0 ± 0.08 (5)	1.7 ± 0.07 (5)
Guinea pig	α-Ketoglutarate	3.1 ± 0.21 (4)	1.7 ± 0.14 (4)

The hydrolysis of adenosine-5′-phosphate, adenosinetriphosphate, and glucose-6-phosphate by the mitochondria was also studied; no increased rate of hydrolysis was found with the mitochondria from pathological livers. Thus, the low P/O ratios do not appear to be attributable to an increased dephosphorylation.

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References and Notes

1. J. W. Harman and M. Feigelson, *Exptl. Cell Research* 3, 509 (1952).
2. A. Fonnesu and C. Severi, *Giorn. biochim.* 2, 326 (1953).
3. W. F. Loomis and F. Lipmann, *J. Biol. Chem.* 173, 807 (1948).
4. A. Fonnesu and C. Severi, *Arch sci. biol. Italy* 38, 597 (1954).
5. ———, in preparation.
6. C. Martius and B. Hess, *Arch. Biochem and Biophys.* 33, 486 (1951).
7. Full details of this study are in preparation.
8. W. C. Schneider, *J. Biol. Chem.* 176, 259 (1948).
9. Sigma Chemical Company.
10. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* 66, 375 (1925.)

2 May 1955

2-Methyl Hydrocortisones: A New Series of Steroids with Enhanced Potency and Prolonged Action

Steroid chemists have recently developed a number of synthetic analogs of the adrenocortical steroids, several of which have been found to possess remarkable biological properties. The 9-α-halogen steroids developed by Fried and Sabo (*1*) have been found to possess a greater biologic potency than their nonhalogenated analogs with respect to all properties thus far studied. The sodium-retaining activity of these halogenated corticoids has been found to be enhanced out of proportion to the increase in "glucocorticoid" activities. The Δ-1 series of corticosteroids developed by Herzog *et al.* (*2*) have been found to possess greater biologic activity than their natural analogs with respect to properties dependent on the presence of an 11-oxygen group (for example, anti-inflammatory activity). On the other hand, those properties that do not depend on the presence of an 11-oxygen group (for example, sodium-retaining activity) have not been enhanced by the Δ-1 modification (*3*).

Very recently, the development of still another series—the 2-methyl analogs of

the corticosteroids—has been achieved by Hogg *et al.* (*4*). Byrnes and associates have found two of these steroids to be more potent than their nonmethylated analogs both in glycogen-depositing activity and in sodium-retaining activity as tested in the rat (*5*).

The present report (*6*) represents the initial use of these steroids in two additional species—man and the dog. We have studied in normal human subjects, in patients with Addison's disease, and in adrenalectomized dogs the comparative pharmacology of hydrocortisone (F), 2-methylhydrocortisone (methyl F), 9-α-fluorohydrocortisone (FF), and 2-methyl, 9-α-fluorohydrocortisone (methyl FF). In the dogs, additional observations were made on the effects of desoxycorticosterone (DOC) and aldosterone.

In human subjects, the oral administration of single doses of methyl FF (0.025 to 1.0 mg), FF (0.2 to 1.0 mg), methyl F (10 to 400 mg) and F (100 mg) induced retention of sodium and loss of potassium. More precise assays of these properties, performed in adrenalectomized dogs by a method previously reported (*7*), are summarized in Table 1. In brief, the 2-methyl compounds are many times more potent than their nonmethylated analogs. Methyl FF is seen to be more potent than aldosterone, and thus to be the most active sodium-retaining and potassium-losing steroid known at the present time.

The mechanism whereby the methylated steroids affect cation excretion was investigated in dogs. Methyl FF and, to a lesser degree, methyl F were capable of producing decreases in sodium excretion despite concomitant increases in glomerular filtration rate, indicating that these steroids increase the reabsorption of sodium by the renal tubules.

The decrease in circulating eosinophils that follows the administration of steroids may be used as one index of their "glucocorticoid" activity. Observations in both man and the dog indicated that the methylated steroids were only slightly more potent than the nonmethylated compounds during the first 4 hours following treatment. Eosinopenia persisted much longer, however, with use of the methylated steroids.

