Chapter 3

Results

1. Amino Acid Analysis : Preparation of Standard Curve

A typical high performance liquid chromatogram of OPA-derivatives of a standard mixture of 8 selected amino acids (conc. 400 pmol each) : aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Glun), glycine (Gly), taurine (Tau), alanine (Ala) and gamma-aminobutyric acid (GABA); is shown in Fig. 7. The complete elution of the amino acids was achieved within about 20 min. The area of each peak in the chromatogram were directly proportional to the amount of the OPA-derivatives of each standard with linearity realiable in the range 40 pmol to 1600 pmol amino acid content (Fig. 8). Table 1. expressed the coefficient of variation (C.V.) of the peak area of each amino acid.

2. Perfusion Experiments

The amount of amino acids liberated into perfusate solution was strongly dependent on the location of the tip of the push-pull cannula. Fig. 9 shows an example of chromatogram of perfusate obtained from a successfull experiment, whose histological section shown in Fig. 10. The histology revealed a scar from tissue damage caused by the cannula tip to be

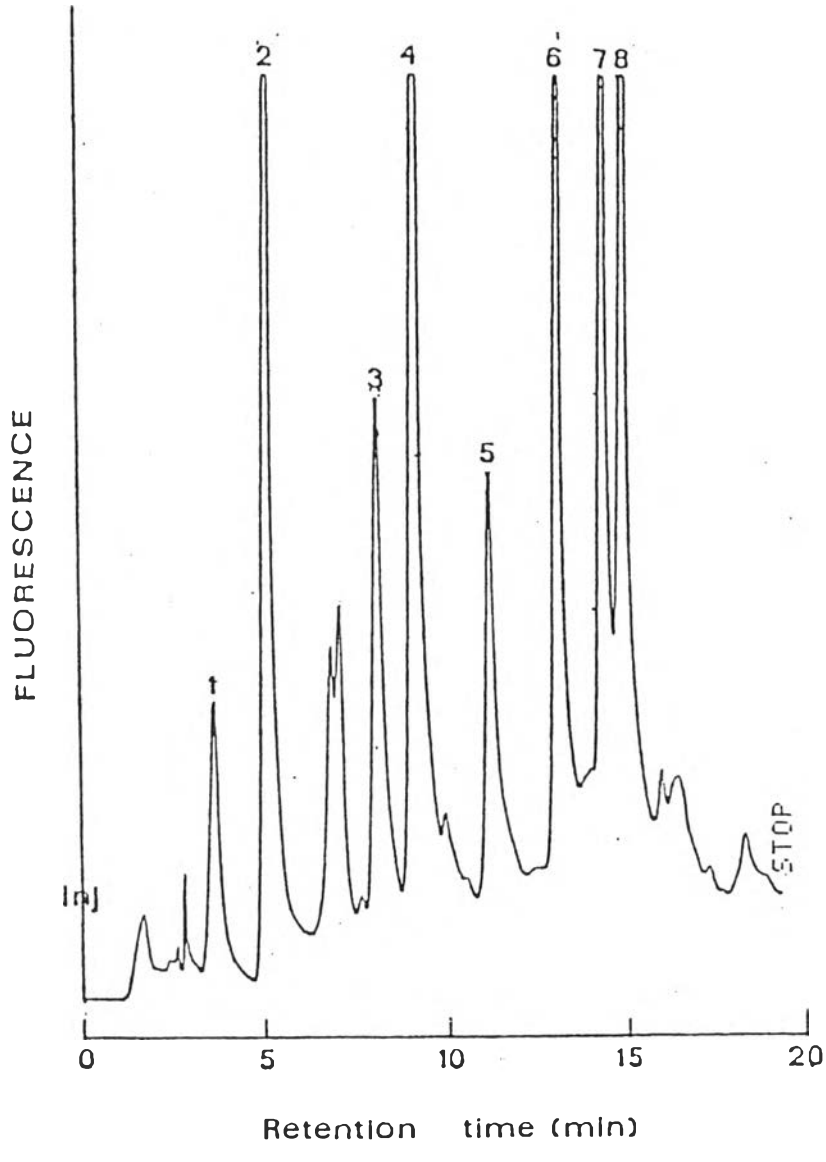


Figure 7. High-performance liquid chromatogram of a standard mixture of amino acids (Ca 400 pmol of each). Peaks : 1 = aspartic acid ; 2 = glutamic acid ; 3 = serine ; 4 = glutamine ; 5 = glycine ; 6 = taurine ; 7 = alanine ; 8 = GABA

