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172. Tomitaro Kita and Hiro-o Kamiya : Studies on Central Depressants. VI.[1] Enzymic Studies on Brain Transamidinase and One Possible Metabolic Pathway of 4-Amino- and 3-Hydroxy-4-aminobutyric Acid.

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Borsook and Dubnoff[2] observed that the mammalian kidney catalyzes a synthesis of guanidinoacetate from glycine and arginine. The name "transamidination" was given to this type of reaction. This transamidination reaction was studied by several persons,[3~7] and the character of its enzymic reaction has become successively clear. In regard to its reaction mechanism, there is the hypothesis of Ratner and Rochovansky,[8] but the hypothesis advocated by Walker[9] after energetic investigation seems to be more generally accepted.

Subsequently, Walker has continued his researches with interest in the pathway in which creatine is synthesized from guanidinoacetate by the action of transmethylase, and in the interaction[10] *in vivo* as for biosynthesis of guanidinoacetate due to the action of transamidinase. The existence of this transamidinase mainly in the kidney, liver, *etc.* has been recognized, but as to whether it exists in the brain tissue or not, there is scarcey an investigation except that by Pisanoand, and Udenfriend, who in 1958, exper imented on the crude brain homogenate of the rabbit and monkey using glycine-[2-^{14}C] and 4-aminobutyric acid [^{14}C], suggested[11] the presence of this substance in the brain. But as yet, it has not been clear in details.

In this paper, the enzyme was obtained from the rabbits whole brains by Walker's extracting method of kidney transamidinase.

The following materials were used as amidine donors, that is, arginine, guanidinoacetate, and 4-guanidinobutyric acid which were reported to exist in the brain,[12,13] and 3-hydroxy-4-guanidinobutyric acid whose pharmacological action has been investigated,[14] and on the other hand such amino acids as glycine, 4-aminobutyric acid, *etc.* were used as amidine acceptors.

According to Purpura and his co-workers 4-guanidinobutyric acid is the most active[15] in the series of compounds which influence the electric activity on cerebral cortex.

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It has been found that in the brain there is brain transamidinase which is essentially about the same as kidney transamidinase experimented by Walker, and that amidine radical of 3-hydroxy-4-guanidinobutyric acid is possible to be transferred into glycine to form 3-hydroxy-4-aminobutyric acid and guanidinoacetate.

Materials and Methods

4-Guanidinobutyric and 3-hydroxy-4-guanidinobutyric acids were both hydrochloride (4-guanidinobutyric acid hydrochloride, m.p. 184°, 3-hydroxy-4-guanidinobutyric acid hydrochloride, m.p. 165°); 4-aminobutyric and 3-hydroxy-4-aminobutyric acids were free amino acids. All these four were obtained from the Ono Pharmaceutical Co., Ltd. On the other hand, L-ornithine hydrochloride (m.p. 230~232°) was secured from the Kyowa Hakko Kogyo, Co., Ltd., and other materials which were of extra pure grade, were commercial products.

1. Purification Procedure of Transamidinase in Rabbit Brain

A healthy rabbit was killed by air emboly, and its brain was removed very carefully without delay. Then crude enzyme was prepared in the method shown in Chart 1.

