enzymatic formation of such anhydrides has not been observed. This communication presents evidence for the enzymatic synthesis of try-AMP.

The total yield (15 mg.) of try-activating enzyme from 5 kg. of beef pancreas[5] was incubated with try-3-C^{14}, Mg^{++}, and ATP (Table I). The reaction mixture was lyophilized and extracted with glacial acetic acid; on addition of ether to the extract a precipitate formed, leaving try-3-C^{14} in solution. Paper ionophoresis of the precipitate gave a radioactive band (A) corresponding to authentic try-AMP.[14] Elution of A yielded an alkali-labile compound that reacted with hydroxylamine to form a C^{14}-hydroxamate, which on paper chromatography in five solvents gave R_F values identical with try-hydroxamate. The negatively charged band (B) has not yet been identified. In a similar experiment with added try-AMP (1 μmole), about 10 times more C^{14} was found in band A, suggesting exchange between try and try-AMP. This was demonstrated by incubating try-3-C^{14} (0.08 μmole; 5×10^5 c.p.m.) and try-AMP (1 μmole) with enzyme followed by addition of hydroxylamine; C^{14}-try-hydroxamate (3420 c.p.m.) was identified as described above.[15] We have found that this enzyme also catalyzes ATP synthesis from P-P and AA-AMP anhydrides (*e.g.*, AMP anhydrides of D-try,[16] L-try, D-phenylalanine,[16] L-phenylalanine, L-isoleucine, L-leucine, L-glutamine, glycine, L-alanine, L-proline, L-tyrosine, L-valine). Thus, incubation of AA-AMP (1 μmole), P-P (2 μmoles), $MgCl_2$ (10 μmoles), and enzyme (0.4 mg.) in 1 ml. of 0.1 M tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.2) gave 0.1 to 0.2 μmole of ATP in 5 minutes at 37°. Benzoyl-AMP, carbobenzoxy-try-AMP, and β-alanine-AMP were inactive.[17] The low specificity with respect to ATP synthesis contrasts markedly with the strict specificity for L-try in hydroxamate formation.[5] The present data indicate net synthesis of a compound with the properties of try-AMP in the absence of hydroxylamine as trapping agent, and thus support the concept that try-AMP is formed in the activation reaction.[18] It would appear that any mechanism proposed for the activation phenomenon should account for the observations that the activating enzyme preparation catalyzes exchange between try and try-AMP, and synthesis[19] of ATP from P-P and various AA-AMP anhydrides.

TABLE I: PAPER IONOPHORESIS[a]

Compound	Position of band (mm./2 hours)	C.p.m.
Tryptophan standard	− 5.0	
Try-AMP standard	−30.2	
Band A	−30.2	3100
Band B	+12.1	450

[a] Reaction mixture consisted of enzyme[5] (15 mg.), ATP (10 μmoles), $MgCl_2$ (10 μmoles), DL-try-3-C^{14} (0.08 μmole; 5×10^5 c.p.m.), crystalline pyrophosphatase (10 γ) in 2 ml. of 0.05 *M* tris-(hydroxymethyl)-aminomethane buffer (*p*H 8.0); incubated at 37° for 30 minutes and processed as described in text. Precipitate was analyzed ionophoretically (R. Markham and J. B. Smith, *Biochem. J.*, **52**, 552 (1952)) in 0.05 *M* ammonium formate buffer (*p*H 4.5) at 20 volts per cm. for 2 hours.

(14) Prepared as described by Castelfranco, *et al.*, accompanying paper.

(15) A value of 101 c.p.m. was obtained non-enzymatically.

(16) The optical purity of the AA moiety was greater than 99.5%; A. Meister, *et al.*, *J. Biol. Chem.*, **192**, 535 (1951).

(17) L-Try-inosinate did not form inosine triphosphate.

(18) W. C. Rhodes and W. D. McElroy recently have shown enzymatic synthesis of adenyloxyluciferin by firefly luciferase (personal communication).

(19) Such synthesis recently has been reported by Novelli (*Proc. Natl. Acad. Sci.*, **44**, 86 (1958)) and with yeast methionine-activating enzyme by Berg (*Fed. Proc.*, **16**, 152 (1957); personal communication).

