Anaerobic Studies of Steroidogenesis Using Perfused Calf Adrenals. (22386)

GEORGE ROSENFELD.* (Introduced by E. P. Vollmer.)
(With assistance of Willard D. Bascom.)
Physiology Division, Naval Medical Research Institute, Bethesda, Md.

In earlier studies with isolated calf adrenals(1), it was observed that suitable conditions for the biogenesis of corticoids were afforded if the glands were perfused at body temperature with a well-oxygenated artificial medium. However, when oxygenation was omitted, negligible production of adrenocortical steroids occurred. In subsequent studies of adrenal intermediary metabolism in which 2, 3, 5-triphenyl tetrazolium chloride (TTC) was employed in place of oxygen as a hydrogen acceptor(2), no corticoids were produced despite concurrent oxidation of various Meyerhof-Embden or Krebs cycle intermediates added to the perfusion medium. Rapid utilization of these substrates was manifested by appreciably larger deposition of formazan (reduced TTC) in the substrate-exposed adrenals than in contralateral control organs. These findings suggested that "molecular oxygen or a reactive derivative, *e.g.*, •OH, HO_2•, H_2O_2, as opposed to hydroxyl ions derived from water, was essential for formation of one or more of the corticosteroid oxygen functions(2)." Recently, Hayano and coworkers(3), employing H_2O^{18}, demonstrated that molecular oxygen, but not water, was utilized by the adrenal 11β-hydroxylase system in C-11β-hydroxylation of steroids. It will be recalled that in the steroidogenic reaction sequence, this biomodification is usually preceded by 17α-and/or 21-hydroxylation(4,5). Accordingly, if the failure of actively metabolizing glands to elaborate compounds F and B† were attributable solely to an inability to perform 11β-hydroxylation under anaerobic conditions, one might anticipate an accumulation of 11-desoxy precursors, *viz.*, Compound S and DOC. Absence of these or other steroidal intermediates suggested that among the large variety of enzymes participating in formation of corticoids from acetate or cholesterol, there may be other systems which require molecular oxygen. The main objective of the present investigation was to test this hypothesis by perfusing calf adrenals anaerobically with the artificial medium containing TTC and either (a) ACTH or (b) model steroids as substrates.

Materials and methods. Weighed calf adrenals, varying from 2.5 to 5.0 g, were excised at a local slaughterhouse, and dissected free of periadrenal fat and connective tissue. They were kept in an ice-cold, citrate-saline solution until cannulated and perfused via the adrenal vein. Details of the perfusion technic have already been presented(1). Control groups of 2 left and 2 right glands were perfused at 37.5°C in a multicycle system with a liter of artificial medium, continuously treated with a stream of 95% O_2/5% CO_2 and composed of the following substances, in grams per liter: glucose 2.00, NaCl 8.2, KCl 0.20, $CaCl_2$ 0.20, $MgSO_4$ 0.13, Na HCO_3 0.60, Na H_2PO_4 0.05. Experimental groups, comprising the 4 contralateral organs and differing in weight by less than 4%, were simultaneously perfused in another apparatus with a liter of the same medium. However, the latter was continuously treated with a stream of nitrogen and contained 2.5 grams of dissolved 2, 3, 5-triphenyl tetrazolium chloride (TTC). Use of this non-toxic, water-soluble tetrazolium salt as a physiological tool depends upon its interaction as a hydrogen acceptor with intracellular reductases, such as dehydrogenases, and its subsequent enzymatic reduction to an insoluble formazan. It is probable that TTC ($E'_o = -0.08$ volt) is reduced by a variety of dehydrogenase systems requiring

* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† These compounds and cortisone (E) are the major components of the steroidal secretion of the perfused calf adrenal(1).

TABLE I. Corticoid Output of Contralateral Groups of ACTH-Stimulated* Calf Adrenals Perfused *In Vitro* with an Oxygenated or TTC-Fortified† Artificial Medium.

Treatment of perfusion medium	μg/hr/4 glands			
	Cortisol (F)	Cortisone (E)	Corticosterone (B)	F, E & B
O_2	858	217	304	1379
N_2 + TTC	0	0	26	26
O_2	571	216	545	1332
N_2 + TTC	0	0	51	51
O_2‡	925	156	259	1340
N_2 + TTC‡	30	8	0	38

* 25 I.U. of ACTH (Armour's *ACTHAR*).
† 2.5 g/l of 2, 3, 5-triphenyl tetrazolium chloride.
‡ Mean values for 2 perfusion experiments in which the control (O_2) and experimental (TTC) effluents were combined before analysis.