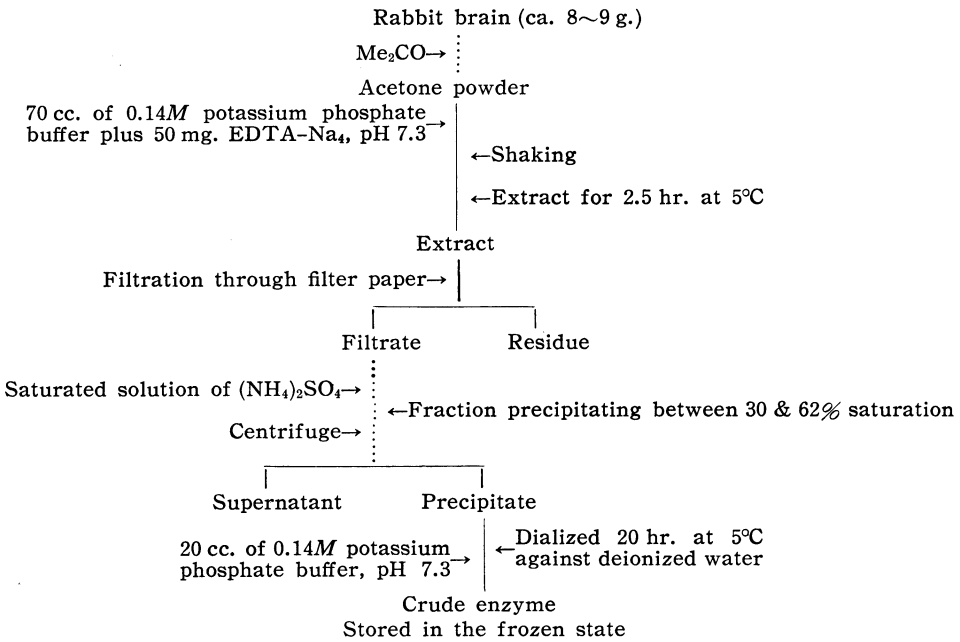

Chart 1. Purification Procedure of Transamidinase in Rabbit Brain

2. Systems of Reaction Mixtures, Donors, Acceptors, and Inhibitors

Reaction mixture was prepared in the system shown in Table I.

Donors, acceptors, and inhibitors were prepared in accordance with Walker's methods and used in the quantity or in the final concentration shown in Table. 1. The addition order when 2-thiouracil plus ferricyanide are used as inhibitors, is as follows :

First, 2-thiouracil was added after adding buffer to enzyme. Then, 5 min. later, ferricyanide was added to it. In succession, after 85 min. of preincubation, substrate was added and was reacted.

3. Purification of Samples for Chromatographic Assay

Reaction mixture was prepared according to the manner shown in Table. 1. and after incubation in the order of the manner shown in Chart 2, samples for chromatographic assay in 0.3 cc. were prepared.

Table I. Systems of Reaction Mixtures and the Proportions of Donors, Acceptors and Inhibitors

Reaction Mixtures

A Buffer + Enzyme + Water

B **Donor / Acceptor** Buffer + Enzyme + Water (+ Inhibitor)

C Donor / Acceptor Buffer + Water + Water

0.25 cc. / 0.25 cc. 1.3 cc. 0.2 cc.

Donor / Acceptor Buffer + Enzyme + Water : complete system

Donors

L-Arginine hydrochloride	15 mg.
4-Guanidinobutyric acid hydrochloride	15 〃
3-Hydroxy-4-guanidino butyric acid hydrochloride	15 〃
Guanidinoacetate	10 〃

Acceptors

L-Ornithine hydrochloride	5 〃
4-Aminobutyric acid	3 〃
3-Hydroxy-4-aminobutyric acid	3 〃
Glycine	2 〃

Inhibitors

Added compounds	final concentration
Cu^{2+}	10^{-4}
$Cu^{2+}+CN^-$	$10^{-4}+10^{-3}$
p-Chloromercuribenzoata	10^{-4}
p-Chlormercuribenzoate+CN^-	$10^{-4}+10^{-3}$
2-Thiouracil	5×10^{-3}
2-Thiouracil+Ferricyanide	5×10^{-3} $+2.5\times10^{-2}$
L-Ornithine	3.6×10^{-2}

Buffer : final 0.14*M*, potassium phosphate buffer pH : 7.4

Reaction mixture

Incubation 34°C, 20 hr.→ ←A few dorps of toluene

Centrifugation 3000 r.p.m., 10 min.→ ←8 cc. of dehyd. EtOH

Supernatant Precipitate

Evaporation 50~70°C→

Sample for Chromatographic assay 0.3 cc.

Chart 2. Preparation of Samples for Chromatographic Assay

Results

1. Chromatographic Assay

At first, the detection of several materials which were concerned in this reaction was carried out by the use of paper chromatography with various developers and coloring reagents, and their coloring condition, and Rf value were examined. The results are shown in Table II.