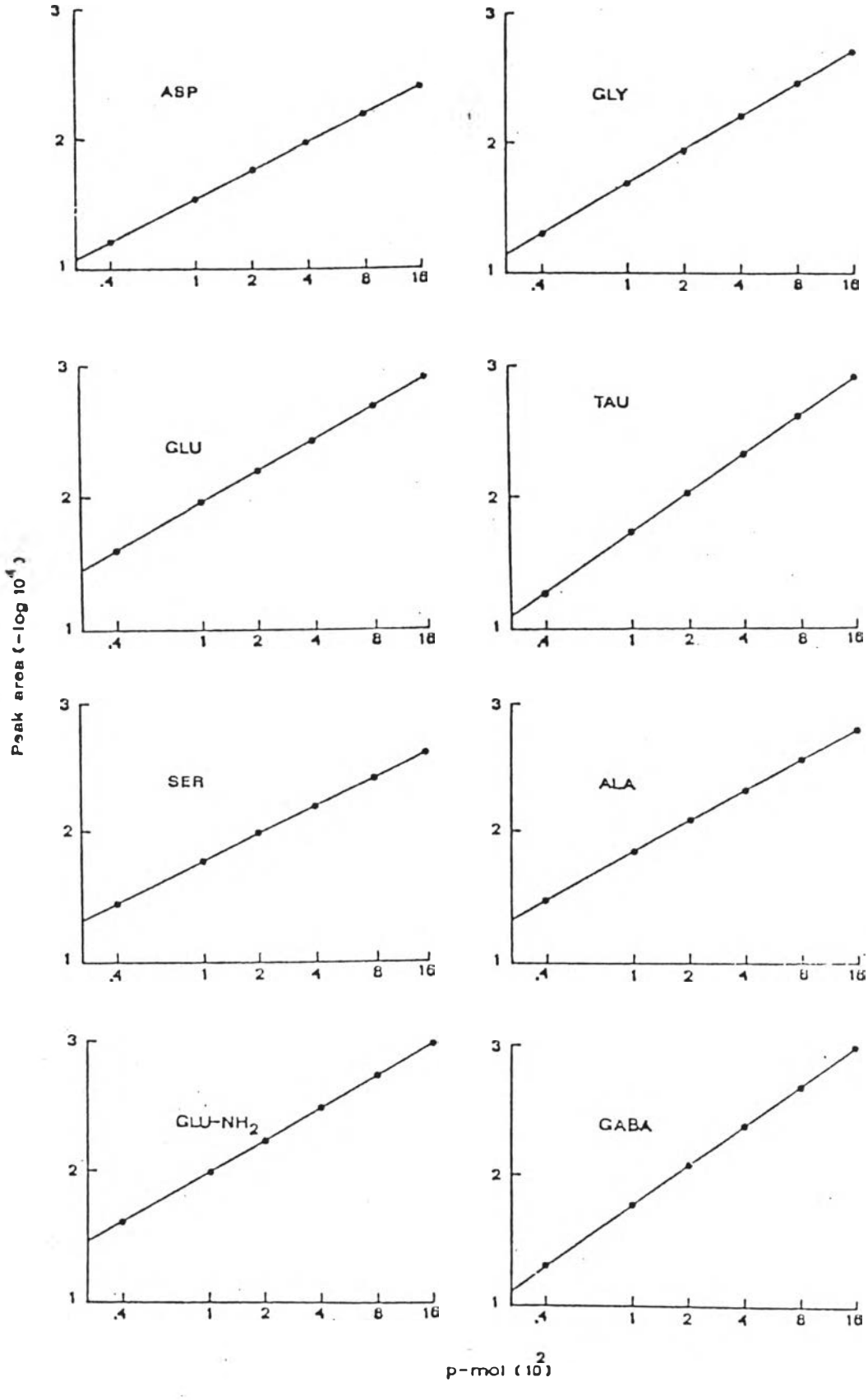
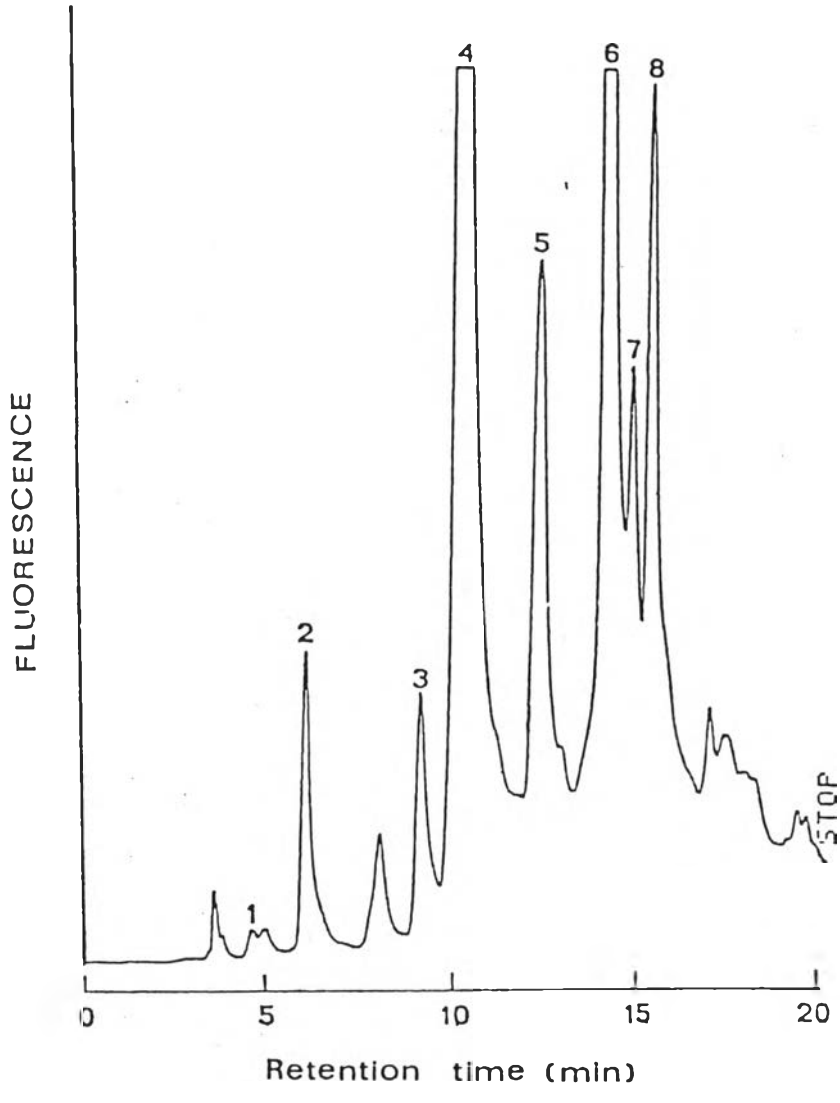


Figure 8. Standard curve of amino acids measurement. Peak area refers to area under each of amino acids peak.

Compound	C.V. (%)
Aspartic acid	3.45
Glutamic acid	9.04
Serine	6.21
Glutamine	7.50
Glycine	10.12
Taurine	10.67
Alanine	9.85
GABA	6.14

Table 1. Coefficient of variation (C.V.) of the peak area. Concentration of each compound is 400 p-mole ; number of determination = 4.



1.	aspartic acid	=	18	pmol
2.	glutamic acid	=	43	pmol
3.	serine	=	60	pmol
4.	glutamine	=	1080	pmol
5.	glycine	=	680	pmol
6.	taurine	=	1224	pmol
7.	alanine	=	138	pmol
8.	GABA	=	160	pmol

Figure 9. Sample chromatogram of the superfusate vestibular nucleus. Showing separation of 8 amino acids in the sample. Amount of amino acids shown under the chromatogram were referenced from standard curve.

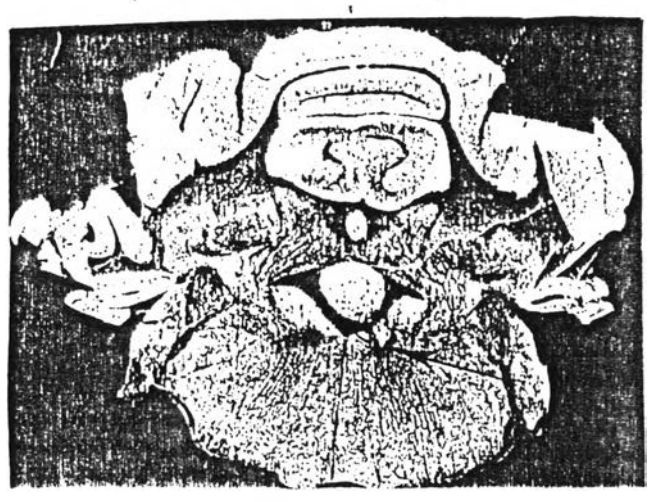


Figure 10. Coronal sections of rat's brain through plane -3.6 (according to atlas by Pellegrino, L.J. et al., 1979) from a successful experiment. Showing the scar caused by the cannula tip.

located within the limit of the vestibular muclei complex. The chromatogram obtained from an incorrect location of the cannula is also shown in Fig. 11, which suggested much less quantities of the amino acid released. Fig. 12 show a histological section with the tip of cannula located outer the vestibular nuclei.

