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AMCHA

CONTENTS

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AN ACTIVE STEREO-ISOMER (TRANS-FORM) OF AMCHA AND ITS ANTIFIBRINOLYTIC (ANTI-PLASMINIC) ACTION IN VITRO AND IN VIVO

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The Research Projects on Plasmin and Antiplasmin

(Received for publication December 30, 1964)

INTRODUCTION

The purpose of this paper is to report the evidence that a trans-form isomer of AMCHA 4-aminomethyl-cyclohexane-1-carboxylic acid) shows a very strong antifibrinolytic action in vitro and in vivo. Basing upon the knowledge of stereo-chemistry, the active agent is, in our laboratories, called trans-AMCHA.

This finding was led from our successive studies on AMCHA, which had been carried out co-operatively with a group of Japanese chemists directed by Shimizu. Details of these chemical studies will be published elsewhere by Shimizu *et al*, however the brief communication of the chemical aspects had been presented by S. Okamoto *et al* in 1964****.

The antifibrinolytic activity of AMCHA was first reported by S. Okamoto *et al* in 1962[1,2], but the existence of two kinds of stereo-isomers of AMCHA had been theoretically expected for some time. In 1963, the chemists of our line, Shimizu *et al*, successfully isolated two stereo-isomers, cis-form and trans-form respectively. The physico-chemical studies on the isolated isomers indicated that the antifibrinolytically active isomer belongs to trans-form. Later, in 1964, the authors learned through a private communication with Melandor *et al* that they also had found an antifibrinolytically active isomer of AMCHA, but they reserved in deciding its molecular shape. In fact, it seemed that their results tempted them to regard the active isomer as a cis-form, instead of trans-form.

Accordingly, the authors understand that the antifibrinolytic activity of trans-AMCHA was claimed solely by our group. The active isomer belonging to trans-form was used in these studies and its action in vitro and in vivo was

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**** This communication was orally published in the Xth International Congress of Hematology held in Stockholm, September 1964.

compared with EACA or cis-form of AMCHA. The results obtained so far give us as such an impression that trans-form of AMCHA might be a goal of our studies lasted so long in searching for the more adequate agent against hyperfibrinolytic disorders.

MATERIALS AND METHODS

Cis- and trans-forms of 4-aminomethyl-cyclohexane-1-carboxylic acid (cis- and trans- AMCHA) were synthetized by Shimizu and his group. The structure conformations of those two isomers were confirmed by infrared spectrum, nuclear magnetic resonance and X-ray diffraction. Epsilon-amino caproic acid (EACA) was furnished by Daiichi Seiyaku Co., Ltd.

Streptokinase (SK) used in the experiments was a commercial preparation, Varidase, Lederle Laboratories; thrombin, Thrombin topical, bovine thrombin preparation, Mochida Seiyaku Co., Ltd.; fibrinogen, bovine fibrinogen, Cohn's fraction I, Armour Laboratories.

Borate saline buffer of pH 7.75, containing H_3BO_3 11.25 g, NaCl 2.25 g and $Na_2B_4O_7 \cdot H_2O$ 4 g in 1 *l* was used.

Antifibrinolytic assay with fibrin plate method.

Standard and heated fibrin plates were prepared by the methods of Astrup and Müllertz[3] and Lassen[4]. Final concentration of fibrinogen in the system was adjusted to be 0.2%. Before adding thrombin to form fibrin, varied amounts of active ingredients were dissolved into the fibrinogen solution. In the case of testing the inhibitory action on the fibrinolysis produced by SK, the plates were made adding particularly, 0.5 ml of human euglobulin solution to 12 ml of fibrinogen thrombin mixture. The human euglobulin used was prepared by dilution and isoelectric precipitation, and the precipitate was dissolved with buffer solution of original plasma volume. Fibrinolytic activity was recorded as the perpendicular diameter products in mm^2 of the lysed areas after 18 hours at 37°C.

Plasminogen activator: Plasminogen activator solution without inhibitor was prepared from aceton powder of pig heart by the method of Astrup and Albrechtsen[5].

Antifibrinolytic assay with clot lysis time.

Retardation of the lysis time of fibrin clot with SK was measured to estimate the antifibrinolytic action in blood. Details had been described in the previous paper[2]. Reaction mixture was as follows, 0.1 ml of serum of blood sample, 0.45 ml of borate buffer (pH 7.75), 0.1 ml of saline solution containing 100 units of SK (in the case of rabbit blood, 1000 units/0.1 ml SK was used), 0.05 ml of

saline solution containing 5 units of thrombin and 0.3 ml of 0.33% fibrinogen solution. The required time for complete lysis of the formed fibrin clot was measured at 25°C.

RESULTS

1. The fibrinolytic activity of two isomers of AMCHA.

The antifibrinolytic activity of cis- and trans-form of AMCHA was estimated using the fibrin plate method. Trans- and cis-AMCHA of 10^{-2} M in physiologic saline were prepared respectively, and both solutions were mixed as shown in table 1. The fibrin plates containing human euglobulin and 10^{-3} M AMCHA in final concentration were also prepared. A 0.03 ml of SK solution containing 30 units or 3 units, or pig heart activator solution was placed on the fibrin plates. The plates were incubated at 37°C, after 18 hours lysed area was measured. The results were summarized in table 1.

Table 1

Comparison of the Antifibrinolytic Activities Between Two Isomers of AMCHA

Mixing ratio of both isomers (10^{-3} M)		Lysed area (mm^2) by streptokinase		Lysed area (mm^2) by pig heart activator
Trans	Cis	30 u.	3 u.	
4	0	68	25	105
3	1	112	25	136
2	2	--	56	206
1	3	187	76	298
0	4	300	168	440
0	0	300	144	468

The figure in the table represents an average value of the lysed areas of 2 points on 2 different plates.

Trans-AMCHA of 10^{-3} M showed strong inhibitory action against fibrinolysis produced by SK and also pig heart activator, the same concentration of cis-AMCHA, however, showed no inhibitory effect. The increasing inhibition was observed with increasing ratio of trans-form to cis-form.

2. A comparison of the antifibrinolytic activity of trans-AMCHA with that of EACA.

The lysed area on fibrin plate produced by 0.03 ml of SK, containing 3 units or 0.3 units, was measured as the above described method. At the point of 50% inhibition, about 10 times of EACA was required to show the same inhibitory action of trans-AMCHA (Fig. 1).

The inhibitory activity against pig heart activator was also estimated.

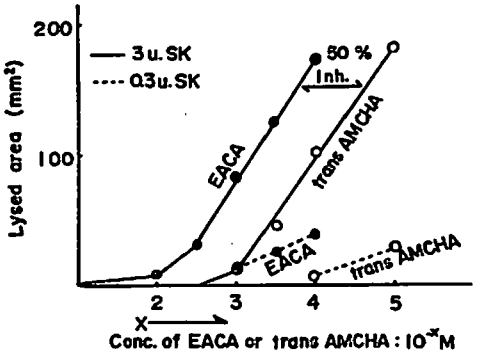

Fig. 1 Inhibitory effect of EACA or trans-AMCHA on fibrinolysis caused by streptokinase.
Each point represents an average value of lysed areas of 2 points on 2 different plates.

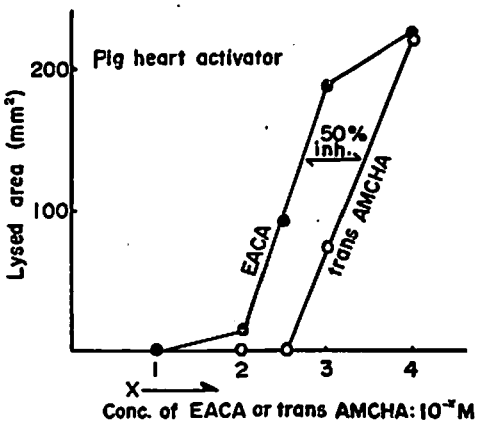

Fig. 2 Inhibitory effect of EACA or trans-AMCHA on fibrinolysis caused by tissue activator.
Each point represents an average value of lysed areas of 2 points on 2 different plates.

(Fig. 2) At the point of 50% inhibition trans-AMCHA was 7–8 times stronger than EACA.

The inhibitory action against plasmin preparation (Lyovac Merck) was estimated. Plasmin solution of 0.03 ml, containing 30 MSD units or 15 MSD units, was placed on heated fibrin plates with various concentrations of trans-AMCHA or EACA. The inhibitory action of both agents here was slight and not much difference was observed between the results obtained by trans-AMCHA and EACA.

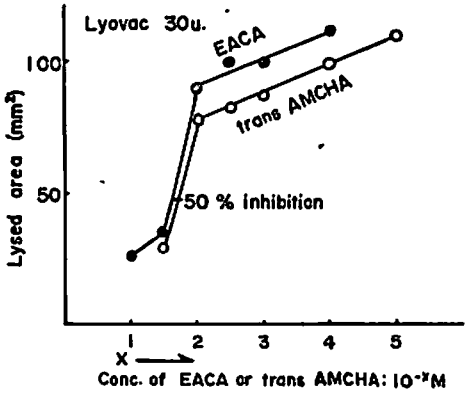

Fig. 3 Inhibitory effect of EACA or trans-AMCHA on fibrinolysis caused by plasmin (Lyovac Merck).
Each point represents an average value of lysed areas of 2 points on 2 different plates.

3. The activity of trans-AMCHA after incubation with blood plasma.

A 0.2 ml of 1% trans-AMCHA was mixed with 1.8 ml of human plasma, and the admixture was incubated at 37°C for 3 hours. The sample of 1 ml was taken before and after the incubation, and 10 volumes of 1% picric acid solution was added to remove protein. After centrifugation, the picric acid in supernatant was removed by letting it pass through the column (2×5 cm) or Dowex 2–X8. Each effluent was diluted in various concentration with borate saline buffer (pH 7.75). The same volume of standard plasma was added to each diluted sample, and 0.1 ml of the admixture was taken. Then it was mixed with 0.45 ml of borate buffer, 0.1 ml of 1000 units/ml SK, 0.3 ml of 0.33% fibrinogen and 0.05 ml of 100 units/ml thrombin. The reaction mixture was incubated at 37°C, and the lysis time of formed clot was measured. The results were shown in table 2. The retardation of the lysis time was not changed before and after incubation with human plasma. The possible change of inhibitory action of trans-AMCHA by blood plasma was denied.

Table 2
SK-activated Fibrinolysis Time (min.) with Trans-AMCHA Effluent

Dilution of effluent	4×	8×	32×	128×	control (without effluent)
Before incubation	16.5	11.5	8.0	6.0	5.5
180 min. after incubation	16.0	11.5	8.25	6.0	5.5

4. The activity of trans-AMCHA given to rabbits.

Intravenous administration: Ten mg per kg trans-AMCHA, mixed-AMCHA (containing ca. 33% trans-form) or EACA were intravenously injected into 3 male rabbits respectively. Blood samples were drawn by venipuncture at the times mentioned in Fig. 4 and the fibrinolysis of circulatory blood was estimated by the lysis time of fibrin clot with SK. The inhibitory action was expressed as percentage of the retardation of the lysis time. The strongest retardation of the lysis time was observed after injection of trans-AMCHA.

Oral administration: Ten mg per kg of trans-AMCHA or EACA were orally given to 2 male rabbits respectively. The SK activated fibrinolysis was estimated at each blood sample drawn at the times mentioned in Fig. 5. A very remarkable and prolonged retardation of the lysis time was observed after trans-AMCHA administration, even after 18 hours slight inhibition was yet observed.

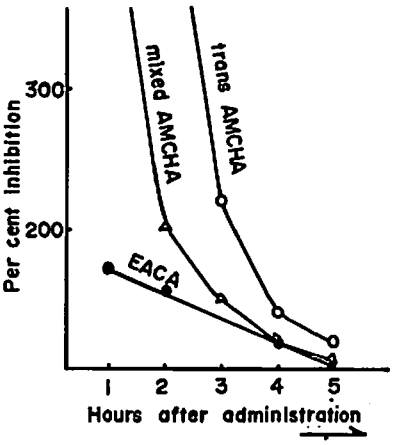

Fig. 4 Antifibrinolytic effect of the intravenous administration of EACA, mixed-AMCHA or trans-AMCHA to rabbits.
Ten mg/kg of EACA, mixed-AMCHA or trans-AMCHA was intravenously administered to 3 rabbits respectively. Ordinate indicates percentage of the retardation of the lysis time estimated by the SK activated fibrinolysis.
Each point represents an average value of 3 blood samples obtained from 3 different animals.

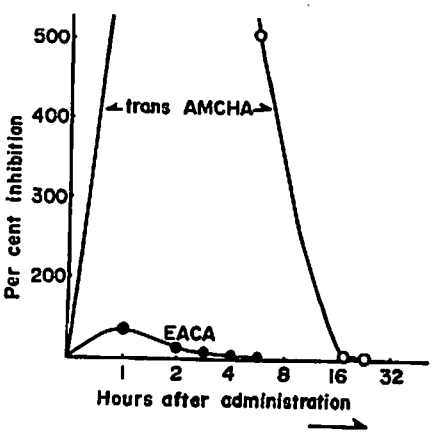

Fig. 5 Antifibrinolytic effect of the oral administration of EACA or trans-AMCHA to rabbits.
Ten mg/kg of EACA or trans-AMCHA was orally administered to 2 rabbits respectively. Ordinate indicates percentage of the retardation of the lysis time estimated by SK activated fibrinolysis.
Each point represents an average value of 2 blood samples obtained from 2 different animals.

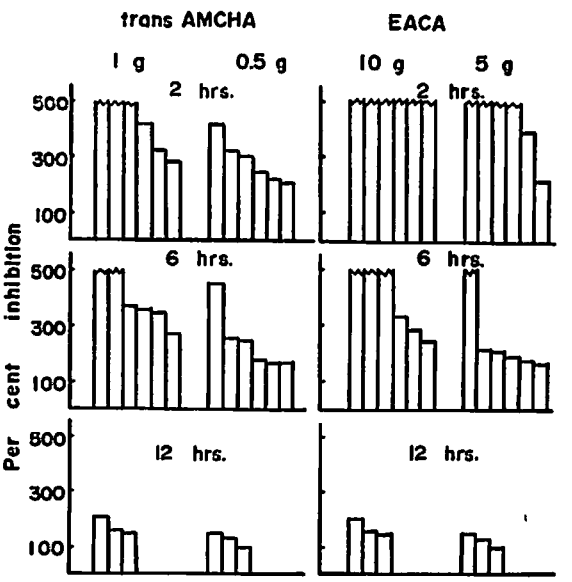

Fig. 6 Antifibrinolytic effect of the oral administration of EACA or trans-AMCHA to men.

5. The activity of trans-AMCHA orally given to man.

One or 0.5 g of trans-AMCHA was orally given to 12 adult healthy persons, and the SK activated fibrinolysis of the serum samples was estimated 2, 6, 12 hours after administration. To compare the antifibrinolytic activity of the agent with that of EACA, 10 or 5 g of EACA was also given to the other 12 healthy persons. The results are shown in Fig. 6. The inhibitory activity was expressed as percentage of the retardation of the lysis time. The results obtained seemed to indicate that the effect of 1 g of trans-AMCHA may have the same effect of 5 or 10 g of EACA. The oral administration of 0.5–1.0 g of AMCHA did not produce any unfavorable disturbance in those persons so far observed.

DISCUSSION

A number of the homologues of EACA had been studied by our projects in order to know the relation between chemical structures and their antifibrinolytic activities. Results thus obtained could be summarized in a simple conclusion that the antifibrinolytic activity of these homologues depends on the distance between NH_2 and COOH radicals of a molecule. This implied that EACA has the most adequate distance between NH_2 and COOH among its close homologues.

AMCHA has a rather solid cyclohexane-ring, instead of the carbon-chains of EACA. Besides, two different conformations of the cyclohexane-ring are existing; boat-form and chair-form as shown in Fig. 7. The antifibrinolytically active isomer is trans-AMCHA having cyclohexane of chair-form, the distance between NH_2 and COOH being longer than the another isomer. The inactive isomer is cis-AMCHA having cyclohexane of boat-form where the distance is shorter. Thus, it is thought that the antifibrinolytic activity of the isomers would be

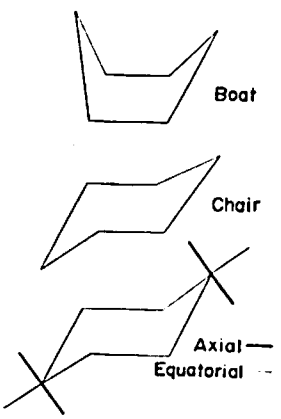

Fig. 7 Models of the stereo-isomers of AMCHA.

depending on stereoconformations of the cyclohexane having NH_2 and COOH. This may raise some new interests on the mode of action of the inhibitors having conformation isomers.