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STERIC CONSIDERATIONS IN THE ENZYMATIC COURSE OF THE HYDROXYLATION OF STEROIDS*

Sir:

Experiments to determine the mode of attack of the steroid hydroxylases on their substrates have been carried out. The two major possibilities considered in hydroxylation reactions involving $>C<^{H}_{H}$ groupings were: (1) a direct replacement of the H from the position to be hydroxylated, or (2) a Walden type inversion with the removal of the H from the alternate position to the one hydroxylated. For this purpose pregnane-3,20-dione-11α,12α-H^3[1] was incubated with *Rhizopus nigricans*[2] and the 11α-hydroxylated analog isolated; progesterone-11α,12α-H^3 prepared by the method of Holysz[3] from pregnanedione-11α,12α-H^3 was perfused through surviving bovine adrenal glands[4]

TABLE I

INCUBATION OF PREGNANEDIONE-11α,12α-H^3 WITH RHIZOPUS NIGRICANS

Compounds isolated	Counts/min./μM[a]
Pregnanedione, m.p. 121–122°[b]	3.25×10^7[c]
11α-Hydroxy-pregnanedione, m.p. 123–125°	0.97×10^7

PERFUSION OF PROGESTERONE-11α,12α-H^3 THROUGH BOVINE ADRENALS

Compounds isolated	Counts/min./μM
Progesterone, m.p. 124–125°	6.90×10^6
17α-Hydroxy-progesterone, m.p. 219–223°	6.93×10^6
Corticosterone, m.p. 179–181°	6.70×10^5
Hydrocortisone, m.p. 213–215°	6.73×10^6

[a] All counts were determined in a Packard Tri-Carb liquid scintillation counter Model 314. [b] All melting points were observed on a Fisher–Johns hot stage and are corrected. [c] Accuracy ±3%.

and the 11β-hydroxylated products, corticosterone and hydrocortisone, isolated. Resolution of incubated material in both experiments was effected by silica gel column chromatography using mixtures of benzene and ethyl acetate for elution. All steroids were purified to a constant count by additional column or paper chromatography, when necessary, and by repeated crystallizations. Iden-

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

(1) Synthesis and discussion of H^3 distribution to be published.

(2) S. H. Eppstein, D. H. Peterson, H. Marian Leigh, H. C. Murray, A. Weintraub, L. M. Reineke and P. D. Meister, THIS JOURNAL, **75**, 421 (1953).

(3) R. P. Holysz, *ibid.*, **75**, 4432 (1953).

(4) O. Hechter, A. Zaffaroni, R. P. Jacobsen, H. Levy, R. W. Jeanloz, V. Schenker, and G. Pincus, "Recent Progress in Hormone Research," Vol. VI, Academic Press, New York, 1951, p. 215.

tifications were established by comparisons against infra-red spectra of known standards. Compounds isolated from the above experiments are listed in Table I together with their specific activities.

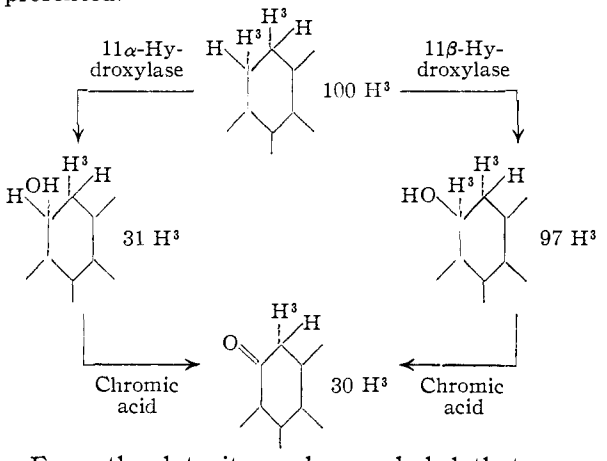
The results showed a loss of 69% of the total count of pregnanedione-H^3 on 11α-hydroxylation, and essentially no loss (2–3%) on 11β-hydroxylation. Mild chromic acid oxidation according to the method of Poos[5] of the hydroxylated products to their keto analogs (Table II) showed a loss of 70% of the total count in the case of the 11β-hydroxylated structure, while there was essentially no change (1%) in counts in the 11α series.

TABLE II

CHROMIC ACID OXIDATION OF 11-HYDROXYLATED STEROIDS

11α-Hydroxy-pregnanedione	11-Keto-pregnanedione
m.p. 123–125°	m.p. 159–161°
8.43×10^4 counts/min./μM	8.49×10^4 counts/min./μM
Corticosterone-21-acetate	11-Dehydro-corticosterone-21-acetate
m.p. 156–157°	m.p. 180–182°
3.21×10^4 counts/min./μM	0.98×10^4 counts/min./μM

A schematic representation of the results is presented.



From the data it can be concluded that enzymatic steroid hydroxylations proceed by a mechanism in which there is a simple replacement of the hydrogen in the position to be hydroxylated. This is the second instance noted in points of similarity in the mechanism of reaction of the steroid hydroxylases, irrespective of source, the first being their ability of the utilization of molecular oxygen directly in the formation of the hydroxyl function.[6,7] Further mechanism studies involving this group of enzymes are now in progress.

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(5) G. I. Poos, G. E. Arth, R. E. Beyler and L. H. Sarett, THIS JOURNAL, **75**, 422 (1953).