3. Spontaneous Release of Endogenous Amino Acids

When the vestibular nuclei was continuously superfused with artificial CSF, the amount of amino acids release was detected 30 min after the onset of superfusion through the end of a 100 min collection period. The first 30 min of superfusion was discarded and considered as a washout materials from tissue damage. The pattern of spontaneous endogenous amino acids release is shown in Fig. 13 and Table 2. The release of each amino acid did not change significantly throughout the experiment. The amino acids detected in this experiment, as identified by the corresponding peak numbers in the chromatogram were Asp, Glu, Ser, Glun, Gly, Tau, Ala and GABA.

4. Effect of High K^+ Concentration on Amino Acids Release

In order to evoke the release of the endogenous amino acids, experiments were carried out by perfusion with artificial CSF containing high K^+ concentration, either 50 mM or 100 mM. After sample No. 3 of baseline

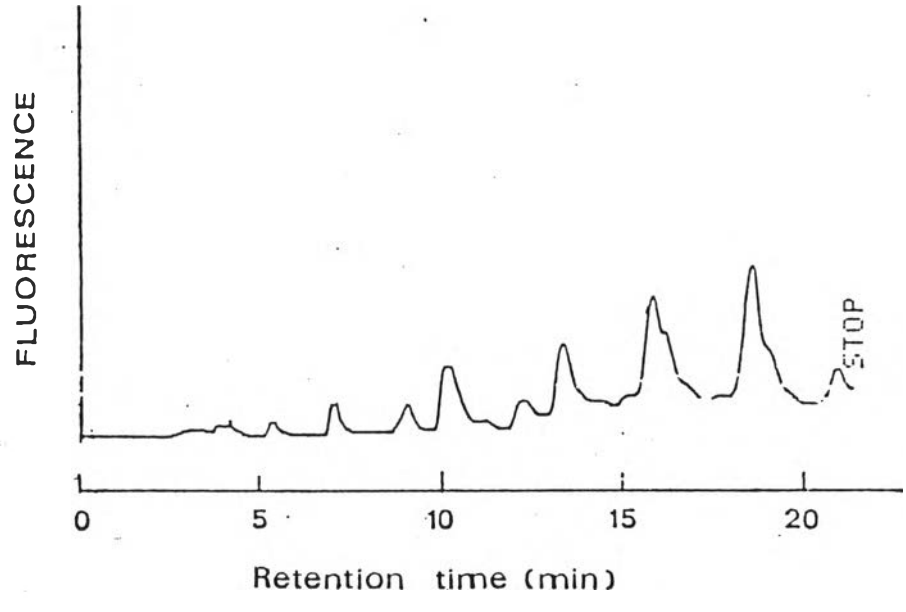


Figure 11. Chromatogram of the perfusate sample from incorrect placement of the push-pull cannula.

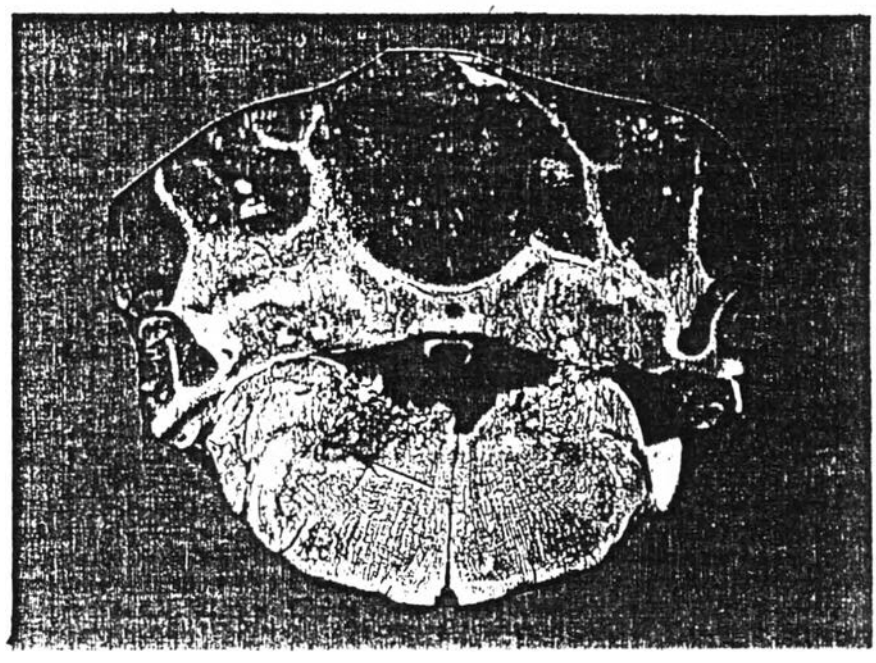
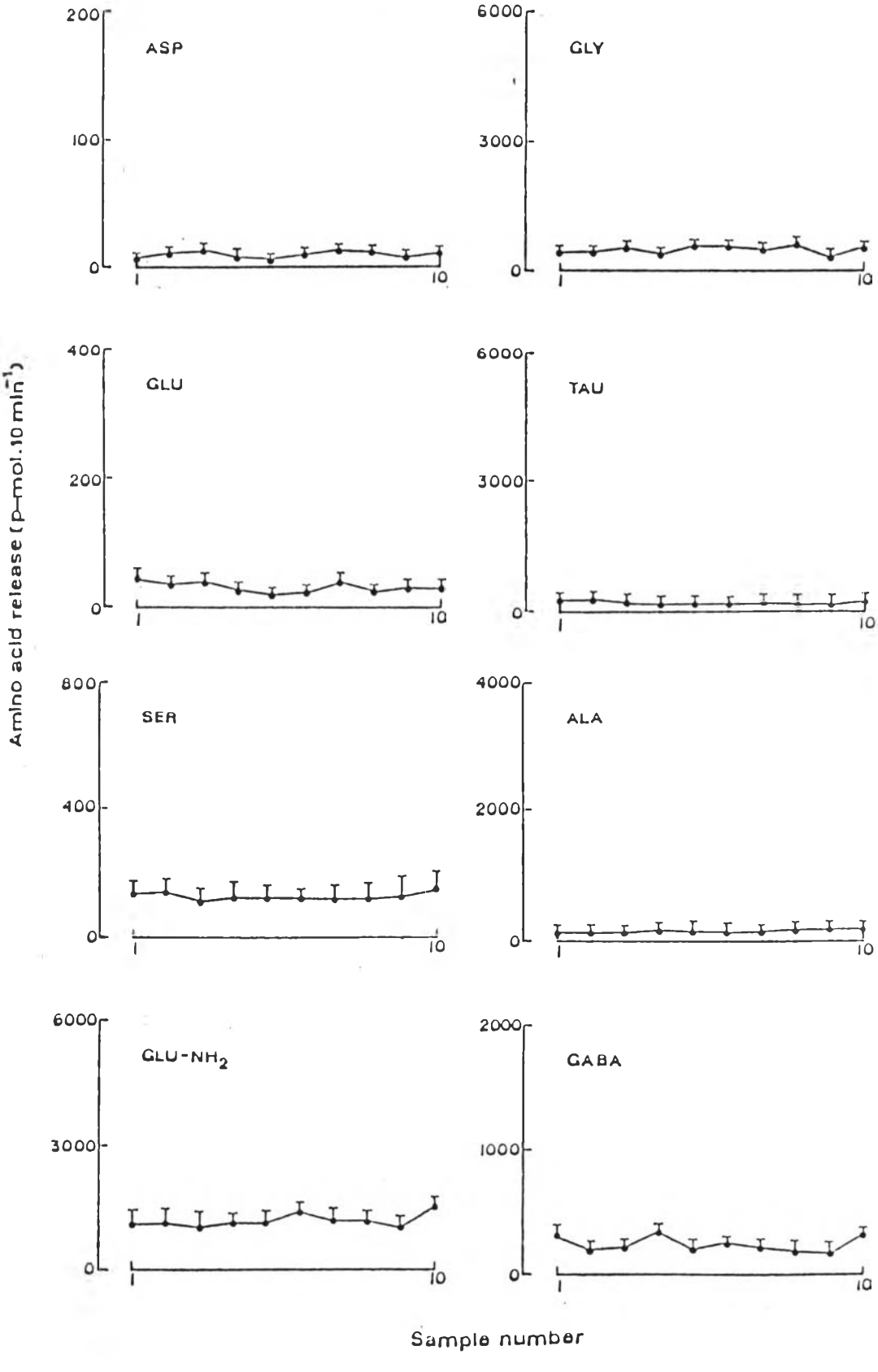