The mode of action of trans-AMCHA was nearly the same with EACA so far examined, but the antifibrinolytic activity of trans-AMCHA on the activation of plasmin was far stronger than EACA. Trans-AMCHA of low concentration well inhibited the fibrinolysis activated by SK or pig heart activator, and cis-AMCHA had no inhibitory action. The high concentration of trans-AMCHA or EACA was required to inhibit the activity of a plasmin preparation (Lyovac) on the heated fibrin plate. When cis-AMCHA was admixed with trans-AMCHA, the former did not disturb the action of the latter at all. Conversion of trans-AMCHA to the other inactive isomer was not observed by the incubation of trans-AMCHA with human plasma for 3 hours at 37°C.

The potent inhibitory effect on the SK-activated fibrinolysis was demonstrated in the blood samples drawn after the administration of trans-AMCHA to rabbits either orally or intravenously. The inhibition produced by trans-AMCHA was obviously stronger than EACA. Administrating orally trans-AMCHA or EACA to the healthy persons, the inhibitory effect of the both inhibitors were examined in the blood samples drawn at intervals. The results obtained also indicated that the action of trans-AMCHA was 5–10 times stronger than that of EACA, but no side effect was observed by the administration of trans-AMCHA.

These results of trans-AMCHA in vitro and in vivo suggested that it is very promising to replace the position of EACA by trans-AMCHA in clinical application. In addition, a series of studies had been made by our projects using the ordinal AMCHA containing ca. 1/3 of trans-AMCHA and ca. 2/3 of cis-form. The results obtained indicated that the AMCHA was more effective than EACA either experimentally or clinically. Noteworthy was the evidence that the side action of the AMCHA was less than EACA even after long term administration. These results seemed to encourage us to extend the studies toward the clinical application.*

SUMMARY AND CONCLUSION

An antifibrinolytic agent was reported by S. Okamoto *et al* in 1962. The agent was called AMCHA abbreviating its original chemical name, 4-amino-methyl-cyclohexane-1-carboxylic acid. AMCHA, however, has practically two kinds of stereo-isomers (conformation isomers) called trans-AMCHA and cis-

* In fact, the clinical studies are under investigation by our projects or the other Japanese workers.

AMCHA. In this report, the antifibrinolytic activities of these isomers in vitro and in vivo were studied using different fibrinolytic systems.

1) The fibrinolysis by pig heart activator or streptokinase (SK) was well inhibited by a low concentration of trans-AMCHA. Cis-AMCHA showed neither activation nor inhibition on the fibrinolytic system used. Cis-AMCHA did not disturb the action of trans-AMCHA. The inhibitory action of trans-AMCHA was 7–10 times stronger than that of EACA.

2) The fibrinolytic activity of a plasmin preparation (Lyovac) on the heated fibrin plate was inhibited only when the plate contained high concentration of trans-AMCHA or EACA.

3) The antifibrinolytic activity of trans-AMCHA was not decreased by the incubation with human blood plasma for 3 hours at 37°C. Conversion of trans-form to cis-form was not produced by such an incubation.

4) Ten mg of trans-AMCHA or EACA per kg of body weight was administered to rabbits either orally or intravenously. Extremely strong inhibition was observed in the blood samples of the rabbit received the administration of trans-AMCHA. This inhibitory action far exceeded that of EACA.

5) Trans-AMCHA of 0.5 g or 1.0 g and EACA of 5 g or 10 g were orally administered to the healthy adult persons. Each of those 4 groups had 6 persons. Blood samples obtained by venipuncture at intervals were examined by the lysis time of the SK activated fibrinolysis. The results indicated that antifibrinolytic activity of trans-AMCHA was 5–10 times stronger than EACA. Any unfavorable side action of trans-AMCHA was not observed.

6) The results reported here suggest that it is promising to replace the position of EACA by trans-form of AMCHA in clinical application.

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FRACTIONATION OF PLASMINOGEN ACTIVATOR AND PROACTIVATOR IN TISSUE AND BLOOD BY GEL FILTRATION

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The molecular sieve principle, forming the basis for the gel filtration method, has been used for separation of low molecular weight substances. In 1962, Flodin and Killander[1] applied this method to fractionation of human serum protein, and in 1963, Cohly and Shulman[2], Robbins and Summaria[3] tried to purify human plasminogen and plasmin by using this method and column chromatography on DEAE-cellulose. In the present studies, fractionation of plasminogen activator and proactivator in tissue and blood was made by gel filtration using sephadex G-200.

MATERIALS AND METHODS

Human plasma: Dried human plasma (Nihon Seiyaku Co., Ltd.) was used.

Commercial preparations of fibrinogen (Cohn's Fraction I, Armour Laboratory), thrombin (Mochida Co.) and streptokinase (Varidase, Lederle Lab. of American Cyamid Co.) were used in the following experiments.

Plasminogen (Kline's preparation) was kindly donated by A.B. KABI.

Urea solution of 0.4 M and 0.04 M, and the soltion of 0.1 M Tris-HCl (pH 8.0) in 0.2 M NaCl were used as eluants for gel filtration.

Gel filtration method: This was principally the same as the method reported by Flodin *et al*[1]. Sephadex G-200 (A.B. Pharmacia) was prepared by swelling for 24 hours in an excess of 0.4 M urea solution or 0.1 M Tris buffer. The column had the dimensions 2 by 8 or 2 by 30 cm respectively. After the gel was packed in the column, it was washed and saturated by urea solution or the buffer solution overnight. A sample was slowly injected on the gel bed by means of a syringe with a 1 mm polyethylene tube which had been carefully inserted through the solution down to level a few milimeters above the gel bed. In the case of euglobulin solution or plasminogen solution, the sample was placed directly on the gel surface after taking out the buffer solution remaining over the gel. Elution was

performed with a pressure of 15–20 cm Hg for urea solution, and 5–10 cm Hg for Tris buffer. The effluent was collected either in 7 or 5 ml portions in an automatic fraction collector. The procedure was performed at 4°C.

Determination of protein: The protein concentration of the effluent was determined by measuring its optical density at 280 mμ, or by a modified Folin's method[4].

Determination of fibrinolysis: The fibrinolytic activity was determined on a standard and heated fibrin plate by the methods of Astrup and Müllertz[5], and Lassen[6], and lysis areas on the plates were measured. The fibrin clot lysis time[7] by streptokinase-activated plasmin system was measured as described later.

Extraction of tissue activator: Aceton powder (containing plasminogen activator[8]) was prepared from fresh rabbit kidney parenchyma. Two M KCl solution of 15 ml containing 300 mg of the powder was homogenized in a mortar, left for 2 hours at room temperature and centrifuged at 3,000 r.p.m. for 10 min. at 4°C. The supernatant was placed on sephadex gel.

Other methods will be described later.

RESULTS AND DISCUSSION

Separation of tissue activator

Two ml of KCl extract obtained from aceton powder of the rabbit kidney was placed on the gel bed saturated by 0.4 M urea solution, and elution was made by

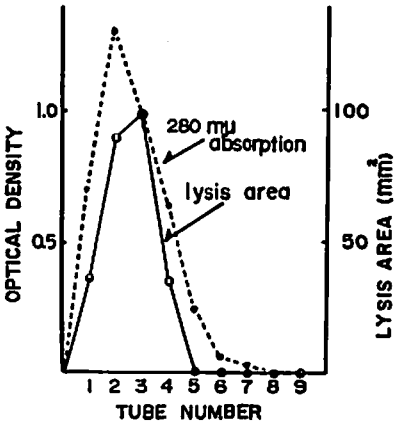

Fig. 1 Elution pattern of tissue activator

Solid line indicates the distribution of fibrinolytic activity and dotted line is the protein distribution.
Column dimension: 2×8 cm
Eluant: 0.4 M urea, each 7 ml taken.

the same solution. Each effluent of 0.03 ml was placed on standard and heated fibrin plates.

Fig. 1 shows the distribution pattern of activity of tissue activator in KCl extract. One peak of fibrinolysis was observed corresponding to that of protein distribution. No lysis was observed on heated plates.

Separation of proactivator

One ml of buffer solution (Tris-HCl, pH 8.0), containing 25 mg dried human plasma, was placed on the gel bed, 2 by 30 cm. Elution was carried out by the same buffer. The effluent was placed on standard fibrin plates, however no lysis was observed. These results indicate that the normal human plasma had practically neither activator activity nor plasmin activity. Then, 0.1 ml of the solution, containing 50 u. of streptokinase (SK), was added to 0.9 ml of the effluent, and the mixture was incubated for 10 minutes at 37°C and placed on standard and heated plates. The results are shown in Fig. 2.

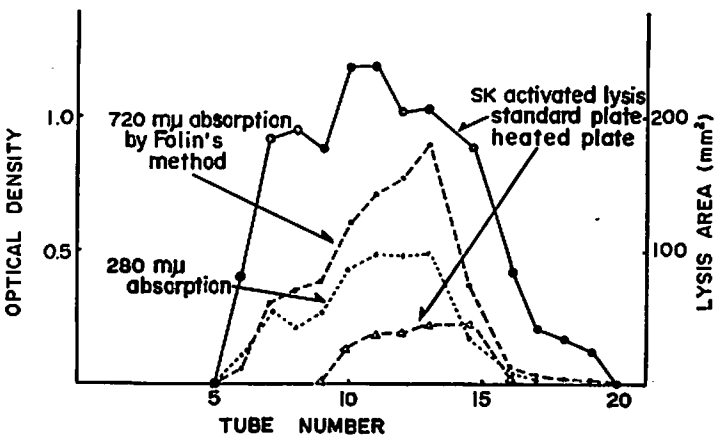

Fig. 2 Elution pattern of normal human plasma

△·····△ shows the distribution of plasminogen, examined by heated fibrin plates.
Column dimension: 2×30 cm
Eluant: 0.1 M Tris-HCl, pH 8.0, each 5 ml taken.

There may be two peaks of SK-activated lysis in the distribution of fibrinolytic activity examined by standard plates. One is seen in fraction number 6 to 9 and the other 10 to 20. Roughly speaking this fibrinolytic pattern seems to go parallel with that of protein distribution which was determined by absorption at 280 mμ or modified Folin's method[4]. However fibrinolysis on heated plates was observed in the fraction number 10 to 16. It was indicated that the effluent of these tubes from fraction number 10 to 16 contained plasminogen and pro-

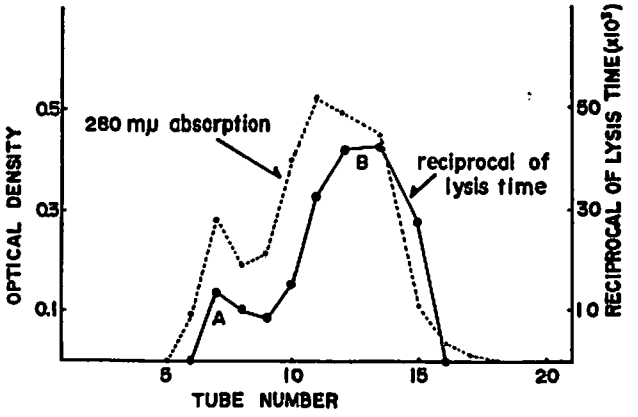

Fig. 3 Elution pattern of normal human plasma
Fibrinolytic activity was examined.
Column dimension and eluant are the same as in Fig. 2.

activator, thus the proactivator being converted to activator by the presence of SK.

Fig. 3 shows the relationship between the distribution of absorption at 280 mμ and the test of SK-activated fibrin clot lysis.

Thrombin solution (100 u./ml) of 0.05 ml and 0.3 ml of 0.33% fibrinogen solution were mixed with 0.2 ml of the SK-activated effluent and 0.4 ml of phosphate buffer* at 0°C. This reaction mixture was incubated at 37°C and the time required for the complete lysis of the formed clot was measured in minutes. There are two peaks in the protein distribution, and two peaks are observed as well in the curve representing the reciprocal of the lysis time. As the system used contained enough plasminogen, the reciprocal of the lysis time can have a distinct relation to the concentration of proactivator in the effluent. Proactivator supposed to be present in the earlier fractions is named proactivator A and that in the later fractions proactivator B tentatively.

Recently J.H. Lewis[9] reported the studies on the localization of proactivator and plasminogen in the normal human plasma by means of sephadex gel filtration; however the results are not in accordance with those of our experiments shown in Fig. 3. There, two peaks were shown in the fibrinolytic pattern, but Lewis' results indicated only one peak in the normal human plasma. Reasonable explanation could not be found for this discrepancy.

Dissociation of proactivator

* $Na_2HPO_4 \cdot 2H_2O$ (18.9 g) and KH_2PO_4 (3.6 g) dissolved in water to 2 *l*. pH adjusted to 7.4 and 18 g of NaCl was added to this solution.

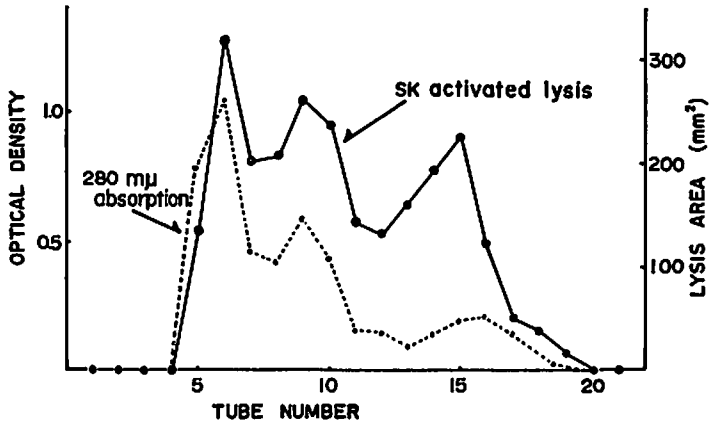

Fig. 4 Elution pattern of normal human plasma.
Column dimension: 2×~~8~~ 30 cm
Eluant: 0.04 M urea, each 7 ml taken.

Two types of proactivators were observed in the normal human plasma as described above, but proactivator may be composed of components of smaller molecular weights, and it may be dissociated in the course of the elution with urea solution. To clarify the point, 0.04 M urea solution was used as eluant, and other procedures were the same as in the case, when Tris buffer was used.

Fig. 4 represents the result. Three peaks were obtained in the distribution of fibrinolytic activity corresponding to those of the protein distribution. The first peak of proactivator may correspond to the proactivator which might not be soluble in 0.04 M urea solution. The proactivator contained in fraction 12 to 20 may be of smaller molecular weight. This suggests that proactivator can be dissociated into smaller particles which are yet fibrinolytically active.

Fractionation of euglobulin

Seventy mg of dried human plasma was dissolved in 1 ml of 0.9% NaCl and the solution was diluted 20 fold with distilled water, and its pH was adjusted to 5.2 with glass electrode by adding 0.5% acetic acid with constant stirring. After centrifugation at 3,000 r.p.m. for 10 min. at 4°C, the supernatant was separated for the other test. The residue dissolved in 0.8 ml of 0.1 M Tris buffer (pH 8.0), and the solution was placed on the gel, after withdrawal of the buffer over the gel.

The results shown in Fig. 5 indicate that there are two peaks in the fibrinolytic activity as well as in the protein distribution of euglobulin. The second peak of protein is not in accordance with the second one of fibrinolysis. The protein distribution of the supernatant of the euglobulin fraction was also

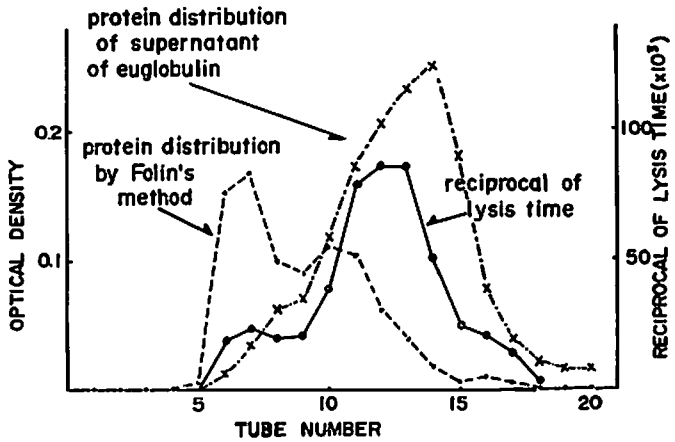

Fig. 5 Elution pattern of euglobulin fraction of normal human plasma.

