

Chapter 3

Results

1. Amino Acid Analysis : Preparation of Standard Curves

Under the chromatographic conditions described, separation of amino acids contents, both of the samples and standard solutions was accomplished within approximately 20 minutes. A sample chromatogram of OPA derivatives of a standard solution containing mixture of 8 amino acids (Ca 400 pmol of each) : aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Gln), glycine (Gly), taurine (Tau), alanine (Ala) and gamma - aminobutyric acid (GABA) is illustrated in Fig. 7. The area of each peak of the chromatogram was directly proportional to the amount of the OPA derivative of each standard with linearity reliable within the range 40 pmol to 1600 pmol amino acid content (Fig. 8). The coefficient of variation of the peak area of each amino acid is shown in Table I.

2. Perfusion Experiments

The amount of amino acids released into the

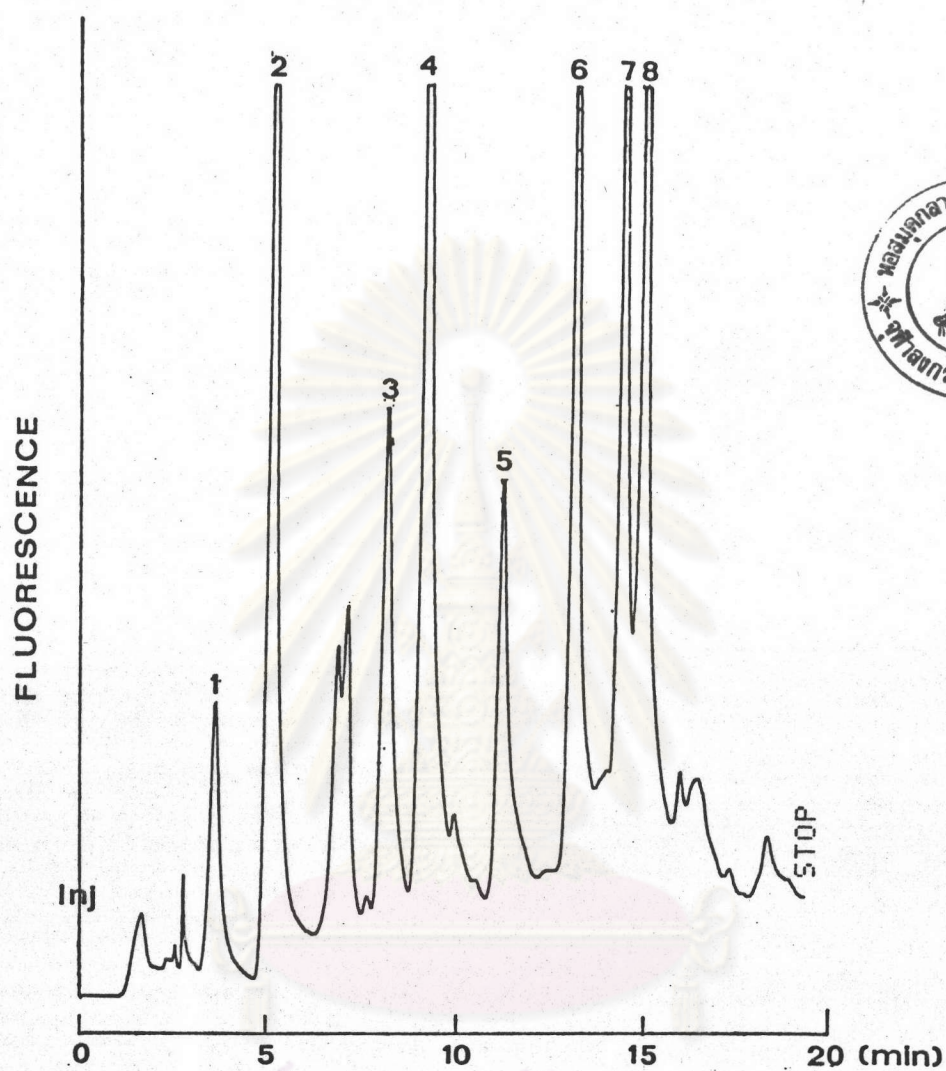


Figure 7. Chromatogram of OPA-derivatives of standard amino acids (Ca. 400 pmol of each). Peak : 1 = aspartic acid ; 2 = glutamic acid ; 3 = serine ; 4 = glutamine ; 5 = glycine ; 6 = taurine ; 7 = alanine ; 8 = GABA.