Figure 12. Coronal section of the rat brain which the cannula tip located outside of the vestibular nuclei, at the arrow.

Figure 13. The spontaneous release of aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Glu-NH_2), glycine (Gly), taurine (Tau), alanine (Ala), and γ-aminobutyric acid (GABA) from the perfusate of the rat vestibular nucleus. The horizontal scale shows number of superfusate sample. Vertical axis shows the amount of amino acids released in pmol per 10 min. Results are the mean and S.E.M. of data obtained in 5 experiments.


ASP
GLY
GLU
TAU
SER
ALA
GLU-NH2
GABA
Amino acid release (p-mol.10 mln-1)
Sample number

amino acid \ sample number	1	2	3	4	5	6	7	8	9	10
Asp $\hat{a}$	7±2	11±3	13±4	9±3	6±1	10±2	13±4	12±3	8±2	11±4
Glu $\hat{a}$	46±12	36±10	40±9	28±7	21±5	23±7	35±12	24±6	32±4	29±5
Ser	140±36	142±36	112±37	120±41	132±27	125±24	112±40	121±47	127±54	148±54
Glun	1159±364	1196±371	1078±450	1165±267	1161±285	1446±228	1272±306	1297±245	1016±209	1558±211
Gly	494±70	483±59	514±78	410±78	593±77	589±76	507±39	677±31	326±88	510±52
Tau	220±44	220±67	186±46	149±34	154±49	132±24	206±58	176±69	181±54	280±97
Ala	118±20	116±44	113±23	160±16	148±13	127±11	114±23	142±27	148±38	150±39
GABA	303±63	202±44	224±17	333±20	209±45	245±32	236±23	213±29	205±52	307±49

Table 2 The spontaneous release of amino acids from superfused vestibular nucleus.
The value in the Table represent the mean total p-mol of amino acids release per minute + S.E. of mean. n = number of observation = 5

to high K^+ artificial CSF. When vestibular nuclei was superfused with solution containing a high K^+ (100 mM) a significant increase of all amino acids were produced, while high K^+ (50 mM) failed to show any significant evoked increase of any of the amino acids. The results are presented in Fig. 14 and Table 3, 4.

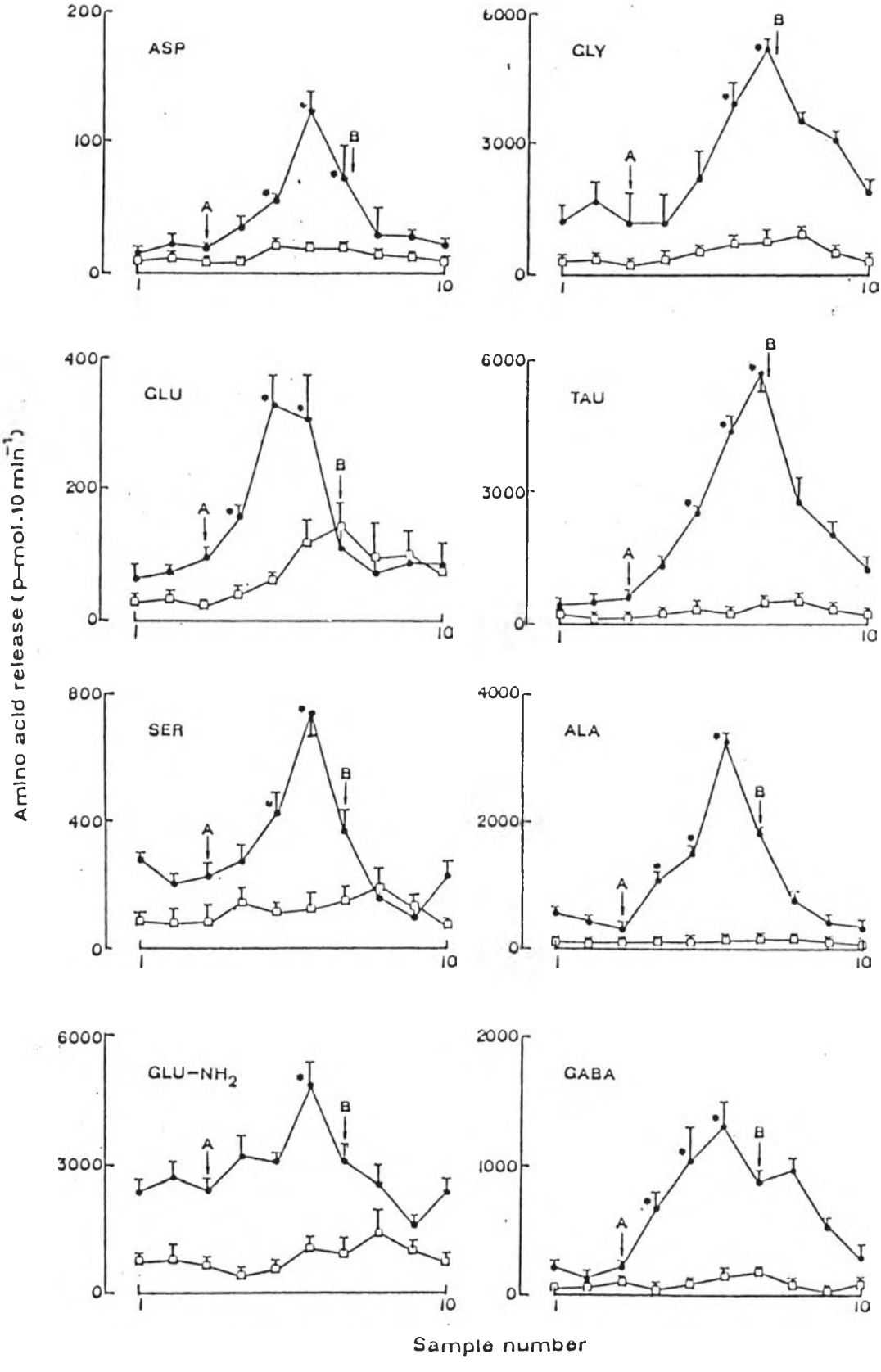
5. Effect of Ca^{2+} on Amino Acid Release