Fibrinolytic activity of the euglobulin was measured by a fibrin clot lysis time. The protein distribution of the supernatant of the euglobulin was also drawn.
Column dimension: 2×30 cm
Eluant: 0.1 M Tris-HCl, each 5 ml taken.

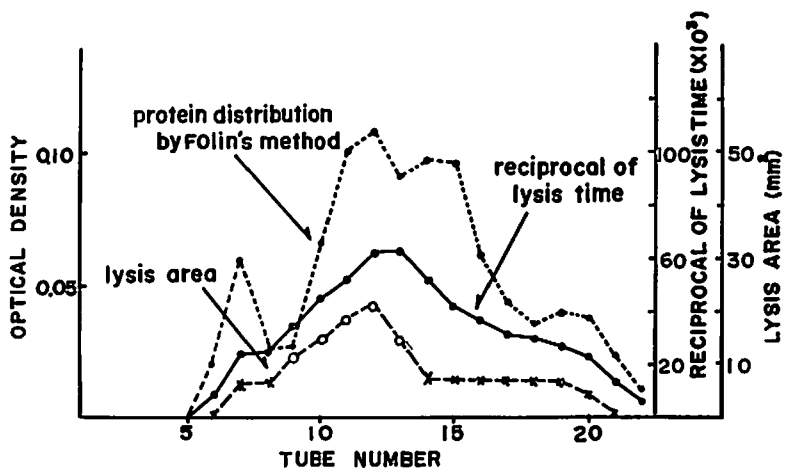

Fig. 6 Elution pattern of purified plasminogen solution.

×—× indicates incomplete lysis on heated fibrin plates and the half of each lysis area is plotted.
Column dimension and eluant are the same as in Fig. 5.

examined. Two ml of the supernatant of the centrifuged solution was placed on the gel and eluted by 0.1 M Tris buffer as usual. The chromatogram of protein in the supernatant is drawn in Fig. 5.

Fractionation of purified plasminogen

Five mg of KABI plasminogen was dissolved in 2 ml of the buffer, and eluted,

and the effluent was activated by SK as usual.

In Fig. 6, the chromatogram of protein distribution and fibrinolytic activity of SK-activated plasminogen were drawn. There are four distinct peaks in the protein distribution pattern, only one peak, however, is found when the fibrinolytic activity was determined by fibrin clot lysis time. The peak corresponding to proactivator A disappeared, and when plasminogen distribution was examined by a heated fibrin plate method, there is also one peak, fairly in accordance with that of the fibrinolytic curve.

Five mg of plasminogen is a very large amount in comparison with that which is present in 25 mg of plasma, and the composition of the proteins in the first peak is different from that of Fig. 2, 3 or 5. The proteins in the first peak in Fig. 2, for instance, are most of globulins, fibrinogen and lipoproteins as Flodin *et al* reported, and those in Fig. 6 will be composed of globulins, which are probably not removed by the purification procedures. Judging from the fact plasminogen activity is detected in the first peak by means of heated fibrin plate, even if weak, its presence in the first peak in Fig. 2 is also considered. However, no lysis was observed in heated plates in the first peak, even if large amount of human plasma (containing 300 mg dried human plasma) was used. Therefore some changes in the structure of plasminogen molecules may be considered to occur during the purification procedures.

Proactivator A which had been present in the normal human plasma, would be removed or reduced to a very small amount during the purification procedures.

In these experiments, proactivator could not be separated from plasminogen completely. In Fig. 2, the proteins in the first peak seem to contain only proactivator but no plasminogen, these proteins, however, may contain so small amount of plasminogen that it could not cause lysis on heated plates.

SUMMARY

1) Fractionation of fibrinolytically active components of blood and tissue was carried out.

2) There are two kinds of proactivators in the normal human plasma, proactivator A and proactivator B.

3) Fibrinolytic components of blood may probably be dissociated to a smaller particle in urea solution, and the smaller one is yet fibrinolytically active.

4) The amount of proactivator A in purified plasminogen is very small, which may be removed during the purification procedures.

The authors wish to express their hearty thanks to Prof, S. Okamoto, Kobe University, for his advice and kind discussions.

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DETERMINATION OF BLOOD FIBRINOLYTIC ACTIVITY BY MEANS OF GEL FILTRATION IN SEVERAL SKIN DISEASES

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Changes of blood fibrinolytic enzyme system in several skin diseases and in experimental dermatitis have been reported by many investigators[1,2,3,4,5]. A number of attempts in the isolation, purification, and characterization of human fibrinolytic enzyme system have recently been performed[6,7,8,9]. The report of Flodin and Killander[10] with regard to a successful chromatographic method for the human serum proteins has indicated that this procedure seemed to be of value as a compliment to other measuring methods of fibrinolytic activity. According to their principle human sera were fractionated by gel filtration in a new type of dextran gel, Sephadex G-200. The Sephadex gels act as molecular sieves allowing separation of protein or other mixtures and estimation of molecular size. In this paper fractionations of human serum proteins by gel filtration and determinations of fibrinolytic activity in each fractions are reported.

MATERIALS AND METHODS

Human serum: Venous blood from patients with skin diseases and other normal adults was obtained from the antecubital vein. The blood was allowed to clot spontaneously and the serum was separated after centrifugation.

Gel filtration method: Gel filtration was performed by the method of Flodin and Killander[10] principally. Sephadex G-200 (A.B. Pharmacia) was dissolved in an excess of 0.1 M Tris-HCl (pH8.0) in 0.2 M NaCl buffer. A column with an inner diameter of 20 mm was filled with the Sephadex suspension to a height of 300 mm. One ml of serum was diluted with two ml of Tris buffer, and one ml of diluted serum was used for the experiment. Eluent consisted of Tris buffer, and elution was performed with a pressure of 5–10 cm Hg. The effluent was

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collected in 5 ml portions in an automatic fraction collector. The procedure was done at 4°C.

Determination of protein concentration: Protein concentration of the fractions was determined by a modified Folin method[11].

Determination of fibrinolytic activity: After activation of fibrinolytic enzyme system in the fractions with streptokinase, the fibrinolytic activity was estimated by the fibrin plate method[5].

RESULTS AND DISCUSSION

Controls: Four normal adults (2 males and 2 females) were selected as controls for the experiments. The typical patterns of normal protein distribution and of normal fibrinolytic activity were showed in Fig. 1. The diluted serum

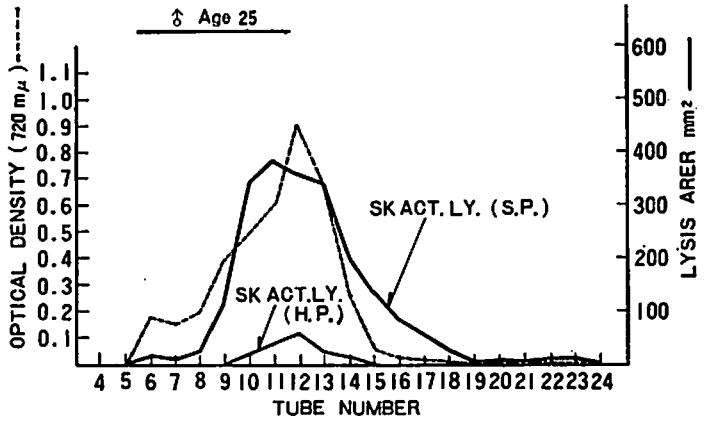

Fig. 1 Normal pattern of protein concentration and of fibrinolytic activity.
SK ACT. LY. (S.P.): streptokinase-activated lysis on a standard plate.
SK ACT. LY. (H.P.): streptokinase-activated lysis on a heated plate.

of a 25-year-old male was fractionated. Since the effluent revealed no fibrinolytic activity on a standard fibrin plate, 0.1 ml of the solution, containing 50 units of streptokinase, was added to 0.9 ml of the effluent, and the mixture was incubated for 10 min. at 37°C and placed on a standard and a heated plate.

On a standard plate the fibrinolytic activity of the fractions showed following distribution; there were two kinds of peaks of streptokinase-activated lysis[12], one was distributed between fraction number 5 to 7, and another was found between 8 to 19. On a heated plate the fibrinolytic activity appeared from fraction number 9 to 15. Both plasminogen and proactivator, contained in the effluent, were considered as a source of fibrinolytic activity.

Case 1. Diagnosis: Urticaria acuta

A 28-year-old male developed pruritic urticarial lesion all over the body. Blood examination, carried out prior to treatment, showed acceleration of fibrinolytic activity (streptokinase-activated lysis) as shown in Fig. 2. Marked fibrino-

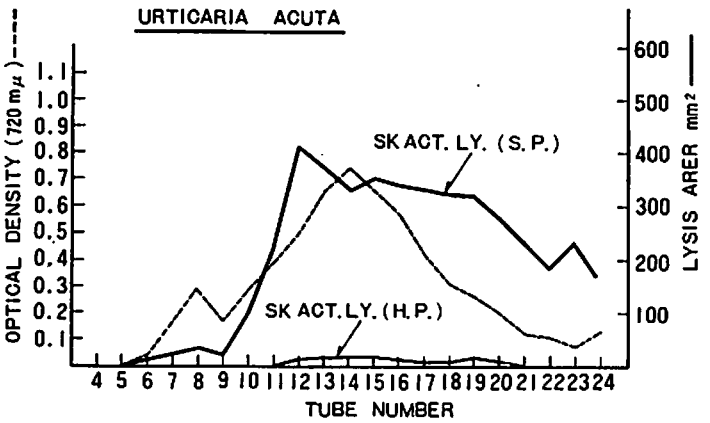

Fig. 2

lytic activity was found from fraction number 14 to 24 on a standard plate. By comparing protein concentration of each fractions with fibrinolytic activity of them the difference between normal and some pathologic condition would become distinct. No remarkable change was seen in the fibrinolytic activity (streptokinase-activated lysis) on a heated plate.

The existence of intensified blood fibrinolytic activity in case of urticaria was already known[1]. The results of the experiment clarified that the substance of fibrinolytic enzyme system, which showed increased streptokinase-activated lysis on a standard plate, was one of the sources of the activity. Proactivator should be considered as the substance.

Case 2. Diagnosis: Fibrinolytic purpura

A 53-year-old male developed repeatedly a few purpuric lesion of extremities of several weeks' duration. Marked hypofibrinogenemia (60 mg/dl) was noted by routine examination. The untreated serum of the patient itself showed conspicuous fibrinolytic activity on a standard plate, and the activity was inhibited by adding epsilon-amino-n-caproic acid[1] in the plate. The activity of the untreated serum, however, disappeared after keeping 24 hours at 0°C and also treating with Sephadex G-200. The fibrinolytic activity (streptokinase-activated lysis) on a standard plate and the protein concentration were shown in Fig. 3. Proactivator, found in the earlier fractions and also in the later fractions,

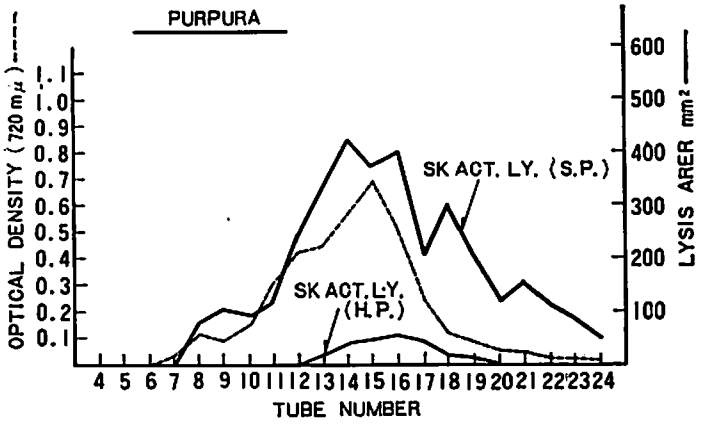

Fig. 3

revealed marked fibrinolytic activity.

Case 3. Diagnosis: Pemphigus vulgaris

A 23-year-old female developed recalcitrant bullous lesions all over the body. Citrated plasma was fractionated with same method in this case. The results were shown in Fig. 4. Very distinct increase in the fibrinolytic activity (strepto-

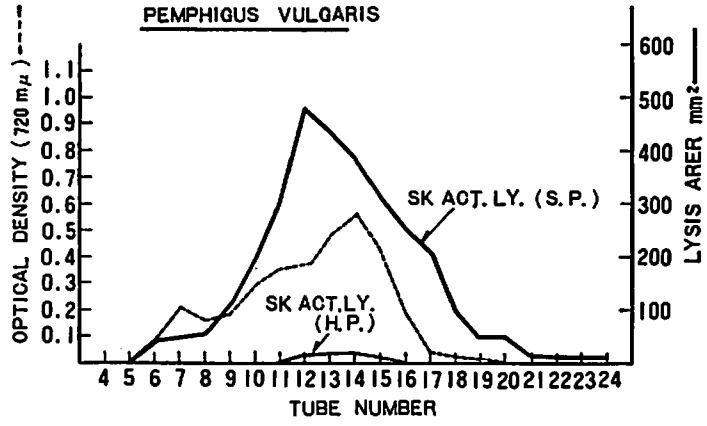

Fig. 4

kinase-activated lysis) on a standard plate was found from fraction number 11 to 17. Though the cause of pemphigus has not been clarified, this pathological fibrinolytic activity due to proactivator seemed to suggest the changes of fibrinolytic enzyme system in case of the disease.

SUMMARY

New method for determination of blood fibrinolytic activity of patient with skin disease by means of gel filtration was discussed.

Each one case of urticaria acuta, fibrinolytic purpura and pemphigus vulgaris showed marked fibrinolytic activity (streptokinase-activated lysis) on a standard plate. The results of the experiments suggested that proactivator should be given much attention in some dermatoses.

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EFFECT OF HISTIDINE OR ITS DERIVATIVES ON THE PLASMA IRON CONTENT*

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It is the well established fact that xanthine oxidase is capable of reducing ferritin iron under anaerobic conditions in the presence of hypoxanthine or xanthine. In this reaction, the reduction of ferritin iron was brought about both by the dehydrogenase activity of xanthine oxidase and by the non-enzymatic reaction with the accumulated uric acid[1]. But the mechanism by which the iron is released from ferritin was not yet elucidated throughly.

In this paper it is indicated that the iron contents in blood plasma are increased remarkably in experimental animals after administration of L-histidine or its irradiated modification.

In an effort to obtain further informations about the mechanism which causes the release of ferritin iron, investigations *in vitro* were also undertaken. And evidence of coupling of ferritin iron reduction with histidine metabolism is presented with the experiments *in vitro* containing partially purified enzyme system from liver, crystalline ferritin and histidine or its derivatives.

EXPERIMENTAL PROCEDURE

Chemicals: Heparin powder was a product from Novo industri A/S, Copenhagen. L-Histidine HCl, urocanic acid, D-histidine, L-glutamic acid, imidazole, 2-imidazolidone (ethyleneurea), imidazole-4, 5-dicarboxylic acid and histamine 2HCl were of chemical pure grade and obtained as commercial products.

L-Histidine modification: The near-ultraviolet irradiated modification of L-histidine was prepared by applying the "mineral light" (cycle 50, wave length of 3650 Å), to the histidine powder or its solution at the distance of 5 cm for 10 minutes.

Preparation of crystalline horse spleen ferritin: This was prepared by the

* Portions of this work were reported briefly in J. Japan. Biochem. Soc. (in Japanese), 35, 629 (1963).

method of Mazur and Shorr[2], and recrystallized five to eight times prior to experiments, ferritin was dissolved in distilled water with the aid of ammonium sulfate, and dialyzed against distilled water until it became free from NH_4^+ and SO_4^{--}. The solution contained 1.56 mg of iron and 1.86 mg of Kjeldahl nitrogen per ml.

Experimental animals: Male rabbits weighing 2 to 3 kg and male Donryu rats were maintained on a diet of Central Experimental Animal Laboratory chow, vegetable and water ad libitum at least for one week prior to experiments.

