4-(4-Cyclohexyl-4-phenylbutyl)-N-methylpiperidine.—A solution of 33.9 g. (0.1 mole) of the nitrile just described in 100 ml. of xylene was added gradually to a vigorously stirred mixture of 12 g. (0.3 mole) of sodium amide in 100 ml. of refluxing xylene. The mixture was refluxed and stirred for ten hours, then cooled and decanted from the excess sodium amide. The benzene solution was washed with water and then extracted with 10% hydrochloric acid. The acid extracts were made alkaline and the base taken up in ether and dried over sodium hydroxide pellets. The ether was removed and the amine distilled under reduced pressure, b.p. 158–163° (0.1 mm.).

The amines prepared in this way are described in Table I.

Hydrochlorides.—Samples of the basic nitriles and amines were converted to their hydrochlorides with hydrogen chloride in alcohol and precipitated with ether. The hydrochlorides were recrystallized from acetone–ether or alcohol–ether combinations and are described in Table I.

Only one racemate of 2-(3-cyclohexyl-3-phenylpropyl)-N-methylpiperidine hydrochloride was obtained in crystalline form.

Methiodides.—The basic nitriles and amines were converted to their methiodides by warming the bases in benzene or ethyl acetate with excess methyl iodide. The methiodides were recrystallized from ethyl acetate–methanol and are described in Table I.

MORRIS PLAINS, N. J. RECEIVED OCTOBER 2, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MARYLAND]

The Action of Rat Liver Tissue on Mixtures of Methionine and Ethanolamine

BY F. P. VEITCH AND GUNTER ZWEIG

This paper describes experimental work which shows that when methionine and ethanolamine are incubated with rat liver slice or homogenate, the presence of D-amino acid oxidase in this tissue causes an apparent disappearance of methionine as judged by the McCarthy–Sullivan method for methionine. In the light of this finding, reports of *in vitro* transmethylation from methionine to ethanolamine to form choline based on the disappearance of methionine from the reaction mixture are subject to grave doubt. The validity of the reported isolation of choline as the reineckate from such reaction mixtures is also shown to be questionable.

The reported[1–5] *in vitro* synthesis of choline from methionine and ethanolamine using rat or guinea pig kidney or liver, slices or homogenates as a source of a transmethylase enzyme or enzyme system led us to attempt to isolate this enzyme or enzyme system. Consistent failure to isolate such a system or even duplicate the cited reports has caused us grave doubt as to the validity of these reports.

Steensholt[2–5] has used the disappearance of methionine from the reaction mixture, as measured by the McCarthy–Sullivan method,[6] as proof that transmethylation has occurred. Both Steensholt and Barrenscheen[1] report the formation of choline as evidenced by precipitation of a supposed choline reineckate from the reaction mixture. No attempt was made to identify this precipitate by physical properties or analysis. Steensholt recognized that this latter was poor evidence, as ethanolamine also gave a reineckate under the conditions employed for the formation of choline reineckate. We concur in this observation of Steensholt and in addition have shown that the choline reineckate obtained by Barrenscheen in his experiments was in all probability the reineckate of ethanolamine.

We have confirmed the observation of Krebs[7] that rat and guinea pig liver or kidney is rich in D-amino acid oxidase, and further observed that the formation of α-keto-γ-methiolbutyric acid by the action of this enzyme on methionine accounts for the apparent disappearance of methionine, from reaction mixtures containing these tissues, as measured by the McCarthy–Sullivan method. When rabbit liver homogenates, which have been shown to contain little if any D-amino acid oxidase,[8] were substituted for rat liver no methionine disappearance was noted.

Analysis of a sample of pure sodium salt of α-keto-γ-methiolbutyric acid by the McCarthy–Sullivan method revealed that while this keto analog of methionine gives a color in this reaction the color is much less intense than for an equivalent amount of methionine. We have described a method for determining the amount of keto analog present in mixtures of methionine and α-keto-γ-methiolbutyric acid.

Experimental

The experiments herein described were conducted in Warburg flasks where possible and desirable, and any gaseous exchange recorded. Suitable blanks were included in all runs. Samples of D-methionine and L-methionine were obtained through the courtesy of Dr. J. P. Greenstein of the Cancer Institute, National Institutes of Health, Bethesda, Maryland. The sodium salt of α-keto-γ-methiolbutyric acid was furnished by Dr. Alton Meister of the same address and had the analysis: Calcd. for $C_5H_7O_3SNa$: C, 35.3; H, 4.1; S, 18.8; Na, 13.5. Found: C, 35.6; H, 4.1; S, 18.7; Na, 13.5.