coenzymes I or II(6) and that flavoproteins are the immediate electron donors(7). Previous studies(2) have shown that TTC competes with oxygen as a hydrogen acceptor, and that its reduction by the perfused calf adrenal to the stable, water-insoluble, deep-red pigment is essentially enzymatic. Either ACTH (25 I. U. of Armour's ACTHAR) or one of the diverse steroid substrates employed, *viz.*, Compound S, DOC, 21-desoxycortisone, progesterone and dehydroisoandrosterone dissolved in propylene glycol, was added to the circulating fluids of both the experimental and control glands. After a 1-hour perfusion, the effluents were collected and extracted with ethyl acetate. The steroid residues were fractionated by paper partition chromatography, and eluates of the various zones assayed by the Porter-Silber (Compounds F and E), phosphomolybdic acid (Compound B), and Zimmermann (C_{19}-ketosteroids) reactions. The extraction, chromatography, and assay procedures have been described in detail(8,9).

Results. Table I embodies analytical data of 4 experiments in which contralateral groups of ACTH-stimulated calf adrenals were concurrently perfused for 1 hour with the oxygenated or TTC-fortified artificial medium. It clearly demonstrates that glands perfused anaerobically (N_2 plus TTC) secreted little or no Compounds F, E and B in marked contrast to control organs perfused aerobically (95% O_2/5% CO_2). That this disparity in output was due to failure in biosynthesis rather than release of corticoids was shown by analysis of the glands following perfusion. The experimental organs contained amounts of corticoids comparable to those recovered from unperfused glands and totaling about 25% of the residual content of the perfused contralaterals (approx. 110 μg/4 glands).

It must be emphasized that under the experimental conditions TTC does not appreciably destroy, alter or combine with Compounds F, E or B nor does it interfere with their chromatographic mobility, identification, or measurement. Recoveries of 94, 93 and 107% were observed after a liter of artificial medium, containing 2.5 g TTC and a mixture of these steroids (510, 210 and 310 μg, respectively) had been circulated for one hour in a perfusion apparatus without mounted organs.

Table II summarizes the results of 5 conversion studies in which contralateral groups of unstimulated calf adrenals were concurrently perfused for one hour with the oxygenated or TTC-fortified artificial medium containing an added steroid substrate. The major transformation products were isolated and determined as previously described; their identification by infrared analysis and melting point has already been reported(8). Although the aerobically perfused glands 11β-hydroxylated substantial quantities of 11-desoxycortisol and 11-desoxycorticosterone to cortisol and corticosterone, respectively, anaerobically perfused glands exhibited negligible 11β-hydroxylase activity. A similar dependence of the 21-hydroxylating mechanism on the presence of molecular oxygen is evidenced by the 21-desoxycortisone → cortisone conversion study. With respect to 17α-hydroxylation, appreciable amounts of Compound F (17-OH-corticosterone) were recovered following aerobic but not anaerobic perfusion of progesterone. Although Compounds F and B are the quantitatively major end-products, small amounts of 17α- and 11β-hydroxyprogesterone have also been reported (10). Control (O_2) chromatograms did reveal a moderate quantity of the latter inter-

TABLE II. Conversion of Steroid Substrates by Contralateral Groups of Calf Adrenals Perfused *In Vitro* with an Oxygenated or TTC-Fortified Artificial Medium.

Steroid substrate and major transformation product(s)	Amt of substrate perfused (mg)	Major product(s) formed in 1 hr (mg)		Specific enzymatic reaction(s)
		O_2	TTC(N_2)	
11-Desoxycortisol (S)	20			
↓				11β-OH
Cortisol (F)		15.5	.3	
11-Desoxycorticosterone	30			
↓				11β-OH
Corticosterone (B)		13.4	.4	
21-Desoxycortisone	20			
↓				21-OH
Cortisone (E)		10.8	.6	
Progesterone	40			
↓				17α-, 21-, 11β-OH
Cortisol (F)		2.5	0	21-, 11β-OH
Corticosterone (B)		3.9	0	
Dehydroisoandrosterone	25			
↓				Δ^5-3β-OH →
Δ^4-androstene-3,17-dione		1.6	7.5	Δ^4-3-ketone
↓				11β-OH
11β-OH-Δ^4-androstene-3,17-dione		4.4	.1	

mediates but there were no corresponding zones on the experimental ones.

In marked contrast to the preceding enzymatic oxidations, the conversion of the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone readily occurred in the absence of molecular oxygen. Table II demonstrates that the anaerobically perfused organs produced a total of 7.6 mg of Δ^4-androstene-3, 17-dione compounds as compared with the control value of 6.0 mg. Inability to 11β-hydroxylate Δ^4-androstene-3, 17-dione formed corroborates the previous finding that this biosynthetic reaction is oxygen-dependent.