Preparation of blood plasma: Prior to preparation, the inner surface of a syringe was soaked once with heparin (500 units/ml 0.9% NaCl) and it was poured out so that the remaining volume of anticoagulant should have been neglected. About 5 ml of blood was drawn with the syringe either from the heart of rabbits or from the abdominal aorta of rats under pentobarbital anesthesia in the latter case. Blood was well mixed and after centrifugation the plasma was taken out.

Preparation of liver homogenates: After decapitation and bled of male Donryu rats, the livers were rapidly removed, and forced through a masher to remove connective tissue. The liver pulp was added to 20 volumes of ice-cold 0.25 M sucrose solution and homogenized for one minute in a Potter-Elvehjem glass homogenizer. The homogenate was then centrifuged at 3000 rpm for 15 minutes and the supernatant was collected. In some cases the supernatant was dialyzed against distilled water at 4°C overnight. All the procedures were undertaken at 0→4°C.

L-Histidine or its derivatives were administered intramuscularly in the case of rabbits or intraperitoneally in the case of rats, as a 2.5% aqueous solution at dose level of 50 mg/kg of body weight.

Measurement of iron: The plasma iron was determined by the method of Barkan and Walker[3].

Measurement of Fe^{++} released from ferritin: One ml of a stock ferritin solution was added to 1 ml of the supernatant of rat liver homogenate, 1 ml of 0.5 M phosphate buffer, pH 7.4, 1 ml of 0.1% *o*-phenanthroline solution and 1 ml of 25 mM substrates. The mixture was incubated for 1 hour at 37° aerobically or anaerobically. After the incubation, 4 ml of a saturated solution of ammonium sulfate and 1 ml of 2 M acetate buffer, pH 4.5, were added to the mixture in order to precipitate ferritin and to obtain an optimal pH for colorimetrical estimation of Fe^{++}-phenanthroline complex. After centrifugation at 3000 rpm for 15 minutes, the supernatant was allowed to stand at room temperature for 1 hour and its colour was read with a photocolorimeter with the use of a No. 50 filter.

against a blank solution which contained all the reagents except ferritin.

RESULTS

In order to differentiate the changes caused by L-histidine administration from those due to bleeding, it was necessary to determine the fundamental effects of bleeding in rabbits on plasma iron content. Five ml of blood was drawn at 3 hours intervals for periods up to 12 hours and plasma iron contents were determined.

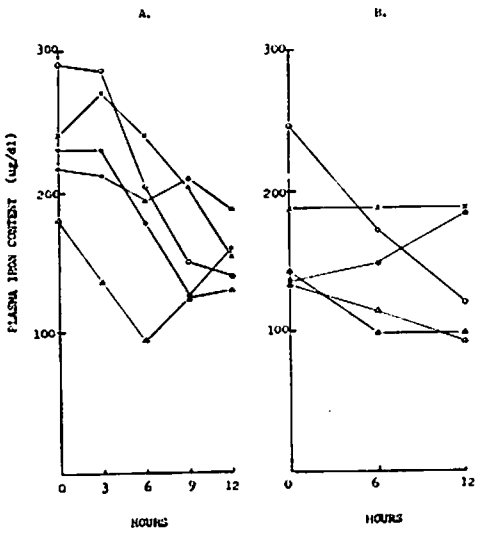

Fig. 1 Effects of bleeding on plasma iron levels of normal rabbits. Five ml of blood was drawn at an interval of 3 hours (A), and at an interval of 6 hours (B), directly from the hearts of rabbits.

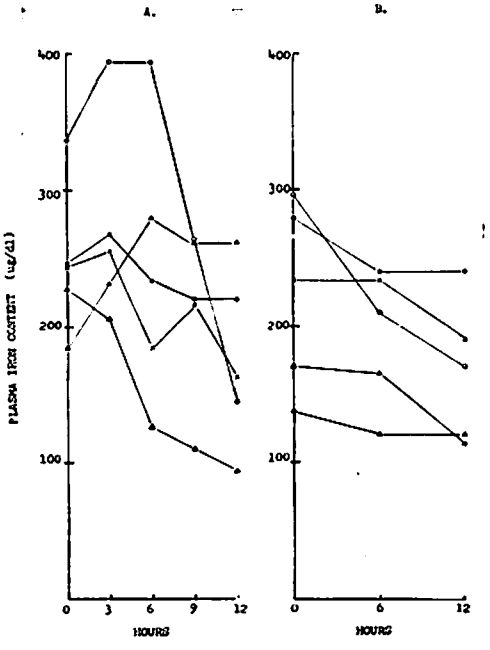

Fig. 2 Effects of L-histidine administration on plasma iron level of rabbits. Fifty mg/kg of L-histidine was administered intramuscularly. The blood was drawn as described in the legend of Fig. 1.

As shown in Fig. 1, the values of plasma iron contents were not altered for the first 3 hours (A), but decreased distinctly after 6 hours (B). When blood was drawn and its iron contents were assayed at 6 hours intervals, decrease in iron contents was smaller than that shown in Fig. 1A (Fig. 1B). These results show that blood plasma iron contents of rabbits have the tendency to be decreased by repeated blood drawings at short intervals.

The effects of L-histidine administration on blood plasma iron content of rabbits were investigated. Five ml of blood was drawn at 3 or 6 hours intervals for period up to 12 hours after administration of L-histidine and its plasma iron

contents were determined.

As shown in Fig. 2A, plasma iron contents of 2 out of 5 rabbits were increased distinctly at 3 hours after administration, the decreases in iron contents were gradual in all cases. The result of similar experiments, in which blood was drawn at 6 hours intervals, are shown in Fig. 2B. Little or no decrease in iron content was observed even at 6 hours after administration.

Blood plasma iron contents were increased at 3 hours after administration of L-histidine modification irradiated with near-ultraviolet n aqueous solution and then restored to the initial level after 6 hours (Fig. 3A). In similar experiments,

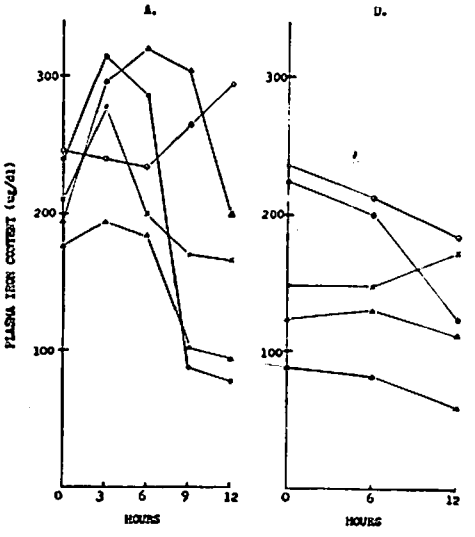

Fig. 3 Effects of L-histidine modification irradiated with near-ultraviolet light in aqueous solution on plasma iron level of rabbits. Fifty mg/kg of irradiated L-histidine was administered intramuscularly. The blood was drawn as described in the legend of Fig. 1.

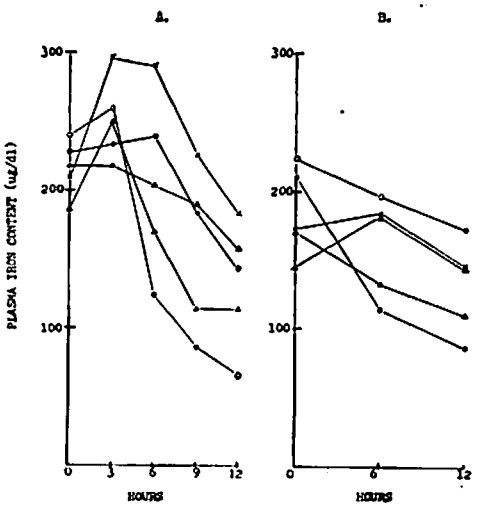

Fig. 4 Effects of L-histidine modification irradiated with near-ultraviolet light in crystal state and in aqueous solution on plasma iron level of rabbits. Fifty mg/kg of irradiated L-histidine was administered intramuscularly. The blood was drawn as described in the legend of Fig. 1.

in which blood was drawn at 6 hours intervals, iron contents were not affected (Fig. 3B).

The administrations of L-histidine modification irradiated in crystal state and additionally irradiated in aqueous solution exerted almost identical effects to those of L-histidine irradiated once (Fig. 4). These data demonstrate that administration of L-histidine or its near-ultraviolet irradiated modification causes increases in plasma iron content of rabbits.

The percentage increases in plasma iron level after 3 hours in each experiment are listed in Table 1.

Table 1

The Percentage Alteration in Plasma Iron Level After 3 Hours in Each Experiment

Substance administered		Rabbit No. 1	2	3	4	5	Average
None	%	−0.1	+12.5	0	−1.8	−23.3	−2.5
L-Histidine	%	+16.8	+8.1	+4.0	−11.3	+27.7	+9.1
Ultraviolet once irradiated L-histidine	%	−4.9	+30.9	+33.3	+50.0	+8.0	+23.5
Ultraviolet twice irradiated L-histidine	%	−8.3	+5.0	0	+41.0	+31.9	+17.2

L-Histidine or its modifications were administered intramuscularly as a 2.5% aqueous solution at dose level of 50 mg/kg body weight.

The irradiated L-histidine modification exerted more remarkable effect than genuine one. Although absolute values of increases in iron were rather minimal, administration of 0.25 mM or 50 mg L-histidine per kg body weight gave rise to a distinct elevation of plasma iron content. In order to confirm the above observations, similar experiments were undertaken with rats. But reliable results could not be obtained, since their plasma iron content was readily affected by their environmental conditions, especially by room temperatures.

Table 2

Influence of the Presence of Oxygen and of Dialysis of Liver Homogenates on Ferritin Iron Reduction

Rat liver homogenate	Gas phase of incubation	Substrate	Fe^{++} concentration Before inc. μg/ml	After inc. μg/ml	Fe^{++} released μg/ml	Fe^{++} liberation due to L-his. μg/ml
Without dialysis	Air	—	9.4	9.9	0.5	5.4
		L-His.	9.4	15.3	5.9	
		—	11.6	12.0	0.4	0.7
		L-His.	11.8	12.9	1.1	
	N_2	—	10.9	15.8	4.9	1.4
		L-His.	10.8	17.1	6.3	
With dialysis	Air	—	4.7	5.3	0.6	5.1
		L-His.	4.2	9.6	5.7	
	N_2	—	4.1	9.4	5.3	9.4
		L-His.	4.1	18.8	14.7	

Final concentration of substrate was 5 mM.

Since Green *et al.*[1] have postulated that plasma iron is originated from ferritin, it would be reasonable to assume that some metabolites of L-histidine might reduce ferritin iron directly, or some enzymatic reactions of L-histidine or its metabolites might involve the reduction of ferrtin iron. In order to verify these assumption, following experiments were undertaken.

Using rat liver homogenate as crude enzyme system, reduction of ferritin iron was investigated with several metabolites of L-histidine or substances that are of similar structure to L-histidine.

In Table 2, influence of the presence of oxygen and of dialysis of liver homogenates on ferritin iron reduction were listed. Non-dialyzed liver homogenates contained large amount of free iron which could be removed easily by dialysis. Although in some experiments, reduction of ferritin iron was remarkable, in most experiments, it was very slight with non-dialyzed homogenate under aerobic conditions. It appears that oxidation of histidine with oxygen would be favorable under these conditions.

Table 3

Iron Released from Ferritin due to L-*Histidine, its Modification and Urocanic Acid*

Substrate		Fe^{++} concentration			Fe^{++} liberation due to subst. μg/ml
		Before inc. μg/ml	After inc. μg/ml	Fe^{++} released μg/ml	
L-Histidine	−	4.7	5.3	0.6	5.1
	+	4.2	9.6	5.7	
L-Histidine*	−	7.5	8.2	0.7	5.4
	+	7.6	13.7	6.1	
U.V. irradiated L-histidine	−	7.3	8.4	1.1	3.1
	+	7.3	11.5	4.2	
Urocanic Acid	−	5.3	6.7	1.4	5.0
	+	7.1	13.5	6.4	
L-Histidine with 0.2 M borate buffer	−	6.7	7.6	0.9	3.6
	+	7.3	11.8	4.5	
L-Histidine with uric acid	−	5.6	6.0	0.4	1.4
	+	5.8	7.6	1.8	

One ml of a stock ferritin solution was added to 1 ml of the supernatant of rat liver homogenate, 1 ml of 0.5 M phosphate buffer, pH 7.4, 1 ml of 0.1% *o*-phenanthroline solution and 1 ml of 25 mM substrates. After aerobical incubation, 4 ml of a saturated solution of ammonium sulfate and 1 ml of 2 M acetate buffer, pH 4.5, were added to the mixture. After centrifugation of mixture, Fe^{++}-phenanthroline in the supernatant was estimated by the use of photocolorimeter.

* The supernatant of liver homogenate at 10,000 g for 15 minutes was used as a crude enzyme.

It was observed that relatively large amount of iron was released even without addition of histidine with non-dialyzed homogenate under aerobic conditions. The results indicate that non-dialyzed homogenate might contain substances which cause acceleration of iron release during incubation.

On the other hand, in the cases of experiment with dialyzed liver homogenate which containes minimum quantity of free iron, the quantity of free iron increased distinctly after aerobic incubation in the presence of L-histidine and the increase was more remarkable under anaerobic conditions.

These results indicate that the more favorable conditions for iron release should be gained by the application of anaerobic incubation with dialyzed liver homogenates. But the aerobic condition will suffice so far to test or compare the iron-releasing ability of various substrates, because there occured remarkable increasing of free iron in the presence of L-histidine under such condition.

Following experiments were carried out under aerobic condition adding each of various metabolic derivatives of L-histidine as a substrate. The results are shown in Table 3.

When L-histidine was used as a substrate, iron was released remarkably. The same results were obtained with the dialyzed supernatant of liver homogenate at 10,000 g, for 15 minutes to remove mitochondria throughly. Near-ultraviolet irradiated L-histidine as a substrate caused slightly lesser iron release than non-irradiated L-histidine did. Urocanic acid also resulted in almost same amount of iron release as that caused by L-histidine. When borate buffer, pH 7.4, was used, slight inhibition was observed in iron release. The iron release was significantly inhibited by the addition of 0.01 M uric acid. When D-histidine, L-glutamic acid, imidazole, 2-imidazolidone (ethyleneurea), imidazole-4, 5-dicarboxylic acid or histamine 2HCl was used as a substrate, no iron release was observed.

DISCUSSION

The results of the above experiments show that the mechanism of increase in plasma iron content *in vivo* by L-histidine would be as follows. Iron is released from ferritin following the reduction by L-histidine metabolite which might be produced in the course of metabolism by urocanicase and xanthine dehydrogenase analogue etc. in the liver, and the released iron enters the plasma via combination with the plasma iron binding globlin. Actual metabolites of L-histidine having such a reducing activity has not yet been found. Possible evidence of these assumption could be deduced from the experimental facts that hydantoinpropionic acid arises from L-histidine via urocanic and imidazolone propionic acids,

and the oxidation of imidazolone propionic acid to hydantoinpropionic acid is mediated by xanthine oxidase[4]. This enzyme is known to be inhibited by borate and uric acids. From these facts, it will be reasonable to assume that the origin of the increased plasma iron is ferritin iron of the liver, and that ferritin iron acts as an electron acceptor when xanthine oxidase operates in the metabolism of L-histidine.

Thus the effect by irradiated modification of L-histidine was compared with that of genuine one. And the implication of stimulating effect in iron release with former was observed specifically *in vivo* (Table I).

It has been reported that the reduction of ferritin iron brought about both by the dehydrogenase activity of xanthine oxidase and by the accumulated uric acid formed by this enzyme[1]. The findings by the author are compatible with that of the above report.

Still the further detailed studies with purified enzyme system and selected substrates should be undertaken to verify these points.

SUMMARY

A distinct increase in blood plasma iron content was caused in animals after administration of L-histidine or its near-ultraviolet irradiated modification.

Using partially purified enzyme system from rat liver homogenate, release of iron from ferritin was observed, when L-histidine or urocanic acid was added as a substrate. D-Histidine, L-glutamic acid, imidazole, 2-imidazolidone (ethyleneurea), imidazole-4, 5-dicarboxylic acid or histamine-2HCl had no effect on iron release.