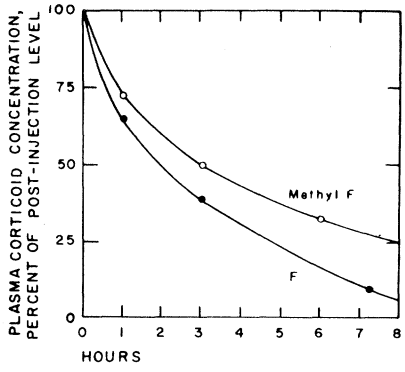

Fig. 1. Plasma 17,21-dihydroxy-20-ketosteroids (unconjugated) levels following intravenous injection of F and methyl F. The steroid concentration 10 minutes after injection is represented at "100-percent" concentration and "zero" hour. Curves are averages of three steroid tolerance tests in each of three subjects.

Whereas the direct biologic effects of a single oral dose of hydrocortisone ordinarily disappear in less than 24 hours, the effects of the 2-methyl derivatives were found to persist for approximately 48 hours in our studies in human subjects. Studies were accordingly designed to determine whether this prolonged action of the methylated steroids could be related to a slower rate of metabolic inactivation by the body.

Following administration of F and methyl F to human subjects, blood levels were determined at various intervals using a modification of the method of Silber and Porter (*8*) for measuring dichloromethane-soluble 17,21-dihydroxy-20-ketosteroids. It was consistently found that methyl F was removed from the circulation at a slower rate than F (Fig. 1). This was true whether the steroids were administered by vein or by mouth.

Following the administration of F to human subjects, one can account for approximately 30 percent of the administered dose by the determination of 17,21-dihydroxy-20-ketosteroids in the urine, using the method of Silber and Porter (*8*). The principal product thus measured is tetrahydrocortisone glucuronide. By way of contrast, following the administration of methyl F one can account for no more than approximately 5 percent of the administered dose using the same chemical methods.

It is suggested that the presence of the 2-methyl group alters the susceptibility of the steroid to enzymatic attack so that the processes by which F is metabolized operate less efficiently, and other processes assume greater prominence. As a result, removal of the methylated steroid from the circulation proceeds slowly, and during the metabolism of the steroid the 17,21-dihydroxy-20-keto configuration is lost. To some degree, the enhanced potency as well as the prolonged action of the 2-methyl steroids might be explained by the slower rate at which the body metabolizes them to inactive forms.

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Table 1. Relative effectiveness of various steroids on excretion of Na^+ and K^+ in the adrenalectomized dog, using DOC as a standard. The figures in parentheses represent 95-percent confidence limits. Ratios of potency are adjusted for molecular weight, and dosages are compared on an equimolar basis.

Steroid	Sodium		Potassium	
	Effect	Potency	Effect	Potency
DOC	Retention	1	Loss	1
Aldosterone	Retention	39 (26–61)	Loss	29 (20–41)
Methyl FF	Retention	49 (19–100)	Loss	155 (75–353)
FF	Variable		Loss	5.5 (3.1–10.7)
Methyl F	Variable		Loss	1.0 (0.5–2.1)
F	Loss		Loss	0.04 (0.026–0.063)

References and Notes

1. J. Fried and E. F. Sabo, *J. Am. Chem. Soc.* 75, 2273 (1953).
2. H. L. Herzog *et al.*, *Science* 121, 176 (1955).
3. M. M. Pechet and F. C. Bartter, *J. Clin. Endocrinol. and Metabolism* 15, 851 (1955).
4. J. A. Hogg *et al.*, *J. Am. Chem. Soc.* 77, 6401 (1955).
5. W. W. Byrnes *et al.*, *Proc. Soc. Exptl. Biol. Med.*, in press.
6. We wish to thank C. J. O'Donovan of the Upjohn Research Laboratories, Kalamazoo, Mich., for generously providing the steroids used in these studies.
7. G. W. Liddle *et al.*, *J. Clin. Invest.* 34, 1410 (1955).
8. R. H. Silber and C. C. Porter, *J. Biol. Chem.* 210, 923 (1954).

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Oral Phosphatase Levels and Caries Activity

Oral phosphatases in human saliva are thought to be principally of bacterial cell origin (*1–4*) and in part of glandular origin (acid phosphatase from the parotid glands, *3*). The significance of these enzymes in saliva is not clear, yet correlations have been suggested between their titers and certain oral debilities (*5, 6, 7*).

Lactobacilli show little phosphatase activity (*2, 4*); however Bray and King (*8*) demonstrated high degrees of phosphatase activity in 14 groups of organisms that are commonly found in the oral environment. Fitzgerald (*4*) has suggested a possible caries-activity test based on the titer of phosphatases in saliva.

Our experiment (*9*) was conducted to determine a possible relationship between