Futher experiment for the calcium-dependent of the release mechanism was obtained by depolarizing vestibular nuclei with high K^+ (100 mM)-Ca^{2+} free solution containing 0.5 mM EDTA. The time course of sample collection was divided into three phase. After three control sample collection, the perfusion medium was changed to the high K^+ (100 mM)-Ca^{2+} free solution. In the final phase perfusing fluid was replaced with control artificial CSF. In such conditions, stimulation with high K^+-Ca^{2+} free failed to evoked release of all amino acids as compared to another high K^+-Ca^{2+} rich solution. (Fig. 15 and Table 4, 5)

6. Effect of Electrical Stimulation on Amino Acids Release

A series of nine experiment were carried out in order to induce release of the endogenous amino acids from vestibular nerve terminal by electrical stimulation. After superfused vestibular nuclei with control CSF for 30 min the ipsilateral vestibular nerve was electrically stimulated for 10 min (duration 0.1

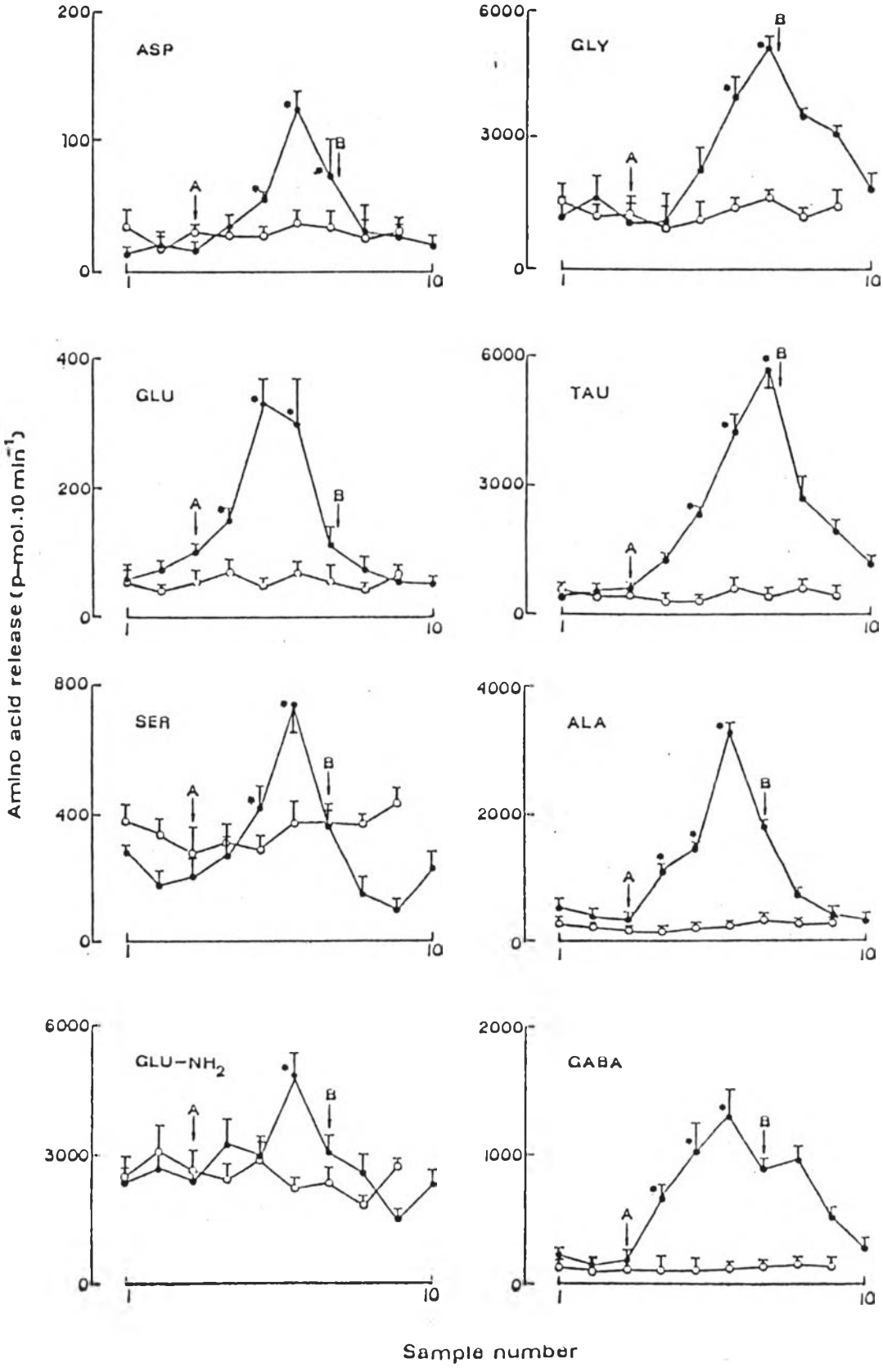
Figure 14. Effect of high concentration of K^+ (50 mM, square symbol ; 100 mM, filled symbols) on the release of endogenous amino acids from the rat vestibular nuclei. The initial superfusion solution was the control artificial CSF, after sample 3 was collected the solution was changed to high K^+ -50 mM and high K^+ -100 mM of each experiment (A). After sample 6 was collected the solution was changed back to control artificial CSF (B). An asterisk symbol adjacent to a point indicates a significant difference from the baseline (student's t-test, $P < 0.05$)