It has been assumed that the origin of the increased plasma iron caused by L-histidine is ferritin iron of the liver and that ferritin iron acts as an electron acceptor coupled with dehydrogenation by the xanthine oxidase in the course of metabolism of L-histidine.

Acknowledgements

I wish to express my appreciation to Dr. H. Hatano, Professor of Dermatology and Dr. K. Sekita, Professor of Biochemistry for their encouragement and guidance. I also wish to thank Dr. T. Sakurada, Assistant Professor of Biochemistry for his suggestions.

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EFFECTS OF SEVERAL EXCITANTS AND DEPRESSANTS ON GLUTAMIC ACID AND ITS METABOLITES CONCENTRATION IN RABBIT AND RAT BRAIN*

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INTRODUCTION

There are various amino acids measured by different qualitatives and quantitatives methods in the brain, such as glutamic acid**, glutamine, γ-aminobutyric acid, aspartic acid, etc. and many reactions such as transamination, decarboxylation, amination, deamination or glycolysis, have proved the existence of relationship to the changes of these amino acids in brain.

These facts suggest us that these amino acids have some close connection with the brain functions. So the quantitative changes of these amino acids is the most interesting problems of today.

Now we want to see whether or no any changes occur when stimulants or anaesthetics which cause the changes of animal brain functions are administered to animals. Now, when administration of stimulants or anaesthetics cause the changes of animal brain functions, is it possible to say that the above-mentioned amino acids provoke the change?

Old reference proved that insulin (Cravioto, Massieu and Izquirodo (1951)[1]) acted on GA and GABA to decrease and on aspartic acid to increase, and Killam and Bain (1957)[2] recognized an evident decrease of GABA by the drugs of thiosemicarbazide in the whole brain of rats.

Roberts *et al.* (1958)[3], investigated the changes in the concentration of free amino acids in the whole brains of mice after receiving single administration of various drugs acting on the central nervous system, but could not detect any change. On the other hand, many investigators who has studied along the same line (Ansell (1954)[4], Cravioto (1951)[1], Dawson (1951)[5], Killam (1957)[2],

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** The following abbreviations are used in this paper:glutamic acid:GA, glutamine: G, γ-aminobutyric acid:GABA, aspartic acid:A.

Woodbury (1957)[6], Noda (1956)[7], Ogura (1959)[8], and Kaneto (1959)[9]) reported that in some of the amino acids such changes did actually occur.

Roberts *et al.* (1958)[3], in the same report, stated that the absence of changes by single administration was because of the fact that estimation was made with whole brain, and that if the estimation was made in parts, positive results might have been obtained. Elliott *et al.* (1959)[10] stated that the changes of amino acids occuring in the locas portion of the brain can not be detected by estimation using homogenized whole brain.

De Ropp and others (1961)[11], investigated the changes of about 10 ninhydrin positive substances in the whole brain of rats at fixed time after single administration of various stimulants and depressants, and stated that it was unable to detect any evident relation between these compounds and the changes of the amino acids in the brain.

On the other hand, Berl and Waelsch (1958)[12], after deviding the brain of adult rats into several parts, made separate estimations of amino acids in each part and demonstrated marked difference among the quantity of the amino acids in each part. Berl, Purpura, Girado and Waelsch (1959)[13] also mentioned that in case of freezing a half of the brain cortex of cats with dry ice, among amino acids in the freezed part, decreases of GA and glutamine were evident, while GABA marked no change.

Häkkinen and Kulonen (1961)[14], investigating the changes of amino acids, especially of GA, G and GABA contents in the whole brain of rats which were received oral administration of ethanol of toxic dose, offered the detailed results of evident changes of the three.

Now, in these reports so far, for the most part, the changes of amino acids in the brain were investigated by using only the whole brain or the brain cortex, and also satisfactory attention was not paid to the quantity, the method and the period of administration of drugs.

In this report of ours, brains of adult rabbits and rats were divided, though anatomically rough, into four parts and each part was measured for its contents of GA, GABA, G plus alanine and A. As control experiments, rabbits were administered with subconvulsive doses of picrotoxin and a relatively greater doses of morphine, sufficient to lower body (rectal) temperature, because the pharmacological action which lower body temperature in rabbits by the two drugs was examined as an indicator for the stimulant or depressant action of the central nervous system. In both cases the changes in the level of the above mentioned amino acids were estimated at the three regular periods after the respective administration of two drugs: 1) the beginning of hypothermia, 2) the lowest fall,

3) the recovery period. As another stimulants and depressants were chosen strychnine, thiosemicarbazide, hydroxylamine and barbiturates. It became clear that amino acids in each part had received some kinds of changes by those drugs. The quantitative changes of each amino acid at the time of stimulation and depression in the brain, caused by the drugs acting on the central nervous system, suggest us the presence of mutual relation between stimulation and depression under influence of drugs.

METHODS

Forty two male adult rabbits and over 100 albino rats (Donryu strain) were used. Two rats were examined as one group of each experiment. Animals were sacrificed by dipping them into ethanol and dry-ice (−60°C), and then the brains were taken out and, while cooling, were divided into 4 parts; namely, cerebrum, diencephalon (including corpus striatum), mesencephalon (including medulla oblongata) and cerebellum. The weight of each part was measured precisely.

Each part of the brain was homogenized using Potter type glass homogenizer and homogenous emulsions were obtained. These emulsions were deproteinized by adding 10–15 cc/g of 70% ethanol and centrifuging (3000 r.p.m. for 10–15 min.). This procedure was repeated once more. After centrifugation the supernatant was distilled under low pressure at 45–50°C. and dried in vacuo, after which amino acids and other substances adhered to the walls of glass tubes. This dried material was dissolved by adding water. The amounts of water were 2.5 cc (or sometimes 2.0 cc) for cerebrum and 1.0 cc for other tissues. To one part of each solution were added 3–5 parts of chloroform and, after vigorous shaking and centrifugation (3000 r.p.m. for 20 min.) it separates into 3 layers.

The top layer, or water layer, was submitted to paper chromatography by spotting on filter paper (Toyo No. 51) of a size of 40×40 cm using micropipettes of 0.03 cc or sometmies 0.015 cc. The round spot on the filter paper was made as small as possible. On the same paper mixture of standard amino acids including all the amino acids to be estimated was also spotted in a volume of 0.03 cc or sometimes 0.015 cc. Doses used for samples were 0.3 mg/cc and 0.6 mg /cc for GABA and 0.4 mg/cc glutamine, and 0.7 mg/cc and 1.4 mg/cc for glutamic acid. In order to develop samples and standard solution the chromatogram chamber for ascending technique was used and the development was carried out for about 15 hours at room temperature (about 20°C). As developing solution 23% phenol containing 0.1% NH_4OH was used and 150–200 cc was poured into solvent through in the chromatogram chamber, and always new solution was used.

After development phenol was evaporated thoroughly and papers dipped into

0.075% ninhydrine-absolute alchohol solution and then the amino acids were completely encolored by a type of heater for 20 minutes at 65°C revised and enlarged a paraffin extension apparatus for histological study, while taking a greatest care that the backgrounds of papers should not be encolored too much.

From the filter papers the colored portion corresponding GABA, GA, G plus alanine and A which were located near Rf 0.76, 0.20, 0.58 and 0.15 respectively, were cut off into test tubes to which was added 75% ethanol containing 0.05 mg/cc of copper sulfate. They were then determined with the use of Beckman DU-type spectrophotometer (wave length 510 $m\mu$) and the amounts were calculated from standard solutions of amino acids which were treated simultaneously.

When phenol is used as an developing solution the problem of overlap of amino acids arises. Glutamine, which has its characteristic staining near the point of Rf 0.58 is contaminated with alanine, threonine and tyrosine. The latter two are so minute in amount that they could be disregarded when tissues weighing about 20 mg or less are used, but alanine is proved to appear in considerable amount judging from the results obtained with two-dimensional development using butanol and glacial acetic acid. Accordingly, the values of the amount of glutamine here described are not true but are apparent ones.

The body temperatures of rabbits were measured by mercury thermometer at rectum.

RESULTS AND DISCUSSION

1. The concentration of GA, G and GABA (sometimes A) in the several parts of the intact animal brains.

The each brain of 12 normal rabbits was divided into four parts, *i.e.*,

Table 1

The Amino Acids in Adult Rabbits Brain

(mg/100 g wet brain)

	Cerebrum	Diencephalon Corpus striat.	Mesencephalon Med. oblongata	Cerebellum
GABA	*24.2± 3.67 **(27.2)	38.4± 4.28 (36.8)	30.3± 5.17 (36.6)	21.2± 4.27 (21.6)
Glut. Acid	139.9±27.36 (135.0)	136.4±13.42 (124.0)	109.2±12.33 (111.9)	130.0± 7.36 (127.5)
Glut.+Ala.	85.8±15.09 (84.7)	80.0±10.91 (83.7)	67.6± 9.78 (69.0)	79.9± 9.99 (84.7)

* mean values from twelve intact animals; mean ± unbiased estimation of s.d.

** mean Values from three animals injected s.i. the physiologic saline solution 1 cc/kg.

cerebrum, diencephalon (including corpus striatum), mesencephalon (and medulla oblongata) and cerebellum, and the contents of GABA, GA and G plus alanine in each part were measured (Table 1).

It is seen from Table 1 that the highest value of GABA is obtained with diencephalon, and decreases in the order of mesencephalon, cerebrum and cerebellum. In the case of glutamic acid the order is cerebrum, diencephalon, cerebellum and mesencephalon. As for the values of glutamine and alanine the order is cerebellum, diencephalon and mesencephalon.

The same experiments were also made on five groups of intact rats, as shown in Table 2.

Table 2

The Amino Acids in Adult Rats Brain (mg/100 g wet brain)

	Ccrebrum	Diencephalon Corp. striatum	Mesencephalon Med. oblongata	Cerebellum
Glut. Acid	82.3*±22.1	109.8±10.4	81.6±7.2	121.1±2.1
Glut.+Ala.	49.8± 5.7	65.3± 2.4	45.6±4.3	80.9±5.7
GABA	29.2± 5.8	42.0±10.3	23.5±6.2	24.2±6.8
Asp. Acid	57.8± 5.3	70.2±10.5	69.3±8.7	62.3±5.1

* mean values from five intact animals mean ± s.d.

The results above mentioned were compared with those in the literatures. The literatures concerning the amino acid in the brains of rabbits are very few and we could find only one (Roberts *et al* 1951[15]), in which it is described that gray matter of thalamus has the value of 41 mg/100 g, frontal cortex 64 mg/100 g, pooled gray matter 64 mg/100 g and white matter 28 mg/100 g. Recently Waelsch *et al.* (1958)[12] examined the distribution of amino acids in the brain of adult rats by column chromatography and showed that thalamus and hypothalamus are the richest in the content of GABA. They emphasized a fact that medulla and pons had always lower quantities of glutamic acid, glutamine and glutathion than the cortex of cerebrum, which is in accordance with our results.

2. Successive changes of the concentration of GA, G and GABA in the several parts of the brain of rabbits following subcutaneous injection of a definite dose of picrotoxin.

   a) The subcutaneous injection of a subconvulsive dose (1.0 mg/kg) of picrotoxin.

Picrotoxin is one of the convulsants and well known for producing characteristic convulsions, and its subconvulsive dose produces marked fall in the body

temperature of animals. Picrotoxin is said to block not only depressor fibers controlling the opener muscle of hind legs but also blocks the inhibiting effect of GABA on the heart of crustacea (Florely (1957)[16]), (Jasper *et al.* (1958)[17]), from their electrophysiological study using cortex of cat, state that picrotoxin does not antagonise with GABA. On the other hand, Robbins (1959)[18], investigating the mechanism of the action of picrotoxin on crustacea, expresses his view that picrotoxin probably competes with GABA at the depressor receptors. Van der Kloot (1960)[19] suggested that GABA is selectively blocked by picrotoxin at every crustacean inhibitory junction without interfering with excitation.

It had been already studied by Tomizawa (1957)[20] and Abe (1943)[21] that pharmacological actions, caused by the definite dose of picrotoxin, lowering the body temperature and increasing blood sugar, were elicited owing to the central effects.

Then, how did this dose of picrotoxin successively alter the concentration of GA, G and GABA in several parts of the rabbit brain?

In approximately 20°C ambient temperature nine rabbits were injected subcutaneously with 1.0 mg/kg picrotoxin and also the change of rectal temperature was measured.

The rectal temperature lowered apparently after the injection of picrotoxin until it showed maximum of lowering an hour after, and then gradually elevating, it almost returned to its beginning temperature three hours after the injection (Fig. 1).

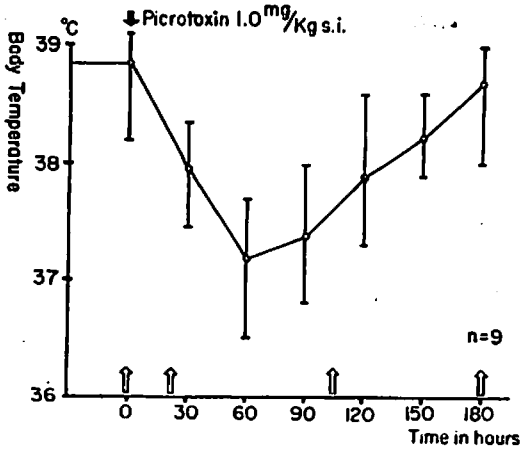

Fig. 1 The hypothermia by the subcutaneous injection of non-convulsive dose of picrotoxin (1 mg/kg) in rabbits. Ordinate is change of body (rectal) temperature. Absciss is time in minutes. Vertical blacketed lines represent mean values, max. and min. values of nine rabbits. White arrows represent the time of sacrificing the animals.

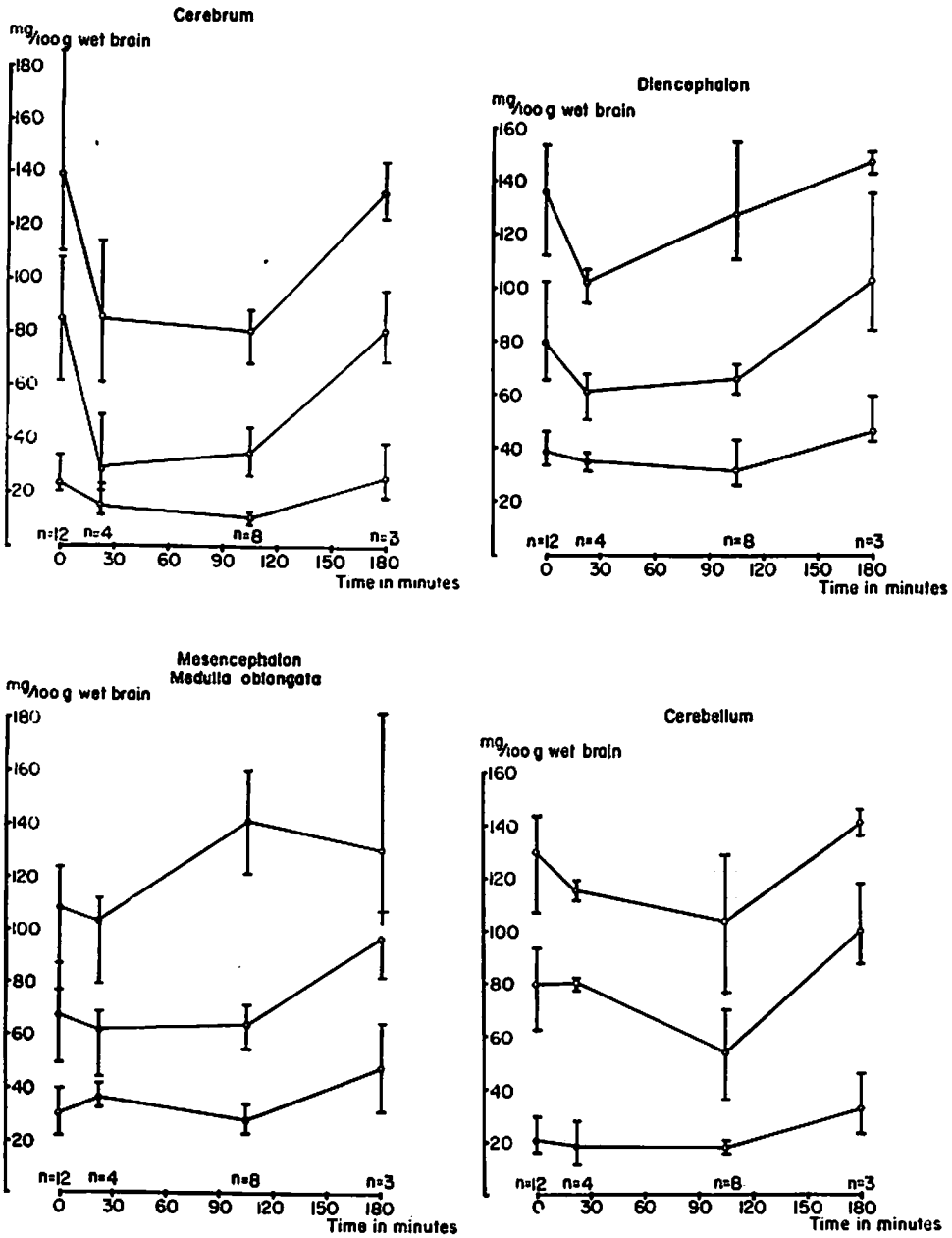

Fig. 2 The changes of the free amino acids in brains after single subcutaneous injection of 1 mg/kg picrotoxin and the comparison with that of normal rabbits. Ordinate is contents of the brain amino acids. Absciss is time in minutes. The upper curves show changes of glutamic acid content, the middle changes of glutamine+alanine, the lower changes of γ-aminobutyric acid. n...means each cases of experiments.

