nommen wurden. Infolge der Empfindlichkeit der Methode genügen 0.2–0.3 ml Blut zur Durchführung von Einzelbestimmungen (ΔE_{366} 0.1 = 2.61 γ ml/Testlösung).

HANS-DIETER HORN
FRIEDRICH H. BRUNS

I. Medizinische Klinik und Institut für Physiologische Chemie der Medizinischen Akademie Düsseldorf (Deutschland)

[1] E. RACKER, *J. Biol. Chem.*, 184 (1950) 313.
[2] H. VON EULER, E. ADLER UND H. HELLSTRÖM, *Z. physiol. Chem.*, 241 (1936) 329.
[3] H. VON EULER, E. ADLER, E. G. GÜNTHER UND H. HELLSTRÖM, *Z. physiol. Chem.*, 245 (1936) 217.
[4] O. MEYERHOF UND P. OESPER, *J. Biol. Chem.*, 170 (1947) 1.
[5] TH. BÜCHER UND H. REDETZKI, *Klin. Wschr.*, 29 (1951) 615.
[6] G. PFLEIDERER UND K. DOSE, *Biochem. Z.*, 326 (1955) 436.
[7] G. BEISENHERZ, H. J. BOLTZE, TH. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT UND G. PFLEIDERER, *Z. Naturforsch.*, 8b (1953) 555.
[8] S. KOLLER, *Graphische Tafeln zur Beurteilung statistischer Zahlen*, Steinkopf, Darmstadt 1953.

Eingegangen den 16. Mai 1956

Hydroxylation of steroids by microorganisms in the presence of $^{18}O_2$*

In a previous series of experiments[1], it was established that in the introduction of an oxygen function in steroids at C-11β by adrenal 11β-hydroxylase that the oxygen of the atmosphere is utilized directly. This finding has been confirmed by SWEAT *et al.*[2]. A continuation of these studies to elucidate the mechanism of enzymic hydroxylations at other position on steroids significant to corticoid activity, namely 17α and 21, were then considered. Unfortunately the preparation of adequately pure adrenal hydroxylases catalyzing these reactions in quantities sufficient for isolation work was found to be prohibitive. Thus, another source of hydroxylating systems was sought. Because of the zealous efforts in recent years by various workers in the isolation of pure strains of microorganisms capable of specific steroid hydroxylations, this avenue was open to us. If the hydroxylation at C-11β as catalyzed by the mold enzyme were to proceed with the utilization of molecular oxygen, as in the case of the adrenal system, the analogy of the similarity of the enzymic mechanisms between microorganism and mammalian systems in the case of hydroxylations at 17α and 21 could be drawn. Thus, these experiments were carried out. In addition, 6β and 11α hydroxylations were also evaluated in conjunction with further projected studies on the mechanism involved.

Microorganisms were grown in 250 ml Erlenmeyer flasks in 50 ml of media. To each flask was added 5–10 mg of steroid precursor in 0.2–0.6 ml propylene glycol or acetone just prior to evacuation of the flask with the aid of a water-vacuum system. Approximately 100 ml of ^{18}O-enriched oxygen, produced electrolytically from $H_2{}^{18}O$-enriched water**, were then introduced into each flask, the remainder of the gas volume being made up with N_2. Incubations were carried out for 24–48 hours at room temperature. The reactions were stopped by the addition of ethyl acetate. The variation in the efficiency of ^{18}O introduction, 82–96% of theoretical, as found in products of the different mold transformations is probably due to small errors accumulated in these experimental manipulations. The finding of 82% of theoretical ^{18}O in the desoxycorticosterone synthesized from progesterone (C-21 hydroxylation) indicated that there is little exchange if any of the α-ketol oxygens with the water of the medium. All manipulations with this compound were carried out under neutral conditions with the exception of a brief wash with 2% $NaHCO_3$ of the ethyl acetate extract.

In every instance examined, ^{18}O from the atmosphere was found to be incorporated into the steroid product. Of interest is the finding that the hydroxylation in the side chain of the pregnane structure apparently proceeds via a mechanism similar to those for the ring. Appropriate controls with $H_2{}^{18}O$ and non-labelled O_2 showed no ^{18}O incorporation.

Studies by various groups of the enzymic requirements for the catalysis of hydroxylations at C-11β[8,9,10], C-17α[8,11,12] and C-21[8,11,13] by adrenal and testis[12] preparations have indicated a remarkable similarity in the need of the individual hydroxylases for TPN or TPNH, oxygen and

* Supported in part by Research Grant C-2207 from the Research Grants and Fellowships, National Institutes of Health, U.S. Public Health Service.