Table II. Rf Values of Compounds

Compounds	*tert*-BuOH-HCOOH-H_2O (70:15:15)	PhOH-H_2O (80:20)	BuOH-AcOH-H_2O (60:15:25)	BuOH-AcOH-H_2O (4:1:1)	Color reaction/color
L-Arginine	0.66	0.71	0.15	0.12	S/orange-red, N/purple-orange, PCF/pink
4-Guanidinobutyric acid	0.66	0.69	0.60	0.58	S/orange-red, N/purple-orange PCF/carmine
3-Hydroxy-4-guanidinobutyric acid	0.63	0.61	0.52	0.56	S/pink-orange, PCF/red
Guanidinoacetate	0.55	0.73	0.33	0.33	S/orange-pink, PCF/orange
L-Ornithine	0.29	0.20	0.12	0.10	N/purple
4-Aminobutyric acid	0.58	0.75	0.42	0.41	〃
3-Hydroxy-4-aminobutyric acid	0.59	0.59	0.38	0.38	N/dark purple yellow
Glycine	0.48	0.42	0.23	0.32	N/purple

S : Sakaguchi reagent N : Ninhydrin R. PCF : Pentacyanoaquoferriate R.

From the results, it has been clarified that it is possible to detect qualitatively various materials by changing its developer or coloring reagent.

2. Details of Enzymic Reaction between 4-Aminobutyric Acid and Glycine by Brain Transamidinase

Its details are shown in Table III.

TABLE III. 4-Guanidinobutyric acid ↖ ↗ Glycine
4-Aminobutyric acid ↙ ↘ Guanidinoacetate

Reaction Mixture	Temp. (°C)	Products: 4-Aminobutyric acid	Products: Guanidinoacetate
4-Guanidinobutyric acid, Glycine + Enzyme + Water : Complete system	34	+	+
Complete system	0	±	±
Complete system-enzyme	34	−	−
Complete system-glycine	34	−	−
Complete system-4-guanidinobutyric acid	34	−	−
Complete system + Cu^{2+}	34	−	−
Complete system + Cu^{2+} + CN^-	34	+	+
Complete system + *p*-chloromercuribenzoate	34	−	−
Complete system + *p*-chloromercuribenzoate + CN^-	34	+	+
Complete system + L-ornithine	34	±	−(Arginine ±)
Complete system + 2-thiouracil	34	+	+
Complete system + 2-thiouracil + ferricyanide	34	−	−

The results of Table III may be summarized as follows :

1) In the complete system of 4-guanidinobutyric acid+glycine and enzyme, the formation of 4-aminobutyric acid+guanidinoacetate was recognized. However, when either 4-guanidinobutyric acid or glycine was lacking, the reaction did not proceed.

2) When enzyme was lacked, the reaction did not proceed.

3) In the presence of cupric ion or *p*-chloromercuribenzoate in addition to the complete system, the reaction was almost entirely inhibited, but by addition of cyanide the reaction was recovered.

4) By addition of a large quantity of L-ornithine, it was inhibited, and a portion of L-ornithine became a substrate and at the same time the formation of arginine 4-aminobutyric acid was noticed.

5) When such a mild oxidant as ferricyanide coexisted with 2-thiouracil, the reaction was almost completely inhibited. The above results were quite in agreement with those of Walker's kidney transamidinase.

3. Various Reactions by Brain Transamidinase

The results of each reaction which can be assayed qualitatively are shown in Table IV.

Rf value mentioned above, was ascertained by mixed and comparative chromatographic technique throughout the experiments.

Disscusion

The present experiments are nothing but qualitative experiments with paper chromatography. Consequently, it is necessary to trap quantitatively amidine derivatives and amino acids which take part in these transamidination reaction with the use of isotope to study further the character of brain transamidinase and the influence of various agents on brain transamidinase.

Table IV.