ASP
GLY
GLU
TAU
SER
ALA
GLU-NH2
GABA
A
B
Amino acid release (p-mol . 10 min-1)
Sample number

	control			50 mM.- K^+			control			
sample number / amino acid	1	2	3	4	5	6	7	8	9	10
Asp â	11±3	12±3	8.8±1.5	8.3±1.4	22.7±3	19.5±1	20.1±4	16.3±1.5	13.5±1.6	10.5±1.6
Glu â	28±8	33±9	24±3	44±11	61±9	117±34	143±36	99±49	97±39	78±37
Ser	84±24	82±38	83±43	142±37	116±21	118±44	149±33	188±51	129±30	69±11
Glun	709±143	772±294	648±184	445±128	557±153	1086±206	991±280	1398±475	1068±125	718±83
Gly	364±149	378±122	294±77	381±154	588±58	715±140	751±239	901±161	495±122	301±83
Tau	216±80	164±49	142±26	238±54	343±81	282±80	498±61	513±80	368±43	224±29
Ala	53±20	48±19	40±20	54±21	124±25	117±71	172±26	130±22	85±15	35±15
GABA	44±3	35±5	93±3	41±7	85±15	148±40	174±25	82±30	24±19	71±12

Table 3 The levels of amino acids release from the vestibular nucleus by stimulated with high K^+ 50 mM. The value in the Table represent the mean total p-mol of amino acid release per minute ± S.E. of mean. n = number of observation = 5

Figure 15. Effect of high concentration of K^+ (100 mM) with Ca^{2+}-dependent (filled symbols) and K^+ (100 mM) with Ca^{2+}-free (open symbols) on the release of endogenous amino acids from the rat vestibular nucleus. The initial superfusion solution was control artificial CSF, after sample 3 was collected the solution is changed to high K^+-Ca^{2+} dependent and high K^+-Ca^{2+} free of each experiment (A). After sample 6 was collected the solution was changed to control artificial CSF (B). An asterisk symbol adjacent to a point indicates a significant difference (student's t-test, $P < 0.05$)


ASP
GLY
GLU
TAU
SER
ALA
GLU-NH2
GABA
A
B
1
10
Amino acid release (p-mol.10 min-1)
Sample number

	control			100 mM - K^+ Ca^{2+}-dependent			control			
sample number / amino acid	1	2	3	4	5	6	7	8	9	10
Asp â	15±3	23±6	19±2	35±7	56±2*	125±13*	73±25	30±18	29±4	22±4
Glu â	64±19	73±9	52±8	150±16*	328±46*	306±72*	110±27	74±13	55±7	53±10
Ser	280±15	180±33	208±46	274±53	427±57*	740±71*	368±60	158±38	101±19	226±47
Glun	2406±227	2702±385	2485±195	3024±514	3092±186	4877±545	3031±480	2602±410	1585±130	2391±248
Gly	1282±380	1688±430	1189±570	1188±650	2209±570	3959±380	5192±178*	3546±89*	3136±94	1889±290
Tau	423±134	501±98	613±54	1331±89*	2482±68*	4303±381*	5750±421*	2748±540	2058±186	1287±185
Ala	568±49	444±59	338±24	1079±33*	1474±43*	3291±52*	1839±69	779±55	416±82	343±52
GABA	229±39	138±53	219±58	667±101*	1037±226*	1306±173*	904±60	981±65	534±55	305±56

Table 4 Evoked release of amino acids from the vestibular nucleus by stimulated with high K^+ 100 mM. The values in the Table represent the mean total p-mol of amino acid release per minute ± S.E. of mean. n = number of observation = 6

* Significant different from that released into the control artificial CSF ($P<0.05$)

	control			100 mM-K^+ Ca^{2+}-free			control		
sample number / amino acid	1	2	3	4	5	6	7	8	9
Asp â	35±10	22±7	30±2	28±4	27±5	37±7	34±11	33±9	32±8
Glu â	66±11	39±4	55±7	79±12	49±9	67±13	57±18	42±9	68±4
Ser	384±45	335±51	270±92	310±65	283±40	367±73	360±45	372±26	435±43
Glun	2413±494	3042±601	2644±478	2489±467	2906±519	2290±203	2376±312	1857±198	2777±113
Gly	1598±490	1284±286	1284±230	1142±480	1159±444	1454±169	1610±105	1241±137	1457±334
Tau	651±109	448±105	463±126	285±160	291±78	613±158	417±83	683±154	429±176
Ala	288±54	201±55	159±25	139±45	194±28	212±18	334±40	301±58	314±79
GABA	142±68	127±83	121±91	114±97	109±77	118±61	124±57	138±27	147±55

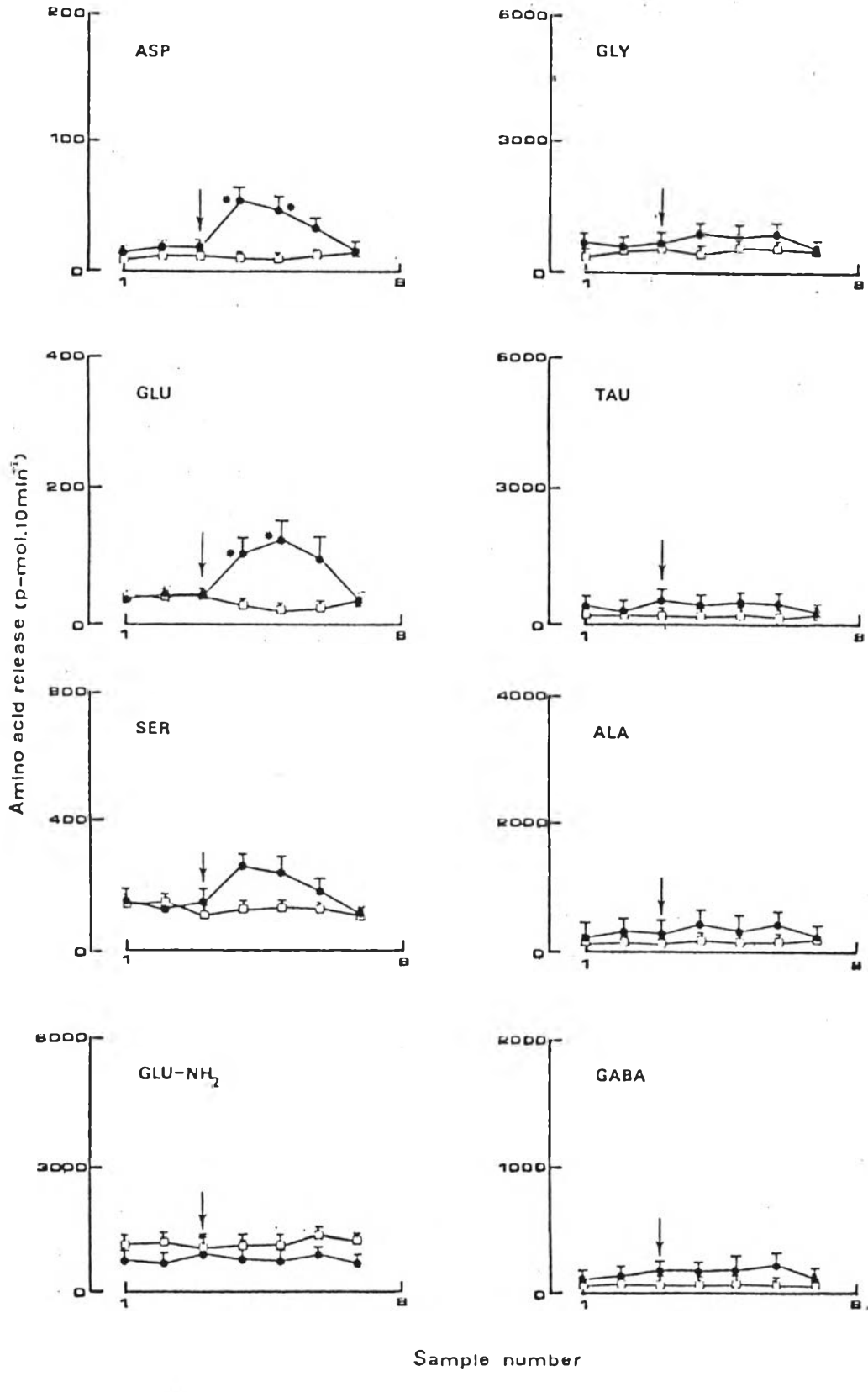
Table 5 The levels of amino acids release from superfused vestibular nucleus with high K^+ 100 mM $-Ca^{2+}$ free solution . The values in the Table represent the mean total p-mol of amino acid release per minute + S.E. of mean. n = number of observation = 3