A number of experiments were run under conditions which duplicated those of Barrenscheen and Steensholt. That is, methionine and ethanolamine neutralized with either phosphoric or hydrochloric acid, were dissolved in McIlvaine's phosphate–citrate buffer (*p*H 7.1) and either 1.0 ml. of a 10% tissue homogenate in Krebs-phosphate Ringer, or approximately 100 mg. of tissue slice added as the source of enzyme. All reactions were conducted at 37°.

At the end of a run the contents of the Warburg flasks were transferred to volumetric flasks, or pooled in cases where isolation procedures were to be followed. In either case the solutions were deproteinized with trichloroacetic acid after which determinations for methionine, choline or isolation procedures were carried out. The results of these experiments are best presented in the form of Table I.

Analysis of Methionine in the Presence of α-Keto γ-Methiolbutyric Acid.—The colored compound formed from methionine in the McCarthy–Sullivan method demonstrated

(1) H. E. Barrenscheen, *et al.*, *Z. physiol. Chem.*, **284**, 228 (1949).
(2) G. Steensholt, *Acta Physiol. Scand.*, **10**, 333 (1945).
(3) G. Steensholt, *ibid.*, **11**, 294 (1946).
(4) G. Steensholt, *ibid.*, **14**, 340 (1947).
(5) G. Steensholt, *ibid.*, **17**, 276 (1949).
(6) T. E. McCarthy and M. X. Sullivan, *J. Biol. Chem.*, **141**, 871 (1941).
(7) H. A. Krebs, *Biochem. J.*, **29**, 1620 (1935).
(8) F. Berheim and M. L. C. Berheim, *J. Biol. Chem.*, **109**, 131 (1935).

TABLE I

Run no.	Time of incubation in hours	Mg. substrate in McIlvaine's phosphate–citrate buffer pH 7.1—1.0 mg. ATP L-Methionine	DL-Methionine	Ethanolamine	Source and amount of enzyme	Methionine disappearing Mg.	Methionine disappearing Mole × 10^{-6}	Oxygen consumed cor. for endogenous respiration mole × 10^{-6}	Ratio moles methionine disappearing:moles O_2 cons.	Keto acid[a] formation	Choline[b] formation
1	4		29.0	40.0	1 ml. 20% rabbit liver homogenate	0	0	0	0	Neg.	Neg.
2	4		29.0	40.0	1 ml. 20% rabbit liver homogenate	0	0	0	0	Neg.	Neg.
3	4		29.0	40.0	1 ml. 20% rabbit liver homogenate	0	0	0	0	Neg.	Neg.
4	4		29.0	40.0	1 ml. 20% rabbit liver homogenate	0	0	0	0	Neg.	Neg.
5	5		21.6	20.0	10% rat liver, homogenate 1 ml.	1.8	12.1	5.27	2.3	Pos.	Neg.
6	5		21.6	None	10% rat liver, homogenate 1 ml.	0.9	6.1	3.48	1.8	Pos.	Neg.
7	5		21.6	None	10% rat liver, homogenate 1 ml.	1.8	12.1	4.55	2.7	Pos.	Neg.
8	5		21.6	20.0	10% rat liver, homogenate 1 ml.	0.6	4.0	3.79	1.1	Pos.	Neg.
9	10		35.8	None	10% rat liver, homogenate 1 ml.	2.5	16.5	5.2	3.2	Pos.	Neg.
10	10		35.8	40.0	10% rat liver, homogenate 1 ml.	3.8	25.3	11.7	2.2	Pos.	Neg.
11	10		35.8	40.0	10% rat liver, homogenate 1 ml.	2.5	16.5	9.3	1.8	Pos.	Neg.
12	17	30.75		40.0	10% rat liver, homogenate 1 ml.	0	0	0	0	Neg.	Neg.
13	17	30.75		None	10% rat liver, homogenate 1 ml.	0	0	0	0	Neg.	Neg.
14	7		60.8	40.0	10% rat liver, homogenate 5 ml.	12.4	83.0	43.0	1.9	Pos.	Neg.
15	7		60.8	None	10% rat liver, homogenate 5 ml.	12.4	83.0	32.5	1.9	Pos.	Neg.
16	4		38.0	40.0	Rat liver slice	14.0	...	...	...	Pos.	Neg.
17	3		74.5	200	Rat liver slice	...	...	...	...	Pos.[c]	Neg.
18	3		74.5	None	Rat liver slice	...	...	...	...	Pos.[c]	Neg.