** $H_2{}^{18}O$ was purchased from the Stuart Oxygen Co., on allocation from the U.S. Atomic Energy Commission.

TABLE I

STEROID HYDROXYLATIONS IN THE PRESENCE OF $^{18}O_2$

Position of —OH introduction	*Microorganism*	*Steroid precursor incubated*	*Steroid product isolated**	*Percent of theoretical ^{18}O found***
11β	*Cunninghamella blakesleeana*[3]	11-Desoxycorticosterone	Corticosterone	89
17α	*Cephalocethium roseum*[4]	Progesterone	17α-Hydroxyprogesterone	96
21	*Ophiobolus herbotrichus*[5]	Progesterone	11-Desoxycorticosterone	82
6β	*Rhizopus arrhizus*[6]	11-Desoxycorticosterone	6β-Hydroxy-11-desoxycorticosterone	83
11α	*Rhizopus nigricans*[6]	11-Desoxycorticosterone	Epicorticosterone	93

* All products were extracted from the incubation medium with ethyl acetate, separated by silica gel chromatography (benzene-ethyl acetate), crystallized, and identified through melting points and comparison of their infrared spectra with standards.

** Based on the introduction of a single oxygen function. ^{18}O was determined by the method of DOERING AND DORFMAN[7].

fumarate. The need for oxygen has now been exemplified. A clue to the requirement for reduced pyridine nucleotide appears in the note on the mechanism of enzyme-catalyzed oxygen transfer by MASON AND ONOPRIENKO[14]. It is the opinion of the authors that fumarate serves in some capacity in the protection of the oxygen-bound enzyme rather than as a reductant for TPN. Additional work is necessary to clarify this point. It is generally agreed that these hydroxylases are peroxidases in nature, as does appear the DPNH-linked phenylalanine hydroxylase of MITOMA[15]. While the latter enzyme has been resolved into two protein components, such a degree of purification has not as yet been achieved with the steroid hydroxylases. A possible exception is the 21-hydroxylase of RYAN[16].

^{18}O analyses were carried out by Mr. MICHAEL A. GREENBAUM of Yale University. The authors are indebted to Dr. D. H. PETERSON of The Upjohn Co. for slants of *Cunninghamella blakesleeana, Rhizopus arrhizus, Rhizopus nigricans,* and *Cephalocethium roseum.* Thanks are due to Mr. ROBERT BIBEAU for valuable aid in the course of these studies.

Worcester Foundation for Experimental Biology, Shrewsbury, Mass. (U.S.A.)

MIKA HAYANO
AKIRA SAITO
DAVID STONE
RALPH I. DORFMAN

1 M. HAYANO, M. C. LINDBERG, R. I. DORFMAN, J. E. H. HANCOCK AND W. v. E. DOERING, *Arch. Biochem. Biophys.*, 59 (1955) 529.
2 M. L. SWEAT, R. A. ALDRICH, C. H. DE BRUIN, W. L. FOWLKS, L. R. HEISELT AND H. S. MASON, *Federation Proc.*, 15 (1956) 367.
3 F. R. HANSON, K. M. MANN, E. D. NIELSON, H. V. ANDERSON, M. P. BRUNNER, J. N. KARNEMAAT, D. R. COLINGSWORTH AND W. J. HAINES, *J. Am. Chem. Soc.*, 75 (1953) 5369.
4 P. D. MEISTER, L. M. REINEKE, R. C. MEEKS, H. C. MURRAY, S. H. EPPSTEIN, H. M. L. OSBORN, A. WEINTRAUB AND D. H. PETERSON, *J. Am. Chem. Soc.*, 76 (1954) 4050.
5 C. MEYSTRE, E. VISCHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 37 (1954) 1548.
6 S. H. EPPSTEIN, P. D. MEISTER, D. H. PETERSON, H. C. MURRAY, H. M. LEIGH, D. A. LYTTLE, L. M. REINEKE AND A. WEINTRAUB, *J. Am. Chem. Soc.*, 75 (1953) 408.
7 W. v. E. DOERING AND E. DORFMAN, *J. Am. Chem. Soc.*, 75 (1953) 5595.
8 M. HAYANO, N. SABA, R. I. DORFMAN AND O. HECHTER, *Rec. Progr. Hormone Research*, XII, in press.
9 M. L. SWEAT AND M. D. LIPSCOMB, *J. Am. Chem. Soc.*, 77 (1955) 5185.
10 J. K. GRANT AND A. C. BROWNIE, *Biochim. Biophys. Acta*, 18 (1955) 433.
11 J. E. PLAGER AND L. T. SAMUELS, *J. Biol. Chem.*, 211 (1954) 21.
12 W. S. LYNN, Jr., *Federation Proc.*, 15 (1956) 305.
13 B. RUBIN, private communication.
14 H. S. MASON AND I. ONOPRIENKO, *Federation Proc.*, 15 (1956) 310.
15 C. MITOMA, *Arch. Biochem. Biophys.*, 60 (1956) 476.
16 K. J. RYAN, *Federation Proc.*, 15 (1956) 344.

Received May 16th, 1956