msec frequency 100 Hz current 1 mA). In a sucessful experiment with correct placement of the stimulating electrode,electrical stimulation produced a significant increase of release of glutamate and aspartate while no significant increase in the efflux of other amino acids was observed. The enhanced release occurred during 3 collected fractions when the nerve was stimulated. A decrease of both amino acids : glutamate and aspartate in the 7th fraction was observed subsequently to the level comparable with the initial 3 control sample. (Fig. 16 and Table 6)

7. The Study of Stimulating Electrode Position

From histological study, identification of electrode position with reference to the brain structure was facilitated by microscopy and photography. Example of the brain section with electrode marking lesion at the vestibular nerve was shown in Fig. 17. Change in glutamate and aspartate release could be detected when the stimulating electrode was placed at the vestibular nerve. The amount of increased release was depend on the location of electrode as shown in Fig. 18. Thus, in some experiments, when the push-pull cannula was well located in the vestibular nuclei but the stimulating electrode was placed near the vestibular nerve, electrical stimulation did not produce enhance amount of the release of either glutamate or aspartate.

Figure 16. Effect of electrical stimulation of vestibular nerve on amino acids release as measured in the superfusate obtained over the experimental periods. The horizontal scale shows number of superfusate sample, each was obtained during 10 min period. Vertical axis shows the amount of amino acids released in pmol per 10 min. Filled symbol shows the release in experimental case, while square symbol represent the control. The position of stimulus indicates as the arrow. An asterisk symbol adjacent to a point indicates a significant difference (student's t-test, $P < 0.05$)


ASP
GLY
GLU
TAU
SER
ALA
GLU-NH2
GABA
Amino acid release (p-mol.10min-1)
Sample number

	control			electrical stimulate		control	
sample number / amino acid	1	2	3	4	5	6	7
Asp a	14±2	17±3	17±2	55±13*	48±11*	31±9	15±3
Glu a	37±4	42±3	42±4	104±13*	126±49*	97±41	33±4
Ser	147±37	125±19	144±33	268±58	243±62	181±46	114±24
Glun	740±216	716±225	910±256	815±196	765±148	917±228	739±172
Gly	682±113	620±131	710±49	917±149	849±219	912±231	589±163
Tau	406±119	274±73	480±144	433±136	475±125	464±179	240±29
Ala	207±63	279±88	259±98	379±160	314±121	369±117	192±68
GABA	101±32	128±35	168±41	160±37	166±42	220±74	104±33

Table 6 Evoked release of amino acids from the vestibular nucleus by electrical stimulation ipsilateral vestibular nurve. The values in Table represent the mean total p-mol of amino acid release per minute ± S.E. of the mean n = number of observation = 9

* Significant different from that evoked released into the artificial CSF ($P < 0.05$)

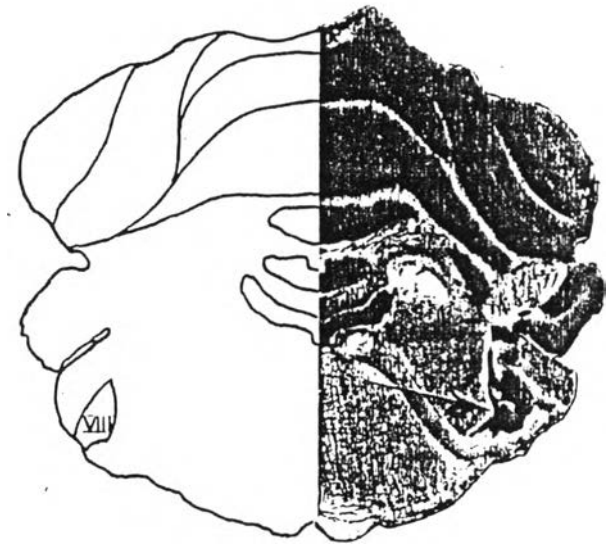
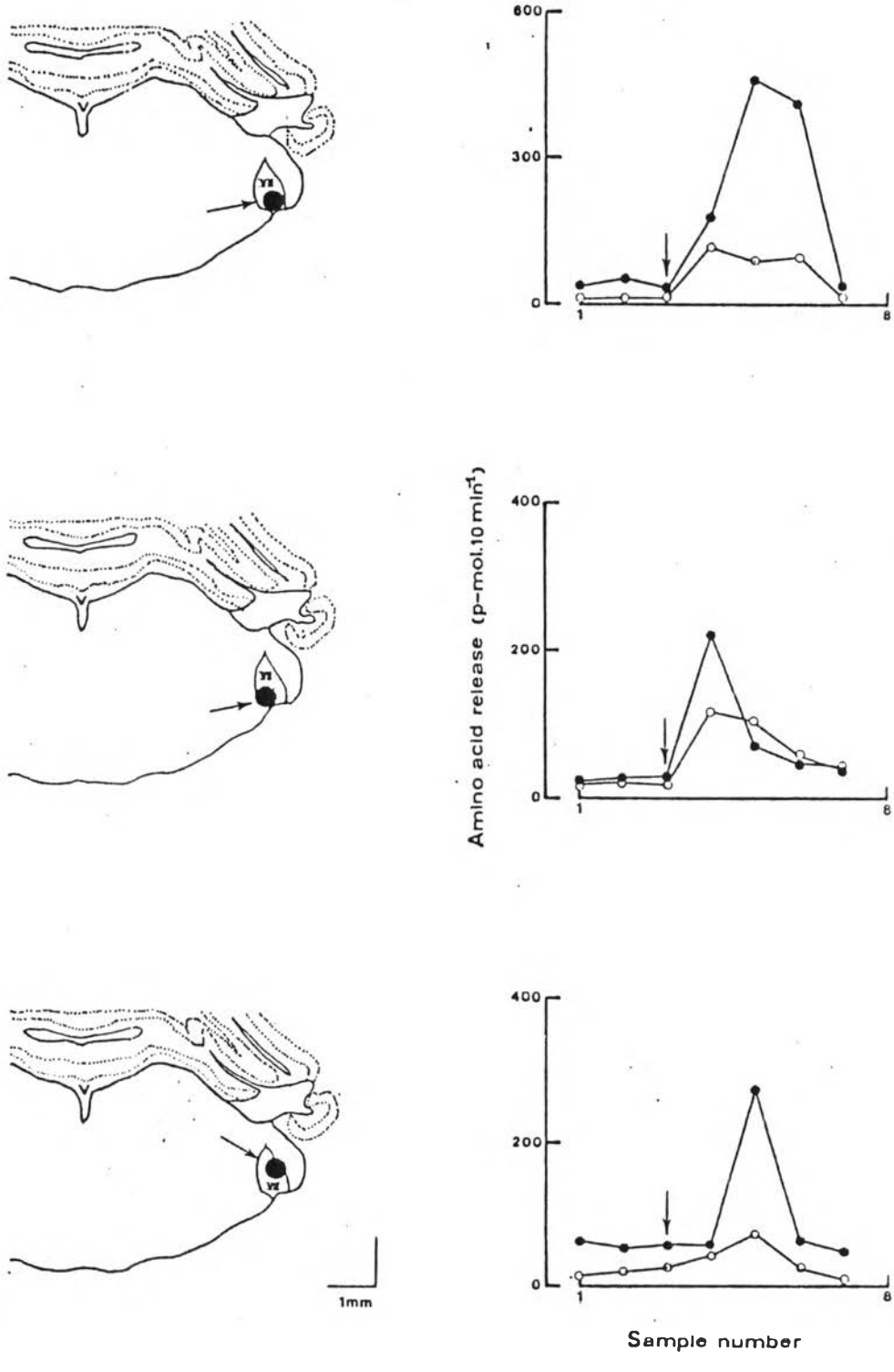