To ascertain whether anaerobically perfused, actively metabolizing adrenals produced appreciable quantities of a C_{19} or C_{21} precursor or intermediate of the adrenocortical hormones, the combined steroid residues of 2 ACTH-stimulation experiments were subjected to a series of successive paper chromatographic fractionations in toluene-propylene glycol(11) and ligroin-propylene glycol partition systems(12). In view of the demonstrated 3β-dehydrogenase activity of the glands under anaerobic conditions, it was reasoned that there might be an accumulation of progesterone (and Δ^4-androstene-3, 17-dione)‡ if oxygen were not needed in the biosynthetic reactions preceding formation of Δ^5-pregnenolone (and dehydroisoandrosterone, the putatively important intermediates in costeroidogenesis). A 72-hour partition in the toluene system followed by an 18-hour resolution of the runoff revealed a weakly positive cortisol zone but no other zones absorbing in the UV or positive to triphenyltetrazolium chloride (TPTZ) or Zimmermann color tests. Thus, no significant amounts of cortisone (E), 11-desoxycortisol (S) or corticosterone (B) were detected. The 18-hour toluene overflow was systematically chromatographed for 48, 24 and 8 hours in the ligroin system. Essentially negative UV scanning and spot tests of these paper chromatograms precluded the possibility of significant amounts of 11β- or 17α-hydroxyprogesterone, 11-desoxycorticosterone (DOC), dehydroisoandrosterone, Δ^4-androstene-3, 17-dione, Δ^5-pregnenolone or progesterone. Pure samples of all the aforementioned steroids were concurrently chromatographed on 1-cm

‡ We have observed at most only minute amounts of C_{19} steroids in the effluent of the isolated perfused calf adrenal.

strips as reference standards and identified. Absence of a substantial quantity of one or more $C_{21}O_2$ steroids in the experimental effluents stands in sharp contrast to the control effluent content which comprised, aside from other quantitatively minor components, a total of approximately 3 mg of Compounds F, E and B. Thus, it would appear that molecular oxygen is required not only in the 17α-, 21-, and 11β-hydroxylation systems but also in one or more of the preliminary enzymatic reactions which result in formation of the $C_{21}O_2$ steroid nucleus.

Discussion. It is noteworthy that the 3β-dehydrogenase system, which does not require an oxygen atmosphere, catalyzes an oxidation reaction entailing a net loss of hydrogen, whereas the 3 oxygen-dependent hydroxylating systems examined catalyze an oxidation resulting in a net increase in the oxygen content of the steroidal end-product. The latter findings recall the observations made by Conn *et al.*(13) of an enzyme system in wheat germ involving a peroxidase which catalyzes the oxidation of TPNH by molecular oxygen, and by Brodie *et al.*(14) of a peroxidase-like system in liver microsomes which requires TPNH and oxygen to hydroxylate aromatic compounds. It is possible that there may be similar peroxidase-like systems in adrenocortical tissue which with TPNH and molecular oxygen selectively introduce hydroxyl groups into the steroid nucleus, perhaps via peroxide formation. This hypothesis appears more plausible if one recalls that the 11β-, 17α-, and 21-hydroxylase mechanisms have all been shown to require TPN or TPNH for optimal activity(4).

In evaluating the present data vis-a-vis the dependence of these hydroxylases on molecular oxygen, it is important to consider the quantitative aspects and nature of the cellular energy produced anaerobically in relation to the oxidative changes studied. Under the experimental conditions, $\Delta F \cong -6000$ cals/mole TTC reduced. Moreover, since oxidation of a methyl group or ring methylene group to the corresponding methylol or secondary hydroxyl function involves a liberation of free energy, it is reasonable to assume that oxidation of C-21 or C-11 would likewise have a negative ΔF and entail liberation of free energy whether coupled to O_2 or TTC as the final electron acceptor. Although it is difficult to estimate the free energy change involved in the C-17α oxidation without an appropriate model for comparison, it seems reasonable that, if not exergonic, at least it does not require an unavailable level of free energy input.

With respect to the intermediary metabolism of the TTC-perfused adrenals, previous studies(2) have demonstrated that all of the added Meyerhof-Embden and Krebs-Johnson carbohydrate intermediates were readily utilized. Thus, whether the energy is derived from the concurrent functioning of the glycolytic and/or the terminal aerobic pathway (15,16), it would appear from the aforementioned considerations of the free energy and oxidation changes that active reduction of TTC would support the steroid hydroxylating mechanisms if they proceeded independently of molecular oxygen.