[a] Formation of a 2,4-dinitrophenylhydrazone as evidence of keto acid. [b] As judged by presence or absence of ppt. with reinecke salt. [c] 2,4-Dinitrophenylhydrazone isolated, m.p. 147°. *Anal.* Calcd. for $C_{11}H_{12}N_4O_6S$: N, 17.07. Found: N, 16.45.

maximum absorption of light at 510 mμ. The extinction coefficient was calculated to be 0.035 cm.$^{-1}$ mg.$^{-1}$/100 ml. (Table II). When α-keto-γ-methiolbutyric acid was treated in the same manner a less intense color per equivalent of methionine developed. The extinction coefficient of this material at 510 mμ was calculated to be 0.008/cm.$^{-1}$ mg.$^{-1}$/100 ml. (Table II).

TABLE II

EXTINCTION COEFFICIENT AT 510 Mμ. COLOR COMPLEX FORMED IN THE MCCARTHY–SULLIVAN METHOD FOR METHIONINE

DL-Methionine, mg.	Extinction coefficient, cm.$^{-1}$ mg.$^{-1}$/100 ml.	α-Keto-γ-methiolbutyric acid, mg.	Extinction coefficient cm.$^{-1}$ mg.$^{-1}$/100 ml.
3.0	0.039	3.01	0.0086
4.0	.030	6.10	.0075
5.0	.035	7.32	.0078
6.0	.037	8.54	.0086
7.0	.033		
8.0	.034		
9.0	.034		
10.0	.034		
Average	.035	Average	.0081

Due to the fact that the molecular weights of methionine (149.21) and that of α-keto-γ-methiolbutyric acid (148.17) differ by less than 1%, the following modification of an equation according to Weissberger[9] may be made.

$$\log I/I_0 = k_1(c_0 - c_2) - k_2c_2 \times d \qquad (1)$$

where k_1 = extinction coefficient of α-keto γ-methiolbutyric acid
k_2 = extinction coefficient of methionine
c_2 = concentration of methionine
c_0 = concentration of methionine at start

The extinction coefficient at only one wave length need be known in order to solve this equation which was used throughout in determining the amount of methionine disappearing during the course of the reaction.

Isolation of the 2,4-Dinitrophenylhydrazone of α-Keto-γ-methiolbutyric Acid.—DL-Methionine, 74.5 mg., and ethanolamine, 200 mg., neutralized with hydrochloric acid were dissolved in McIlvaine's buffer (pH 7.2) and incubated with fresh rat liver slices. At the end of three hours incubation the solution was decanted from the liver slices, deproteinized with trichloroacetic acid and the clear filtrate heated to boiling with 15 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The yellow precipitate which appeared on chilling was removed by filtration and recrystallized twice from alcohol–water solution. After drying *in vacuo* at 100° the material melted at 147–148° (uncor.). *Anal.*[10] Calcd. for $C_{11}H_{12}N_4O_6S$: N, 17.07. Found: N, 16.35.

Waelsch[11] reports the 2,4-dinitrophenylhydrazone of α-keto-γ-methiolbutyric acid as melting at 149° and having a nitrogen analysis of 16.57%.

Choline Reineckate.—Choline hydrochloride, 0.5 g., was dissolved in 100 ml. of water and 100 ml. of saturated aqueous ammonium reineckate, containing about 4 g. of ammonium reineckate, was added. The solution was acidified with four drops of concentrated hydrochloric acid, at which time a pink precipitate appeared. The flask was set aside in a refrigerator. The precipitate was separated by centrifugation and the supernatant liquid decanted.

(9) A. Weissberger, "Physical Methods of Organic Chemistry," Vol. II, 2nd ed., p. 1298, Interscience Publishers, Inc., New York, N. Y., 1949.

(10) In a repeat preparation of this derivative of the keto analog of methionine the analyst reported 16.46 and 16.41% of nitrogen, while the melting point was recorded at 147–148° (uncor.). We can offer no explanation of the low nitrogen values reported here and by Waelsch.

(11) H. Waelsch and J. E. Borek, THIS JOURNAL, **61**, 2252 (1939).

The precipitate was dissolved in 100 ml. of acetone and water added drop by drop until the solution became cloudy. The flask was again kept in a refrigerator overnight. The precipitate was separated by centrifugation and washed with 95% alcohol, the liquid decanted and the precipitate washed with anhydrous ether. The precipitate was isolated by filtration and dried *in vacuo* over concentrated sulfuric acid. The decomposition point was observed at about 254°. No literature values on the decomposition point could be found.

Anal. Calcd. for $C_9H_{20}ON_7CrS_4$: N, 23.20. Found: N, 22.80, 23.50.

Ethanolamine Reineckate.—In one run, made under conditions identical to those employed by Barrenscheen (ref. 1), DL-methionine and ethanolamine were incubated with rat liver homogenate according to the protocol. See Flow Sheet labeled Protocol I. At the end of the incubation period the contents of the various flasks were combined and Barrenscheen's directions for the isolation of choline reineckate followed exactly. A precipitate similar in appearance to that of choline reineckate was obtained. After drying *in vacuo* at 100° this material melted with decomposition at about 170°.