The centers involved in the regulation of body temperature, whose excitation caused by picrotoxin lowered the body temperature, would stimulate until approximately an hour after the injection, but after that the excitation would be gradually mitigated and disappeared to cause returning of body temperature to its beginning about three hours after the injection.

The changes of three amino acids in brain after subcutaneous injection of 1.0 mg/kg picrotoxin and the comparison with that of normal rabbits are shown in Fig. 2.

Results of the above data were treated with the variance analysis (Table 3).

Table 3

Statistical Analysis of Changes in Adult Rabbits Brain caused by Subcutaneous Injection of 1.0 mg/kg Picrotoxin

		F	n_1	n_2	P
Cerebrum	GABA	21.6	3	24	< 0.1%
	Glut. Acid	10.3	3	24	< 0.1%
	Glut.+Ala.	39.6	3	25	< 0.1%
Diencephalon (Corpus striat.)	GABA	11.94	3	24	< 0.1%
	Glut. Acid	4.97	3	24	< 1%
	Glut.+Ala.	9.71	3	24	< 0.1%
Mesencephalon Med. oblongata	GABA	8.47	3	24	< 0.1%
	Glut. Acid	0.398	3	25	> 5%
	Glut.+Ala.	9.37	3	25	< 0.1%
Cerebellum	GABA	2.94	3	24	> 5%
	Glut. Acid	9.12	3	24	< 0.1%
	Glut.+Ala.	14.9	3	24	< 0.1%

F .....variance ratio
n_1 n_2 .....degree of freedom
P .....possibility, by the analysis of variance

It is evident from the Table that the decrease in the amino acids was most marked in the cerebrum which was recognized difference at the significant limit of 0.1%. At 20 minutes after the injection of picrotoxin the body temperature fell by 1°C or so, and marked decrease in the amino acid group was observed. After 105 minutes the body temperature began to rise somewhat from the minimal point, but the decrease in the amino acid was almost as same as that observed after 20 minutes. After 180 minutes when the temperature recovered almost completely, contents of amino acids also returned to normal.

By subcutaneous injection of subconvulsive dose of picrotoxin the amino acids in the diencephalon tissue had the tendency to decrease next to cerebrum. Glutamic acid was significantly lower at the significant limit of 1%, while other two were so at the level of 0.1%. Only one interesting point is that when the body

temperature returned to normal every amino acid had the tendency to increase or even exceed the value at intact state.

In the case of mesencephalon and medulla the changes were somewhat different from the above two; namely, there was no significant change of glutamic acid.

In cerebellum no significant difference was observed regarding the GABA content. At the time of recovery it had the tendency to increase over the value of normal control.

As shown in Fig. 2, the fructuation of the amino acids in rabbit brains was significant except glutamic acid content of mesencephalon and medulla oblongata and GABA content of cerebellum.

These results made it clear that when rabbits were received subcutaneous injection of subconvulsive dose of picrotoxin the concentration of GA, G and GABA in the brain was fluctuated, though the degree of the fluctuation was individual in each part of the brain. It was also recognized that when subcutaneous injection of the subconvulsive of picrotoxin, acting on the center, apparently lowered the body temperature of rabbits, the concentration of GA and G in the parts of cerebrum, diencephalon and cerebellum showed an evident decrease. Above all the fall of the concentration of these amino acids was exceeding in cerebrum, and the concentration of GABA in diencephalon and cerebellum was evidently lowered, too. At the time of nearly complete restoration of the body temperature, having greatly lowered by the dose of picrotoxin, the concentration of the amino acids in four parts of the brain, which had been decreased, almost returned to normal or rather increased.

b) The subcutaneous injection of 3.0 mg/kg of picrotoxin.

When the subcutaneous injection of 3.0 mg/kg of picrotoxin in rabbits provoked clonic convulsions approximately thirty minutes after the injection, the concentration of GA, G and GABA in four parts of the brain was measured in next two terms; four rabbits prior to fall into convulsion a quarter minutes after the injection of the dose of picrotoxin, that is "in a state of preparation for convulsion," and six in state of seizure thirty minutes after the injection.

These results as shown in Fig. 3 were secured.

In the previous stadium which would elicit convulsion by the subcutaneous injection of the convulsive dose of picrotoxin in rabbits, the concentration of GA, G and GABA would decrease intensively with the exception of the part of cerebrum where no significant change was seen. Excessive decrease of these amino acids was indicated in two parts of diencephalon and mesencephalon, medulla oblongata, and the decrease was twice as much as that in case of com-

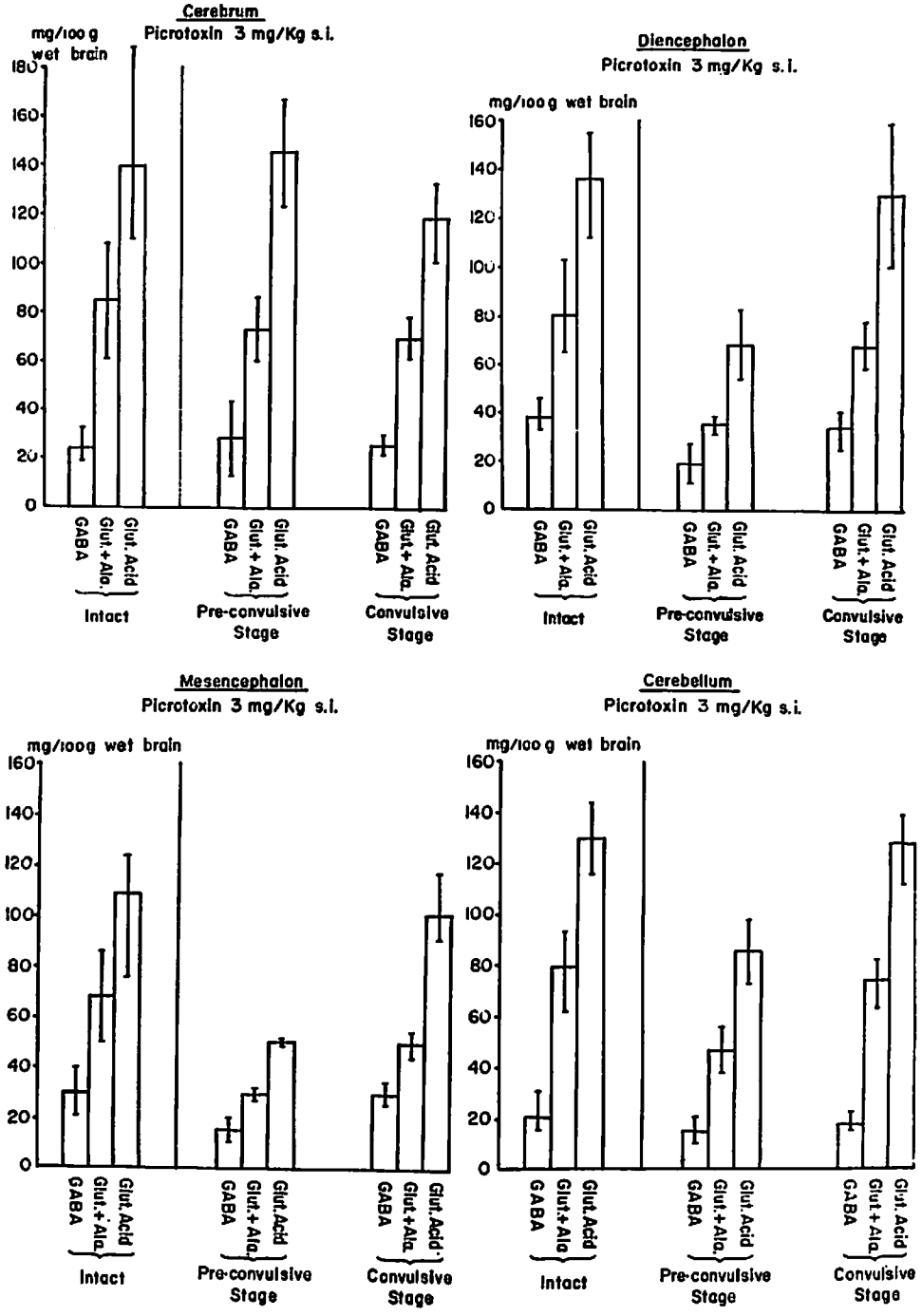
Cerebrum
Picrotoxin 3 mg/Kg s.i.
mg/100 g wet brain
Diencephalon
Picrotoxin 3 mg/Kg s.i.
mg/100 g wet brain
Mesencephalon
Picrotoxin 3 mg/Kg s.i.
mg/100g wet brain
Cerebellum
Picrotoxin 3 mg/Kg s.i.
mg/100 g wet brain
GABA
Glut.+Ala.
Glut.Acid
Intact
Pre-convulsive Stage
Convulsive Stage

Fig. 3

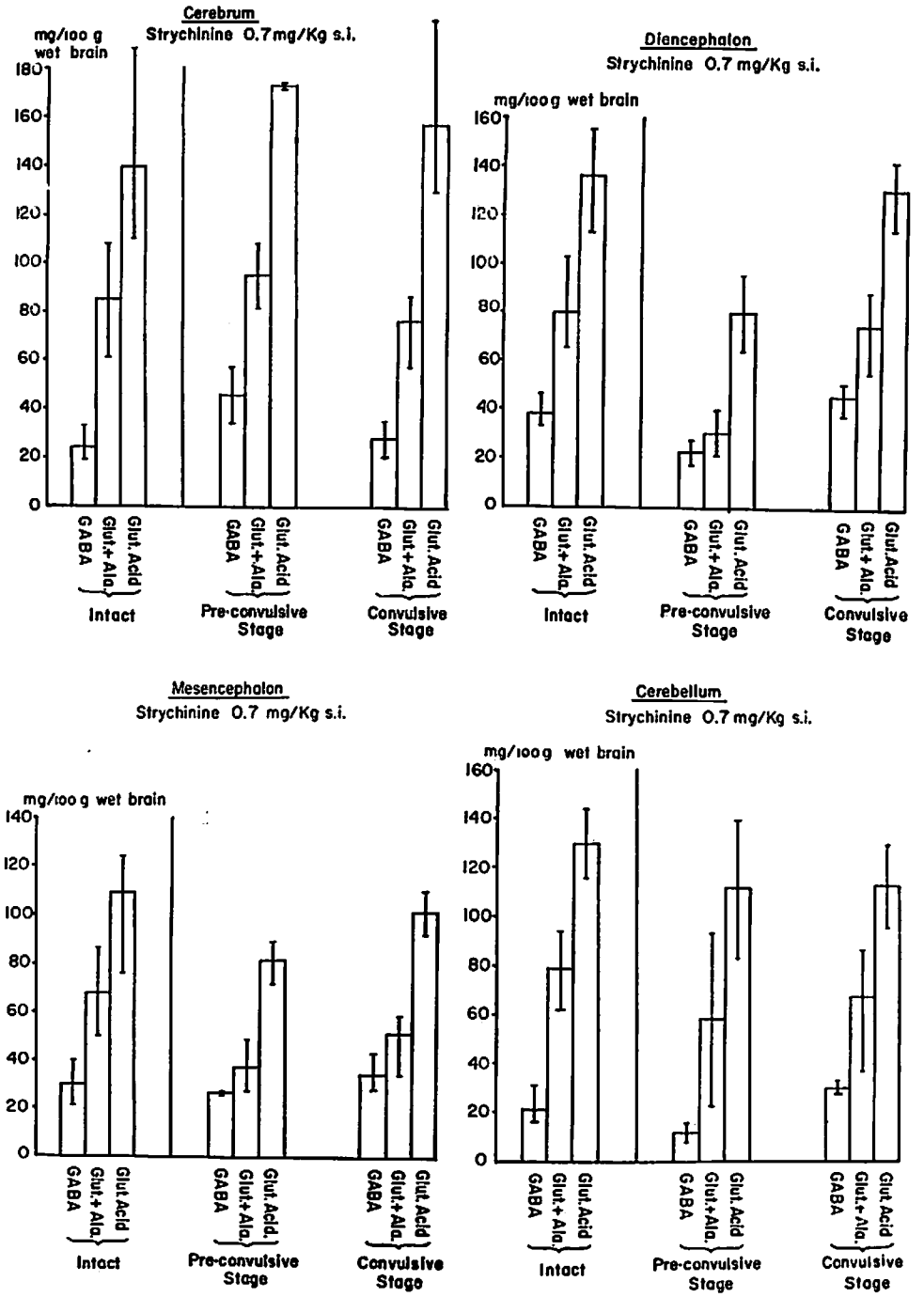
Cerebrum
Strychinine 0.7mg/Kg s.i.
mg/100 g wet brain
Diencephalon
Strychinine 0.7 mg/Kg s.i.
mg/100g wet brain
Mesencephalon
Strychinine 0.7 mg/Kg s.i.
mg/100g wet brain
Cerebellum
Strychinine 0.7 mg/Kg s.i.
mg/100g wet brain
GABA
Glut.+Ala.
Glut.Acid
Intact
Pre-convulsive Stage
Convulsive Stage

Fig. 4

paratively smaller quantity of picrotoxin, 1.0 mg/kg of it. However, when convulsion was induced by the drug, the amino acids which had became less in parts of the brain were almost restored to the normal state or rather increased.

As shown in Fig. 4, the almost similar effects were obtained by the subcutaneous injection of 0.7 mg/kg of strychnine which induces always a typical tetanic type of convulsion (Fig. 4).

3. Successive determinations of contents of three amino acids and their fluctuations following subcutaneous injection of a dose of morphine hydrochloride.

   a) The subcutaneous injection of 20 mg/kg morphine hydrochloride to rabbits.

Among the literatures treating morphine and GABA, Miya *et al.* (1958)[22] are of the opinion that the analgetic effect of morphine has a close relationship with the production of GABA in the brain, judging from the fact that the analgetic effect of morphine is increased by glucose, insulin and various metabolites of sugar (α-ketoglutaric acid, pyro-racemic acid and oxaluric acetic acid). Kaneto *et al.* (1959)[9], who found that the glutamic acid content of whole brain in mice following administration of morphine was increased and also that α-ketoglutaric acid intensified the analgetic effect of morphine. Morphine is well known for its lowering effect of body temperature.

It is also a well known fact that the sedative effects, depressive respiratory effects and the other depressive effects in central nervous system would be caused

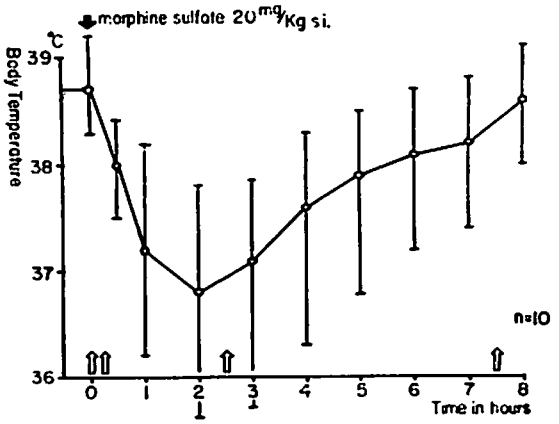

Fig. 5 The hypothermia by the subcutaneous injection of morphine hydrochloride (20 mg/kg) in rabbits. Ordinate is changes of body (rectal) temperature. Absciss is time in hours. Vertical blacketted lines represent mean values, max. and min. values of ten rabbits. White arrows show the time of sacrificing the animals.

by the administration of morphine in man and animal.