Summary. Isolated, ACTH-stimulated calf adrenals, perfused anaerobically (N_2) with TTC as a hydrogen acceptor, failed to elaborate Compounds F, E, B or a $C_{21}O_2$ steroidal intermediate despite an active oxidative metabolism. Specific enzymatic conversion studies with added steroid substrates demonstrated their inability to perform 11β-, 17α-, and 21-hydroxylations under anaerobic conditions although they were able to transform the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone. It would appear that the hydroxylases, which selectively introduce hydroxyl groups in the side chain or nucleus, require molecular oxygen (or a reactive derivative) in contrast to the 3β-dehydrogenase system, which catalyzes the oxidation of an existing oxygen function.

The authors are grateful to H. B. Bond for his valuable technical assistance.

1. Rosenfeld, G., *Endocrinology,* 1955, v56, 649.

2. ———, to be published in *Arch. Biochem. Biophys.*

3. Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H., and Doering, W. von E., *ibid.,* 1955, v59, 1.

4. Hechter, O., and Pincus, G., *Physiol. Rev.,* 1954, v34, 459.

5. Dorfman, R. I., *The Hormones,* Academic Press Inc., 1955, v3, 589.

6. Mattson, A. M., Jensen, C. O., and Dutscher, R. A., *Science,* 1947, v106, 294.

7. Straub, F. B., *Biochem. J.,* 1939, v33, 787.

8. Rosenfeld, G., Ungar, F., Dorfman, R. I., and Pincus, G., *Endocrinology,* 1955, v56, 24.

9. Rosenfeld, G., and Ungar, F., *ibid.,* 1955, v56, 30.

10. Levy, H., Jeanloz, R. W., Jacobsen, R. P., Hechter, O., Schenker, V., and Pincus, G., *J. Biol. Chem.,* 1954, v211, 867.

11. Zaffaroni, A., Burton, R. B., and Keutmann, E. H., *Science,* 1950, v111, 6.

12. Savard, K., *J. Biol. Chem.,* 1953, v202, 457.

13. Conn, E. E., Kraemer, L. M., Liu, P., and Vennesland, B., *ibid.,* 1952, v194, 143.

14. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C., and Udenfriend, S., *Science,* 1955, v121, 603.

15. Hayano, M., and Dorfman, R. I., *J. Biol. Chem.,* 1953, v201, 175.

16. Brownie, A. C., and Grant, J. K., *Biochem. J.,* 1954, v57, 255.

Received March 5, 1956. P.S.E.B.M., 1956, v92.

Effect of Eastern Equine Encephalomyelitis Virus Infection on Phosphate Transfer and Modification by Cortisone.* (22387)

R. D. HIGGINBOTHAM,† E. V. STEWART, L. P. GEBHARDT, AND L. T. SAMUELS.

Departments of Bacteriology and Biological Chemistry, University of Utah College of Medicine, Salt Lake City.

The biochemical approach to the study of viruses and their relation to host cell has produced numerous reports on alterations of host cell metabolism due to virus infection. The most prolific source of information has arisen from investigations concerned with effects of bacteriophage on its host cell, and these have been furthered by use of radioactive isotopes (1-3). Similar investigations in the field of animal viruses have not always been so easily resolved as those with bacteriophage, largely due to heterogeneous mixture of cells in host tissue and the difficulty in distinguishing the direct effect produced by virus on infected cells from indirect effects on neighboring cells and the organism as a whole (*e.g.,* inflammation). Anderson, *et al.*(4), using radioactive phosphate, reported that infection of monkey brain tissue with Lansing poliomyelitis virus produced significant changes in rate of phosphate turnover in infected areas of the brain, and suggested that it was due to an increased metabolic activity. Since these changes were detected in specific activities of total acid-soluble organic as well as in inorganic phosphate fractions, the authors discounted the possibility that these changes could be due to injury. No significant alterations in transfer rates from blood to spinal fluid were observed. On the other hand, Rafelson, *et al.*(5), found no changes in turnover of organic acid-soluble phosphates of day-old mouse brain tissue cultures infected with Theiler's GD VII virus which could be related to virus synthesis, although changes in other phosphate fractions attributable to virus synthesis were observed.

It therefore seemed of interest to investigate the effect of a neurotropic virus on the phosphate turnover in infected mouse brain tissue *in vivo* to determine if the effect of the virus infection was due to an actual metabolic requirement for virus synthesis or merely to an increased rate of transfer associated with inflammation.

Materials and methods. Swiss albino mice

* This and related studies constituted thesis submitted by senior author to the Department of Bacteriology, University of Utah College of Medicine, Salt Lake City, in partial fulfillment of the degree of Doctor of Philosophy. This research was supported in part by contract with U. S. Atomic Energy Commission.

† Present address: Department of Anatomy, University of Utah College of Medicine.