	Reaction mixtures	Temp. (°C)	Products: Glycine	Products: 4-Guanidinobutyric acid
Guanidinoacetate, Glycine ⇄ 4-Aminobutyric acid, 4-Guanidinobutyric acid	Complete system	34	+	+
	Complete system	0	−	−
	Complete system-enzyme	34	−	−
	Complete system + *p*-chloromercuribenzoate	34	−	−
			3-Hydoxy-4-aminobutyric acid	Guanidinoacetate
3-Hydoxy-4-guanidinobutyric acid, 3-Hydoxy-4-aminobutyric acid ⇄ Glycine, Guanidinoacetate	Complete system	34	+	+
	Complete system	0	−	−
	Complete system-enzyme	34	−	−
	Complete system + *p*-chloromercuribenzoate	34	−	−
			Glycine	3-Hydoxy-4-guanidinobutyric acid
Guanidinoacetate, Glycine ⇄ 3-Hydoxy-4-aminobutyric acid, 3-Hydoxy-4-guanidinobutyric acid	Complete system	34	+	+
	Complete system	0	−	−
	Complete system-enzyme	34	−	−
	Complete system + *p*-chloromercuribenzoate	34	−	−
			Ornithine	Guanidinoacetate
Arginine, Ornithine ⇄ Glycine, Guanidinoacetate	Complete system	34	+	+
	Complete system	0	−	−
	Complete system-enzyme	34	−	−
	Complete system + *p*-chloromercuribenzoate	34	−	−
			Glycine	Arginine
Guanidinoacetate, Glycine ⇄ Ornithine, Arginine	Complete system	34	+	+
	Complete system	0	−	−
	Complete system-enzyme	34	−	−
	Complete system + *p*-chloromercuribenzoate	34	−	−

Moreover, physiological significance of these observations is not discussed, but these enzymic investigations may suggest some evaluation in the light of these observations.

Roberts[16] with a view to explain the reaction mechanism of 4-aminobutyric acid chemically using thiosemicarbazide as inhibitor of glutamic acid decarboxylase; or using hydroxylamine as inhibitor of 4-aminobutyric acid transaminase, tried to elucidate the mechanism of convulsion and depression, as a result of increase and decrease of 4-aminobutyric acid levels in the brain, but did not succeed in this attempt.

Therefore, he considers relationship between the nerve excitability and the side metabolic pathway of 4-aminobutyric acid to be more important than brain levels of the acid.

Whether this transamidination reaction can contribute to chemical explanation about the induction or depression of convulsion or not, it is an interesting problem to be decided in future.

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Summary

Following Walker's extracting method of kidney transamidinase, brain transamidinase the whole brain of the rabbit.

Then enzymic reaction was carried out, and reaction mixture was investigated with paper chromatography as a sole experimental technique, and it was found out that the following reaction might be possible.

1. 4-guanidinobutyric acid+glycine $\rightleftharpoons$ 4-aminobutyric acid+guanidinoacetate
2. 3-hydroxy-4-guanidinobutyric acid+glycine $\rightleftharpoons$ 3-hydroxy-4-aminobutyric acid +guaninoacetate
3. arginine+glycine $\rightleftharpoons$ ornithine+guanidinoacetate

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173. Sadao Iguchi and Atsuko Inoue : Studies on Pyrone Derivatives. VII. On the Syntheses of Dehydroacetic Acid Analogue having Aroyl Group in its Side Chain.

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Numerous reports were already published as to the relationship between the chemical structures and antibacterial properties[1~3] of the dehydroacetic acid (DHA) type compounds. Since in the previous studies, our main interest was in synthesizing a compound possessing the acyl group in the 3-position of DHA nucleus [triacetic acid lactone (TAL)], the derivatives having the aroyl instead of the acyl group in the 3-position have never been synthesized. In order to investigate the activity which the carbonyl group in their side chain displays for of various primary amines and their antibacterial activity, some new DHA type derivatives possessing the aroyl group were attempted to synthesize, and the details of these synthesis and their reaction processes are described below.

It was already attempted[3] to synthesize 3-benzoyl derivative of DHA by Friedel-Crafts reaction of TAL (I) and benzoyl chloride in the presence of a suitable condensing agent, for example, a few drops of conc. sulfuric acid, pyridine-piperidine or an equivalent mole of aluminum chloride, but without success.

In the cases when pyridine-piperidine or an equivalent mole of aluminum chloride was used as the condensing agent, a monobenzoate (IIIa), m.p. 91°, was obtained, while when conc. sulfuric acid, a dibenzoate, m.p. 242~243°, was formed.

Treatment of (IIIa) with an excess of aluminum chloride led to the productien of a new compound, m.p. 109~111°. This substance was found to be a 3-benzoyl derivative of DHA (IIa) prepared by Fries rearrangement. It was also proved that the 3-benzoyl derivative (IIa) could be directly prepared from (I) using an equivalent mole of benzoyl chloride in the presence of an excess of aluminum chloride in nitrobenzene.

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