(6) M. Hayano, M. C. Lindberg, R. I. Dorfman, J. E. H. Hancock, and W. v. E. Doering, *Arch. Biochem. Biophys.*, **59**, 529 (1955).

(7) M. Hayano, A. Saito, D. Stone and R. I. Dorfman, *Biochim. Biophys. Acta*, **21**, 380 (1956).

THE STEREOCHEMISTRY OF 7α-HYDROXYLATION IN THE BIOSYNTHESIS OF CHOLIC ACID FROM CHOLESTEROL

Sir:

Recent evidence on the course of enzymatic hydroxylation of steroids at a saturated carbon atom indicates that such reactions occur by a direct replacement mechanism and not by hydration of olefinic intermediates.[1–3] However, the chemical nature of the enzymatic reagent and the type of mechanism involved are still undetermined. The stereochemistry of enzymatic hydroxylation at C_7 of the steroid nucleus has now been examined to provide geometrical evidence regarding mechanism.

During the biochemical conversion of cholesterol to cholic acid a 7α-hydroxyl group is introduced, quite possibly as the initial step since nuclear hydroxylation of cholesterol proceeds side-chain degradation[4] and since 7α-hydroxycholesterol is transformed into cholic acid in the rat.[5] The stereochemical course of this 7α-hydroxylation in rats has been investigated by double-labelling experiments using cholesterol stereospecifically labelled with tritium at position 7[6] and cholesterol-4-^{14}C.[7]

TABLE I

		Activity ratio T/C^{14}	Per cent. retention of tritium in cholic acid
1	Cholesterol-4-^{14}C-7α-*t*	1.75	
2	Cholic acid rat 4	0.31	7.4
3	Cholic acid rat 5	0.12	6.8
4	Cholesterol-4-^{14}C-7α-*t*	1.24	
5	Cholic acid rat 6	0.07	5.6
6	Cholic acid rat 7	0.09	7.2
7	Cholesterol-4-^{14}C-7β-*t*	1.04	
8	Cholic acid rat 9	0.95	91.5
9	Cholic acid rat 10	1.03	100

When cholesterol-[4-^{14}C + 7α-*t*] was administered to rats the isolated cholic acid retained only *ca.* 7% of tritium relative to radiocarbon (Table I).

(1) M. Hayano and R. I. Dorfman, *J. Biol. Chem.*, **211**, 227 (1954).

(2) B. M. Bloom and G. M. Shull, THIS JOURNAL, **77**, 5767 (1955).

(3) M. Hayano, A. Saito, D. Stone and R. I. Dorfman, *Biochem. and Biophys. Acta*, **21**, 380 (1956).

(4) See S. Bergstrom and B. Bergstrom, *Ann. Rev. Biochem.*, **25**, 177 (1956).

(5) S. Linstedt, *Acta Chem. Scand.*, **11**, 417 (1957).

(6) (a) Cholesterol-7α-*t* and cholesterol-7α-*d* were synthesized stereospecifically by the sequence: 7α-bromo-6-ketocholestanyl acetate → 7α-^{n}H-6-ketocholestanyl acetate (Zn-^{n}HOAc) → 7α-^{n}H-6β-hydroxycholestanyl acetate ($NaBH_4$) → 7α-^{n}H-cholesterol ($POCl_3$-C_5H_5N, followed by $LiAlH_4$). Cholesterol-7β-*t* and cholesterol-7β-*d* were prepared by a similar process starting with 5α,7β-$^n H_2$-7α-bromo-6-ketocholestanyl acetate using unlabelled acetic acid in the debromination step. The cholesterol-7α-*d* and -7β-*d* (infrared max. 2102, 2127 cm.$^{-1}$ and 2147, 2160 cm.$^{-1}$, respectively) were analyzed by infrared absorption [E. J. Corey, M. G. Howell, A. Boston, R. L. Young and R. A. Sneen, THIS JOURNAL, **78**, 5036 (1956)] and the isotope orientation was found to be stereospecific within the analytical sensitivity of ±4%. It follows that the tritium labelling is 96 ± 4% stereospecific; (b) see E. J. Corey and G. A. Gregoriou, *Abstracts*, 131st A.C.S. meeting, p. 15-O.

(7) *Ca.* 2 mg. of a mixture of cholesterol-4-^{14}C and cholesterol-7α-*t* or cholesterol-7β-*t* (one microcurie of each isotope) was injected intraperitoneally into rats with bile fistula. The bile was collected and cholic acid was isolated as described earlier [S. Bergstrom and A. Norman, *Proc. Soc. Exptl. Biol. Med.*, **83**, 71 (1953)], further purified by dilution with 100 mg. of unlabelled cholic acid and recrystallization, and analyzed for isotopes according to R. Glascock, *Isotopic Gas Analysis for Biochemists*, Academic Press, N. Y., 1954.