To begin with, in normal ambient temperature, after the subcutaneous injection of 20 mg/kg morphine hydrochloride nine rabbits were investigated to see how rectal temperature decreased. After the injection, the fall of body temperature occured in rabbits and the highest fall was seen in two or three hours after the injection, and then it recovered gradually and returned to its normal temperature in eight hours or so (Fig. 5).

These results showed that the nervous centers, which was effective to lower the body temperature of rabbits, were intensively depressed in less than two hours after the injection by the dose of morphine. After that depressive action elicited by this drug was gradually ceased and in more than eight hours or so it vanished almost entirely.

Rabbits were subcutaneously injected with 20 mg/kg of morphine hydrochloride, and after confirming marked fall in the body temperature, the same rabbits received about 1 week later subcutaneous injection of the same dose of morphine hydrochloride. Rabbits were sacrificed after 10 minutes, 150 minutes and 480 minutes respectively and the contents of GABA and other amino acids in each part of brain were estimated.

Successive changes of GABA, glutamic acid and glutamine plus alanine are demonstrated in Fig. 6. Table 4 shows the results of statistics of small samples, using the analysis of variance (Fig. 6 and Table 4).

Table 4

Statistical Analysis of Changes in Adult Rabbits Brain caused by Subcutaneous Injection of 20 mg/kg Morphine hydrochloride

		F	n_1	n_2	P
Cerebrum	GABA	2.05	3	19	> 5%
	Glut. Acid	0.59	3	19	> 5%
	Glut.+Ala.	1.07	3	19	> 5%
Diencephalon (Corpus striat.)	GABA	17.70	3	18	< 0.5%
	Glut. Acid	3.81	3	18	< 5%
	Glut.+Ala.	6.67	3	18	< 1%
Mesencephalon Med. oblongata	GABA	4.46	3	19	< 5%
	Glut. Acid	17.16	3	19	< 0.5%
	Glut.+Ala.	2.92	3	19	> 5%
Cerebellum	GABA	6.55	3	19	< 1%
	Glut. Acid	12.04	3	19	< 0.5%
	Glut.+Ala.	2.32	3	19	> 5%

In cerebrum tissue, even when the rabbits received morphine sufficient to produce marked fall in body temperature, no significant change took place at any

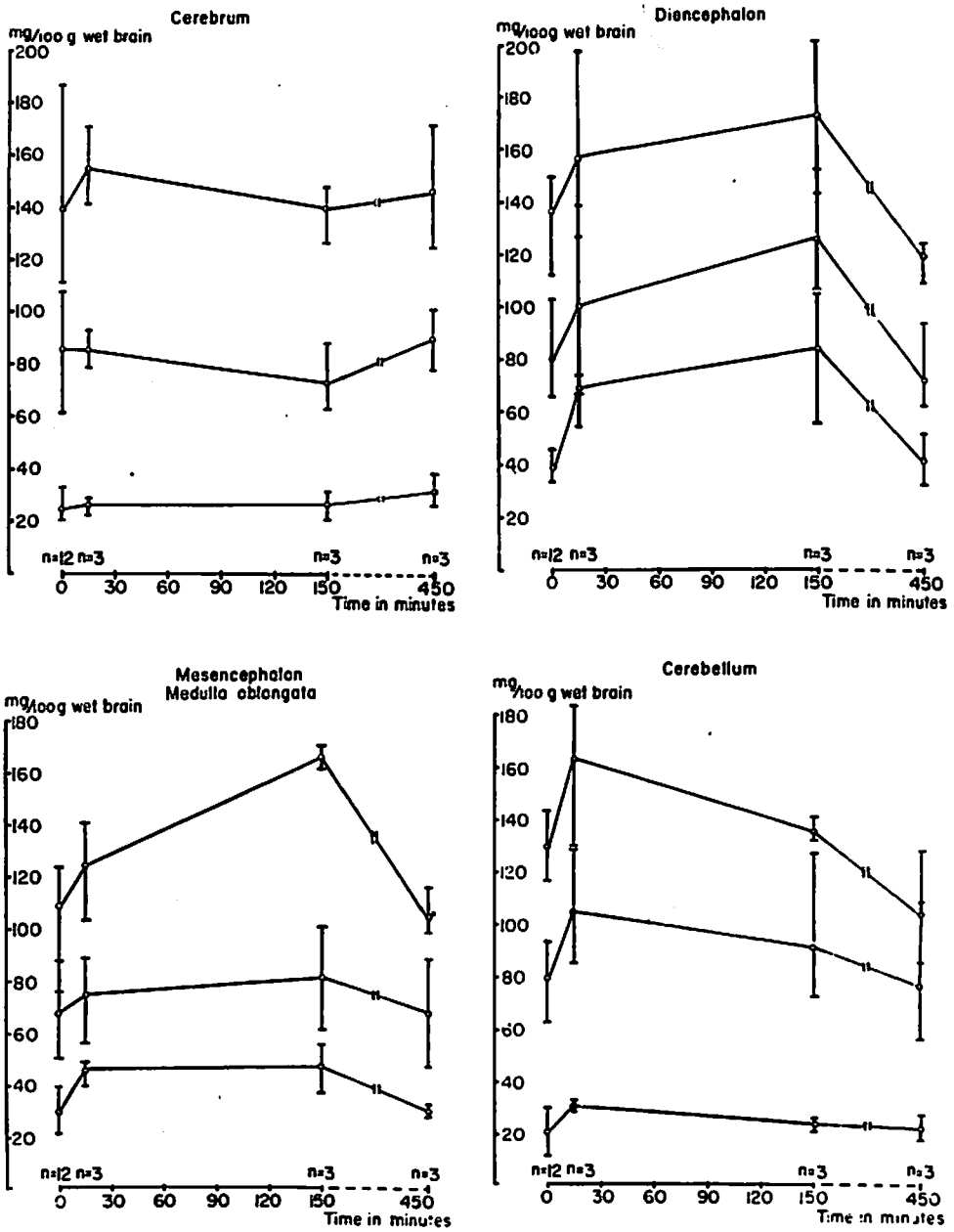

Fig. 6 The changes of the free amino acids in brains after single subcutaneous injection of 20 mg/kg morphine hydrochloride and the comparison with that of normal rabbits. Ordinate is contents of the brain amino acids. Absciss is time in hours. The upper curves show changes of glutamic acid, the middle changes of glutamine+alanine, the lower changes of GABA. n: means each cases of experiments,

time after injection or in any amino acid. In the case of diencephalon all the amino acids showed the tendency to increase significantly after injection, the significance limits being 0.5% for GABA, 5% for glutamic acid and 1% for glutamine plus alanine. Maximal values were obtained 150 minutes after injection in every amino acid, and the restorations of each amino acid content were parallel with the recovery of body temperature. In the case of mesencephalon and medulla both GABA and glutamic acid increased significantly with the significant limits of 5% and 0.5% respectively, while glutamine plus alanine did not increase significantly.

It is interesting that even in the brain of rabbits that were divided anatomically rough, the amino acids tend to increase, except in the tissue of cerebrum, after the administration of 20 mg/kg of morphine hydrochloride.

According to these results, when the nervous centers of rabbits were depressed by relatively larger dose of morphine, the concentration of GA, and GABA in the part of cerebrum was scarcely changed, while an evident increase was seen in the concentration of these amino acids in parts of diencephalon, mesencephalon and cerebellum. Especially in the part of diencephalon the concentration of these amino acids, GABA particularly, showed a remarkable increase.

Drugs	Animals	Site of Injection	Dosis (mg/kg)	Time of Dead After Inj. (m)	Cerebrum				Diencephalon				Mesencephalon Medulla oblong.				Cerebellum			
					G	A	GA B	A	G	A	GA B	A	G	A	GA B	A	G	A	GA B	A
Morphine	Rat	s.c.	20	60	↓	±	±	±	↑	↑	↑	↑	±	↑	↑	↑	±	↑	↑	↑
Barbital	Rat	s.c.	200	90	↑	↑	±	↓	↑	↑	±	↓	↑	↑	±	↓	±	↓	±	↓
Phenobarb.	Rabbit	s.c.	400	125) 150	±	±	↑		±	±	↑		↑	±	↑		±	±	±	
Pentobarb.	Rat	i.p.	40	10	↑	↑	↓		↑	↑	±		↑	↑	±		↑	↑	↓	
Thio-semicarb.	Rat	i.p.	200	60	↑	↑	↓	↓	↑	↑	↓	↓	↑	↑	↓	↓	↑	±	↓	↓
Hydroxy-lamine	Rat	i.p.	15	45	↑	↑	↑	↑	±	±	↑	↓	↑	↑	↑	±	↑	±	↑	±

GA.....Glutamic acid
G .....Glutamine+Alanine
GABA...γ-aminobutyric acid
A .....Aspartic acid
↑ .....increase
↓ .....decrease
± .....unchanged

Fig. 7

b) The subcutaneous injection of 20 mg/kg of morphine hydrochloride to rats.

In the same way as rabbits the concentration of amino acids was investigated in four parts of the rats brain an hour after subcutaneous injection of 20 mg/kg morphine.

As shown in Fig. 7, the concentration of GA in the part of cerebrum only showed a slight decrease and that of G, GABA and A scarcely changed. In the part of diencephalon the concentration of GA, G, GABA and A increase apparently and that of G, GABA and A showed a prominent increase; while in the parts of mesencephalon medulla oblongata and cerebellum the concentration of GA showed little change and an increase was seen in that of G, GABA and A (Fig. 7).

These experimental results showed that the changes of contents of amino acids in several parts of rat brain at the elapses of an hour after the injection of moderate dose of morphine would be almost similar to these of rabbit brain with the exception of a little difference of GA contents of cerebrum between two animals.

c) The subcutaneous injection of 5 mg/kg of morphine hydrochloride to rats.

McIlwain (1955)[23] explained that at concentrations comparable to those causing analgesia, morphine has little effect on various aspects of the metabolism of cerebral and other tissues, and it is, however, at 0.1 mM a potent inhibitor of cholinesterase. Kitao (1960)[24] presented his view that in animal (rabbits) experiment comparatively small quantity of morphine had worked to cause stimulating action just like megimide.

Then, amino acids in parts of the brain of rats were investigated; three cases which elepsed ten minutes after subcutaneous injection of 5 mg/kg of morphine hydrochloride, four cases which elapsed half an hour after the injection and four which elapsed eighty minutes after the injection.

As shown in Fig. 8, in cases of relatively smaller dose of morphine the concentration of GA and G in parts of the brain showed maximum of lowering ten minutes after the injection, and after then gradually recovering, it returned nearly to the normal state in eighty minutes after the injection, but in the part of diencephalon the concentration of GA, being slow in recovery, regained only 66% of lowering at that time.

As for GABA and A, the concentration showed maximum of lowering ten minutes after the injection in the parts of cerebrum, mesencephalon, medulla oblongata and cerebellum and in the part of diencephalon half an hour after the injection, and after then it gradually recovered. But the concentration of all

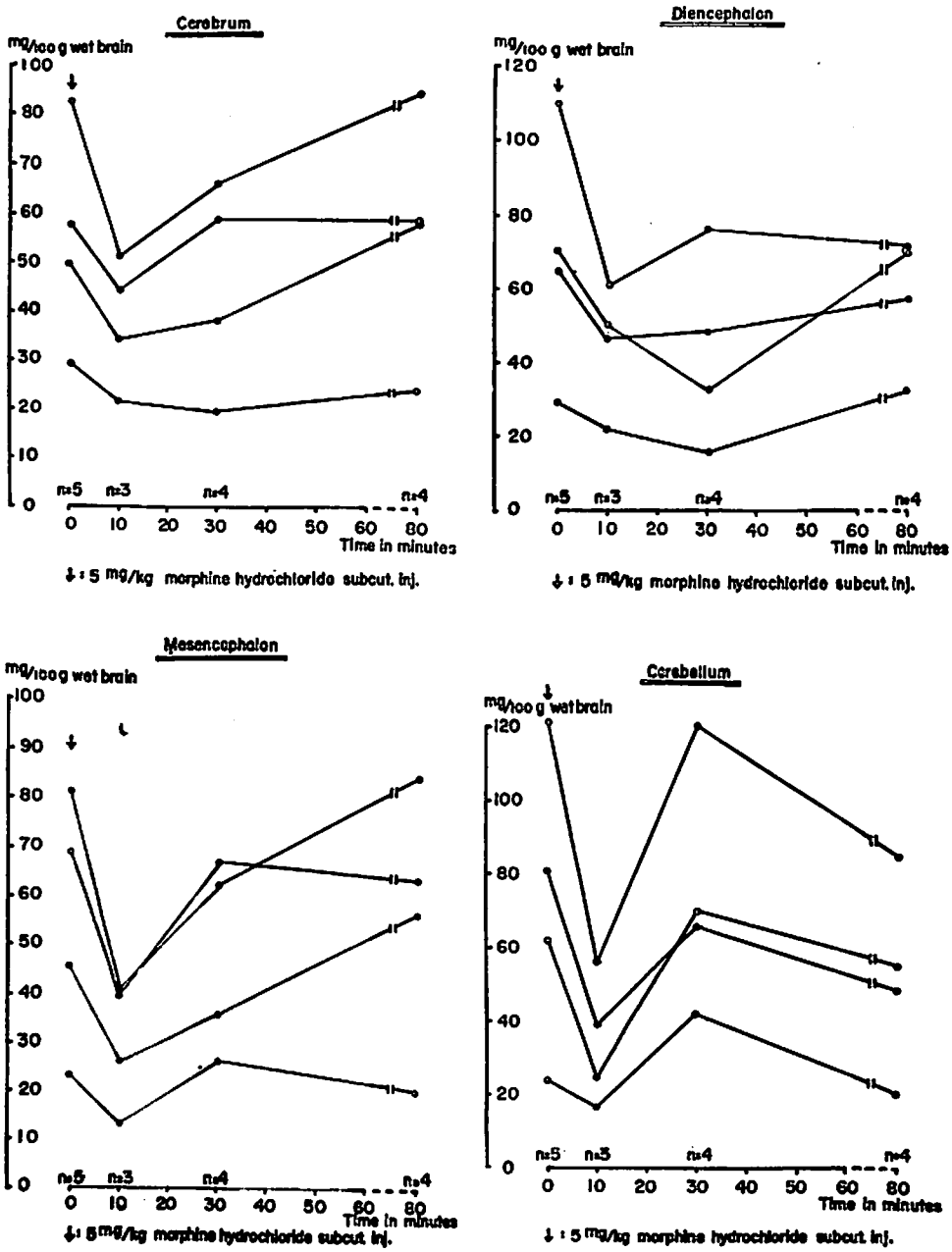
Cerebrum
mg/100 g wet brain
100
80
70
60
50
40
30
20
10
0
n=5 n=3 n=4 n=4
0 10 20 30 40 50 60 80
Time in minutes
↓ = 5 mg/kg morphine hydrochloride subcut. inj.
Diencephalon
mg/100 g wet brain
120
100
80
60
40
20
0
n=5 n=3 n=4 n=4
0 10 20 30 40 50 60 80
Time in minutes
↓ = 5 mg/kg morphine hydrochloride subcut. inj.
Mesencephalon
mg/100 g wet brain
100
90
80
70
60
50
40
30
20
10
0
n=5 n=3 n=4 n=4
0 10 20 30 40 50 60 80
Time in minutes
↓ = 5 mg/kg morphine hydrochloride subcut. inj.
Cerebellum
mg/100 g wet brain
120
100
80
60
40
20
0
n=5 n=3 n=4 n=4
0 10 20 30 40 50 60 80
Time in minutes
↓ = 5 mg/kg morphine hydrochloride subcut. inj.

Fig. 8

amino acids in the part of cerebellum and that of GABA and A in the part of mesencephalon, medulla oblongata lowered more greatly eighty minutes after the injection than half an hour after it (Fig. 8).