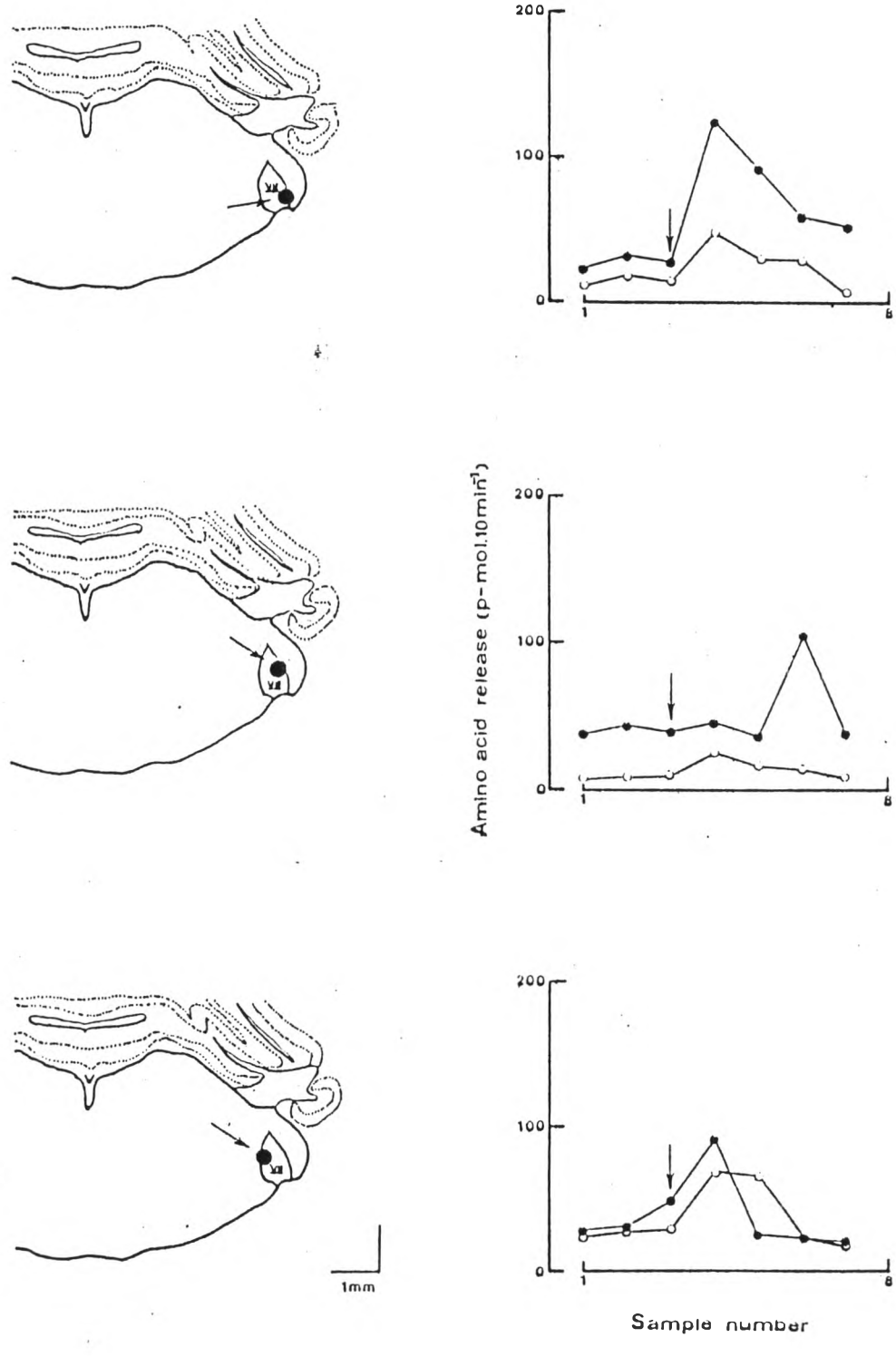
Figure 17. Coronal section of rat's brain. Photograph on the left is printed from tissue slice. Right hand part is diagrammatic drawing traced from section. The electrolytic lesion representing location of stimulating electrode is seen as the dark spot at the arrow.

Figure 18.(A-C) Effect of electrical stimulation at difference point of the vestibular nerve on the release of glutamate and aspartate. The various site of stimulating electrode from brain section are represented on the left part of the figure, the black circle (●) at the arrow compared with the increase release of glutamate (filled symbol) and aspartate (open symbol) from electrical stimulation of the vestibular nerve on the right part of the figure.

VIII = Vestibular nerve

V = Ventricle


600
300
0
1
8
400
200
0
1
8
Amino acid release (p-mol.10 min⁻¹)
400
200
0
1
8
1mm
Sample number


200
100
0
1
8
Amino acid release (p-mol.10min⁻¹)
1mm
Sample number