Anal.[12] Calcd. for ethanolamine reineckate, $C_6H_{14}N_7OS_4Cr$: C, 18.95; H, 3.68; N, 25.79. Found: C, 18.91; H, 3.72; N, 25.04.

An authentic sample of ethanolamine reineckate prepared in this Laboratory melted with decomposition at about 170°.

PROTOCOL I

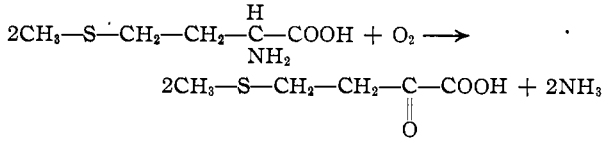
Source of enzyme: fresh rat liver homogenate in Krebs–Henseleit solution; substrate: DL-methionine in Krebs–Henseleit solution, ethanolamine neutralized with dilute HCl to *p*H 7.2; time of incubation one hour

Flask no.	Contents of each flask		Oxygen absorbed less endogenous oxygen:micromoles
2	DL-Methionine	12.8 mg.	1.8
3	Ethanolamine	40.0 mg.	1.6
4	10% Homogenate	1.0 ml.	1.5
5			1.9
6			1.7
7			1.8
		Average	1.7

(12) The microanalyses reported here were performed by Mrs. Mary H. Aldridge and Mr. Byron Baer.

Discussion

Examination of Table I reveals the following: In every experiment where a disappearance of methionine was observed, keto acid formation was also observed. The ratio of methionine disappearing to oxygen consumed in these cases was 2:1 (average of nine experiments). Assuming the reaction for the oxidative deamination of methionine to be



the methionine disappearing can be accounted for by oxidation to the keto analog. Supporting this view are the results with L-methionine (runs 12 and 13) which would not be affected by the D-amino acid oxidase.

The observation of Steensholt[5] that D-methionine is a better methyl donor in transmethylation, when judged by its disappearance as measured by the McCarthy–Sullivan method, must now be interpreted in a different light. The failure of rabbit liver homogenates, known to be low in D-amino acid oxidase, to cause any loss in methionine is in line with our other experimental evidence.

Examination of Table I shows that in no case was any choline produced, though from the amount of methionine disappearing sufficient choline could have been formed by transmethylation to be easily detected. In the only case in which a reineckate precipitate formed, the material was identified as the reineckate of ethanolamine.

COLLEGE PARK, MD. RECEIVED NOVEMBER 7, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Purification of Adrenocorticotropic Peptides by Carrier Displacement Chromatography

BY CHOH HAO LI, LUCIENNE ASH AND HAROLD PAPKOFF

The adrenocorticotropic peptides (ACTH peptides) prepared by peptic digest of sheep ACTH protein have been purified by fractional precipitation with trichloroacetic acid (TCA). It was found that the activity in the digest occurs in a 5–25% TCA precipitate. The ACTH activity in this precipitate can be abolished by further hydrolysis with pepsin and trypsin. Moreover, the 5–25% TCA precipitate can be further purified by the carrier displacement chromatographic technique. Using normal alcohols with 8 to 10 carbon atoms as the carriers, it was possible to obtain an active fraction having a specific activity of about 10–20 times that of the starting material. The amino acid composition of some of the purified fractions was investigated; one of these active fractions was found to have a low cystine content and to be essentially free from methionine and tyrosine.

We recently reported the analysis of a peptide mixture derived from the partial hydrolysis with pepsin of sheep adrenocorticotropic hormone (ACTH), by various chromatographic techniques.[1] It was found that a purified ACTH peptide fraction could be obtained by the carrier displacement procedure of Tiselius and Hagdahl.[2] However, although the fraction was chemically somewhat purified by this procedure, there was achieved no increase of biologic activity. In the present investigation, a plastic column was substituted for the stainless steel which had been used before, since it was thought that the previous difficulties had probably been caused by the reaction of the steel with HCl which in turn inactivated the hormone. When the plastic column was employed, not only was the ACTH activity totally recovered, but there also resulted an enhancement of activity: a fraction was obtained which had a specific activity of about 10–20 times that of the starting material.

Preparation and Properties of Preparation A2.—The 5% trichloroacetic acid (TCA) soluble fraction[3] of the partial

(1) C. H. Li, A. Tiselius, K. O. Pedersen, L. Hagdahl and H. Carstensen, *J. Biol. Chem.*, **190**, 317 (1951).

(2) A. Tiselius and L. Hagdahl, *Acta Chem. Scand.*, **4**, 394 (1950).

(3) Designated as Preparation S.