It became clear that the amino acids in most parts of the brain had been lowered by relatively smaller dose of morphine, but this fact was apparently quite contrary to the results presented when relatively larger dose of morphine was subcutaneously injected to rabbits or rats.

4. Determinations of the amino acids and fluctuations following subcutaneous or intraperitoneal injection of barbiturates to rabbits and rats.

There are no references concerning to the changes of several amino acids in partial brain of animal by the administration of various barbiturates, but there are a few in whole brain; *i.e.*, no significant change of GABA content in mice brain by the subcutaneous injection of barbital sodium (Hisada (1955)[25]), a decrease of GA content and an increase of A content in rabbit brain by the subcutaneous injection of methylhexabital (Suzuki (1955)[26]), a decrease of GA and an increase of A, alanine and GABA in rat brain by the intraperitoneal injection of thiopetone (Dawson (1951)[5], (1953)[27] and Ansell *et al.* (1954)[4]), a decrease of GA, A and an increase of G in rat brain by the subcutaneous injection of diphenylhydantoin (Woodbury *et al.* (1957)[6]).

a) The subcutaneous injection of 0.2 g/kg barbital sodium.

The concentration of amino acids in parts of the brain in four groups of rats was investigated ninety minutes after the subcutaneous injection of 0.2 g/kg of barbital sodium.

As shown in Fig. 7, the concentration of GA increased in all parts of the brain except in the part of cerebellum, showing particularly an evident increase in the parts of diencephalon, and cerebrum, and that of G increased slightly in the parts of cerebrum, diencephalon and mesencephalon, but decreased in cerebellum, and that of GABA showed little change in all parts, and that of A apparently decreased in all parts of the brains (Fig. 7).

b) The subcutaneous injection of 0.4 g/kg phenobarbital sodium.

On investigating the amino acids in four parts of the brain ninety minutes after the subcutaneous injection of 0.4 g/kg of phenobarbital sodium to rabbits, it was seen that, as shown Fig. 7, the concentration of GA scarcely altered in three parts excepting mesencephalon where it increased a little and that of G showed slight increase only in cerebellum while that of GABA increased evidently in three parts except cerebellum, and in the parts of diencephalon and mesencephalon especially large increase was seen (Fig. 7).

c) The intraperitoneal injection of 40 mg/kg pentobarbital sodium.

On investigating the amino acids in every part of the brain ten minutes after the injection of 40 mg/kg of pentobarbital sodium to four groups of rats, it was known that the concentration of GA and G apparently increased in all parts of the brain, and that of GABA caused no significant change in diencephalon and mesencephalon though in cerebrum and cerebellum some decrease occured. Not including cerebellum other parts showed a slight increase of A (Fig. 7).

Above mentioned results showed that on measurement of amino acids in each part of the brain a definite time after in rabbits or rats were administered the barbiturates of three different spieces, concentration of GA increased under administration of two kinds of barbiturates except phenobarbiturate, and there was an evident increase in case of pentobarbiturate being short acting. On the other hand, the concentration of GABA increased under the administration of phenobarbiturate only which was long acting and in case of pentobarbiturate even decrease was seen.

5. The fluctuation of amino acids in the brain when picrotoxin-convulsions were prevented by a certain dose of phenobarbital administered beforehand.

Nonexistence of convulsion was ascertained in rabbits in case of subcutaneous injection of 3 mg/kg of picrotoxin seventy minutes after the subcutaneous injection of 0.4 g/kg of phenobarbital. Another experiments, being made on estimation of amino acids in four parts of rabbit brain were conducted in a preparatory stage for convulsion twenty minutes after the injection of the same dose of picrotoxin.

As shown in Fig. 9, comparison was made among three experimental results, that is, among cases of intact rabbits, cases in rabbits of administration of picrotoxin only and cases in rabbits of administration of phenobarbital followed to pircotoxin. As for the concentration of GA, all of these cases did not show any evident changes in the part of cerebrum, and in parts of diencephalon and mesencephalon the second case (of picrotoxin) and the third one (of phenobarbital plus picrotoxin) showed apparently more decrease than the first one. On drawing a parallel between the second case and the third, the former showed 50% decrease of the concentration of GA, and the latter showed less decrease of only 30%. In the part of cerebellum this kind of relation was not observed, through a decrease of the concentration of GA was able to be recognized.

In regards to the concentration of GABA, similar relation to GA was seen. But in the concentration of G there was no such relation that had been observed in GA and GABA, though it was not clear whether this fact was owning to the compound, glutamine plus alanine, mentioned before (Fig. 9).

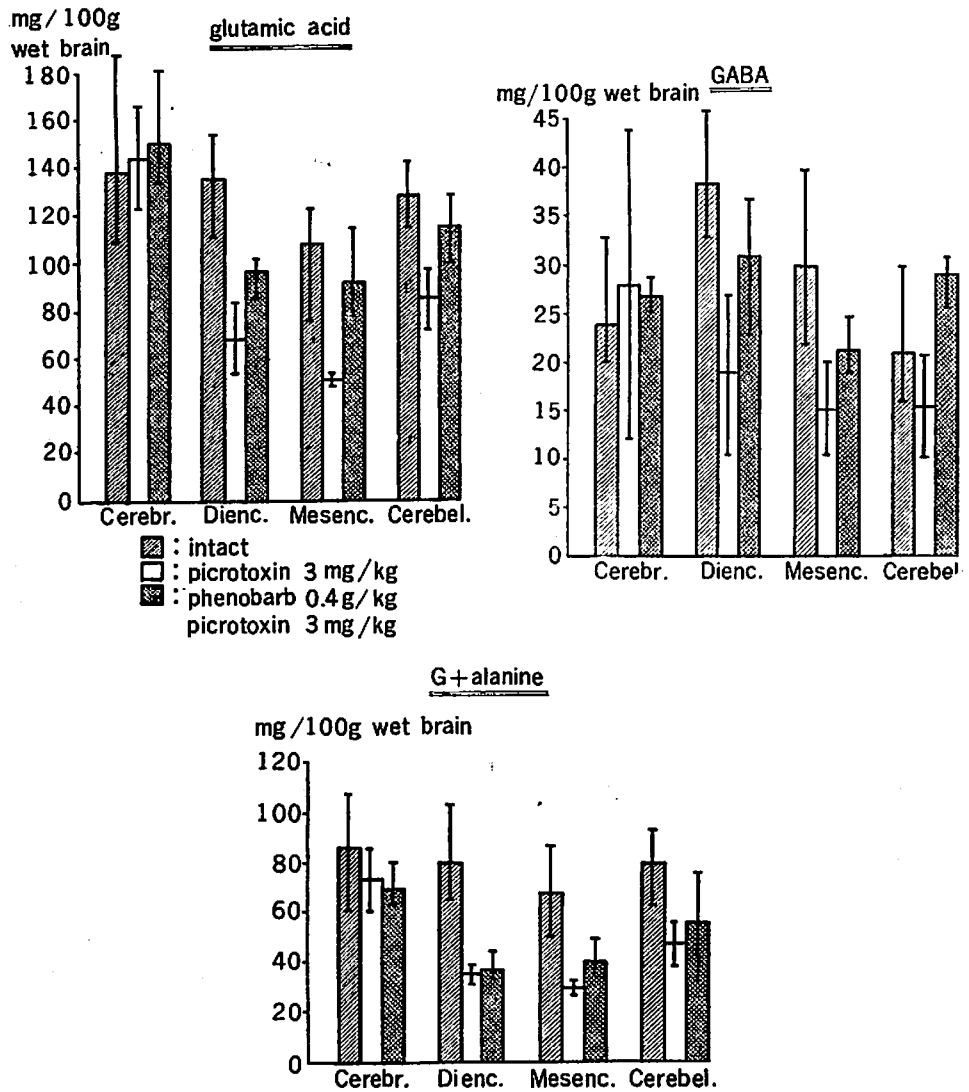

Fig. 9

These experimental results indicated that just prior to the stadium which would elicit convulsion by a definite dose of picrotoxin, the concentration of GA and GABA induced a decrease of 50% or more in the parts of diencephalon and mesencephalon and when the picrotoxin convulsion would be prevented by a definite dose of phenobarbital GA and GABA contents in the two parts decreased in the smaller quantity than when picrotoxin convulsion was evoked. These phenomena possibly suggests that there is some correlation between convulsion caused by picrotoxin and GABA contents in brain.

6. The intraperitoneal injection of 200 mg/kg of thiosemicarbazide.

Killam and Bain (1957)[2] made following experiment: after intraperitoneal injection of 200 mg/kg of thiosemicarbazide, rats were decapitated, and then taking away the cortex of cerebrum and cerebellum and grossly removing white substance, these samples were weighted and free amino acids were analysed with paper chromatography. But whether thiosemicarbazide caused convulsion in them was without description. It was stated by them that the experiment resulted in a decrease of GABA only and the concentration of GA, G and A scarcely altered.

Then same experiment was tried, when rats were intraperitoneally injected 200 mg/kg of thiosemicarbazide, they were in passive position with polypnea until seventy minutes after the injection and more intensifying polypnea they were in excited condition from ten to twenty minutes after the injection. Approximately thirty minutes after the injection some showed tremor in face, and approximately fifty minutes after tremor extended to the whole body but clear clonic convulsion was not yet.

Amino acids in four parts of the brain were investigated an hour after the injection in four groups of rats, which were injected intraperitoneally the same dose of thiosemicarbazide and caused no clonic convultion, but only excited symptomes. The concentration of GA showed eminent increase in all parts, and that of G increased apparently in three parts except cerebellum, while that of GABA and A decreased in all parts (Fig. 7).

Killam and others (1957)[2] said that a decrease of the concentration of GABA was owing to the fact that thiosemicarbazide had blocked vitamin B_6, co-enzyme of glutamic acid decarboxylase, and consequently glutamic acid decarboxylase was inhibited.

Data of experiments suggested that an increase of the concentration of GA and G would have originated in blocking pathway to GABA in consequence of inhibition for glutamic acid decarboxylase. Since Killam and others inquired merely into the whole brain as mentioned before, difference between Killam's experimental results and ours concerning the concntration of GA should have resulted from lack of his investigation in parts of the brain.

7. The intraperitoneal injection of hydroxylamine.

Baxter *et al.* (1960)[28] reported that hydroxylamine would be an inhibitor of GABA-transaminase in the brain, and after intraperitoneal injection of 4.8 mg /kg and 12 mg/kg hydroxylamine to rats, in four parts of the brain, that is, diencephalon, colliculi, olfactory lobes, cortex and cerebellum, the concentration of GABA was investigated, which showed apparent increase in all parts.

Then, amino acids were measured in each part of the brain forty five minutes after intraperitoneal injection of 15 mg/kg hydroxylamine. In the part of cerebrum the concentration of not only GABA but also GA, G and A caused an increase, but in diencephalon that of GABA showed imperceptible increase, that of GA and G was unchanging and that of A decreased. In mesencephalon that of GA and G as well as GABA increased and that of A was changeless. Lastly in cerebellum that of GABA showed a slight increase and other three scarcely changed (Fig. 7).

Above mentioned results may be summarized as follows:

1) When excitement and depression by subconvulsive and convulsive dose of picrotoxin, by relatively large quantity of morphine and barbiturates was elicited in the brain, in case of excitement, amino acids had a tendency to decrease in all parts of the brain, and especially in the parts of diencephalon and mesencephalon a decrease of the concentration of GA and GABA was so eminent that as much decrease as 50% was seen before causing convulsion. As for the state of depression, although tendency of amino acids was toward increase, the alternation was not so intense as in the former. These amino acids decreased or increased approximately in accordance with the degree of excitment or depression, and the alternation was varied in direct proportion to the dose of administration.

2) When picrotoxin and strychnine brought about convulsion, amino acids in each part of the brain would cause no change but before eliciting convulsion, that is, in the previous stadium to convulsion they would show the most intensive decrease.

The convulsion induced by these drugs would be always followed by depression and in depressive state the concentration of amino acids which had decreased would tend to increase on the contrary.

3) Though subconvulsive dose of picrotoxin induced an intensive decrease of the concentration of amino acids in cerebrum, in the preparatory stadium of convulsion, little change was observed.

4) When relatively large quantity of morphine was injected, the concentration of amino acids in cerebrum scarcely changed, an evident increase was seen in diencephalon and mesencephalon. In case of relatively small quantity of morphine, however, amino acids in each part of the brain had a tendency to decrease. It will be necessary to testify in future experiments whether decrease of amino acids in the brain, induced by comparatively small quantity of morphine, showed superiority of phenomenum of excitement to that of depression.

5) Barbiturates except pentobarbiturate do not induce an evident change of amino acids in each part of the brain. It is impossible to explain the ways, but

this fact should indicate that localization of side chain in each barbiturate produces a wide difference among appearance of its own pharmacological action, and that barbiturates localize the attack point upon the brain.

6) Killam *et al.* (1957)[2] reported that the appearance of convulsive action elicited by thiosemicarbazide, originated in a decrease of the contents of GABA in the brain, and others agreed to them. However, there is a disparity between this action and the fluctuation of glutamic acid metabolism in the brain in case of administration of picrotoxin. This differences suggests that picrotoxin has a separate attack point from that of semicarbazides, and that picrotoxin attacks the point which is close to the attack point of tricarboxylic acid cycle in the brain (Häkkinen *et al.* (1961)[14]).

GENERAL DISCUSSION

It became evident from the experimental results mentioned above that some excitants and depressants, which acted on the central nervous systems, caused the fluctuation of glutamic acid and the substances of glutamic acid metabolism in every part of the brain corresponding with the appearance of pharmacological action. It, however, will be impossible to affirm basing only on these results that the appearance of this action, that is, brain function of excitement and depression, is chiefly owing to the alternation of amino acids in the brain.

As touched in "Introduction," a definite conclusion is not found in looking over references on these studies, for they present us various results concerning existence of the alternation. In the most studies so far the investigation of contents of amino acids was made in cortex of cerebrum or in the whole brain, being not made in parts of the brain, in order to explain the alternation of amino acids in the brain. This method of study was criticized by Roberts (1958)[3] and Elliot (1959)[10] as being unexact and we also testified that.

Besides, in many references close attention was not paid to the dose, the period and the number of times of the administration when drugs were used in experiments. For instance, De Ropp and others (1961)[11] reported that they used rats as experimental animal and the alternation of the contents in the whole brain was investigated only once after one time administration (except reserpine) of approximately twelve kinds of excitants and depressants. It is the dose of drugs administered that must be discussed now. They administered strychnine at the rate of 4 mg/kg and picrotoxin at the rate of 12 mg/kg. In our experiments subcutaneous injection of 0.7 mg/kg of strychnine nitrate elicited convulsion and as for picrotoxin subcutaneous injection of 3 mg/kg did so. Another question is whether they ascertained the fact that amino acids contents in the brain at a

time when administration of these two medicines had caused convulsion were scarcely changed. Nothing was described about that. We have used 1–3 mg/kg of reserpine* to rats and mice, whereas as much as 16–50 times of the drug was used by them. According to references, 5 mg/kg is the maximum effective dosis of reserpine sedation. Though they detected an increase of the concentration of G when 100 mg/kg of chlorpromazine was used, in our experiments* one hundredth of the dose was sufficient to observe an increase in the concentration of GA and G (glutamine+alanine) and a decrease in that of GABA and A. Investigating alternation of amino acids in the brain after administration of 10–240 mg/kg of morphine, they recognized an increase of the concentration of GABA, yet how much dose induced an increase is obscure. These results corresponds to our data presented by observation of successive changes in rat brain after subcutaneous injection of 5 mg/kg of the drug.

We do not always point our mistakes in all drugs used by De Ropp and others. However, the dose of administration should become the first subject of discussion, and then it must be considered that their investigation of amino acids which made only once in the whole brain might produce insufficient results.

On the other hand, Häkkinen and Kulonen (1961)[14] observed the fluctuation in the concentration of amino acids in the course of successive investigation in the whole brain of rats which received oral application of a large quantity of ethanol. Their reports were closely inquired in experiments and were founded on statistics calculation of precision. They found that during the period of ethanol intoxication an increase was observed in the content of γ-aminobutyric acid, glutamic acid and aspartic acid in the brain and a decrease in glutamine. These changes closely resemble our experimental findings with various depressants and excitants, reported above, except as regards glutamine. However in our data glutamine+alanine were measured instead of only glutamine.

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