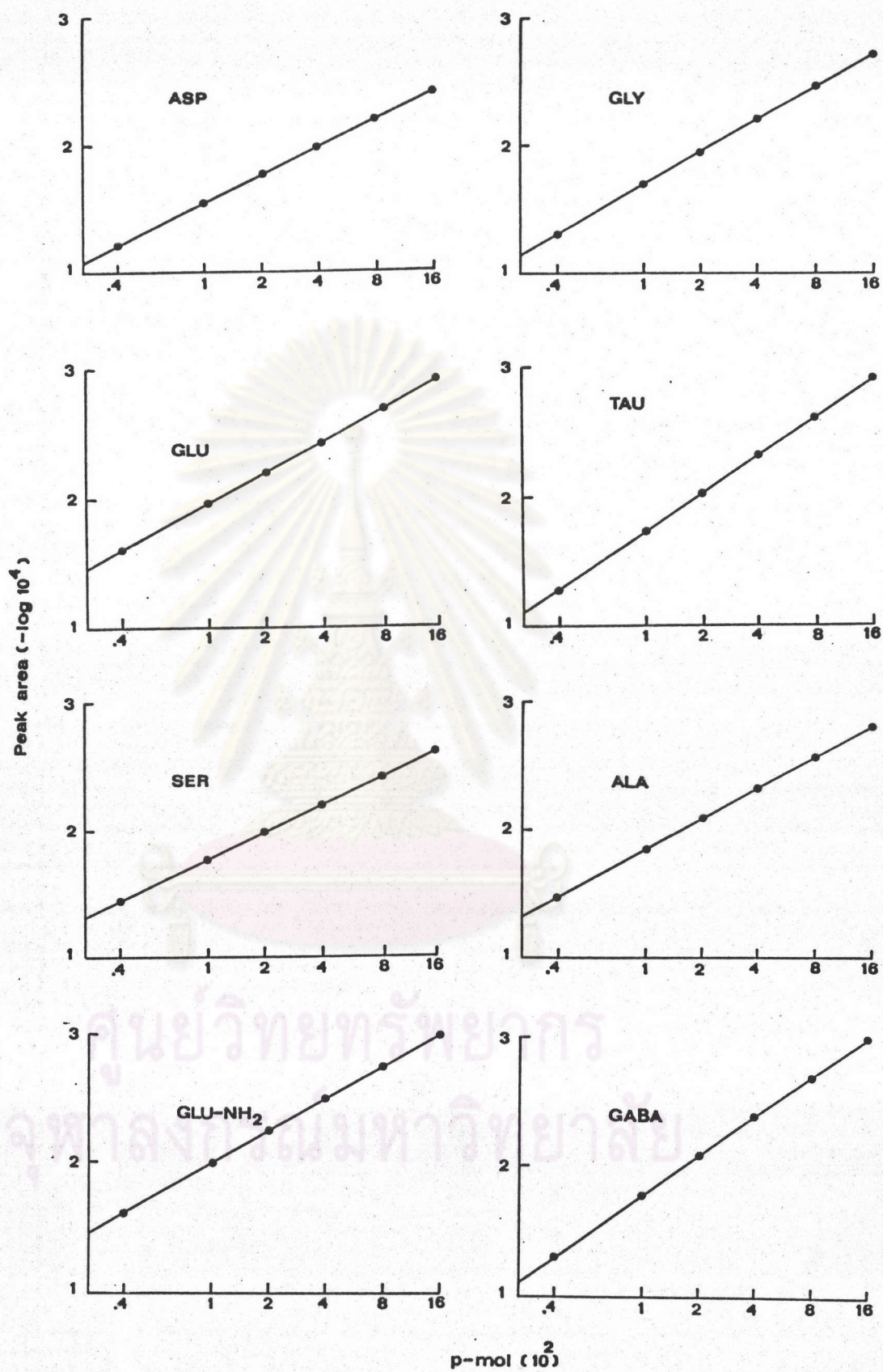


Figure 8. Standard curve of amino acids measurement. Peak area refers to area under each of amino acids peak.

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.

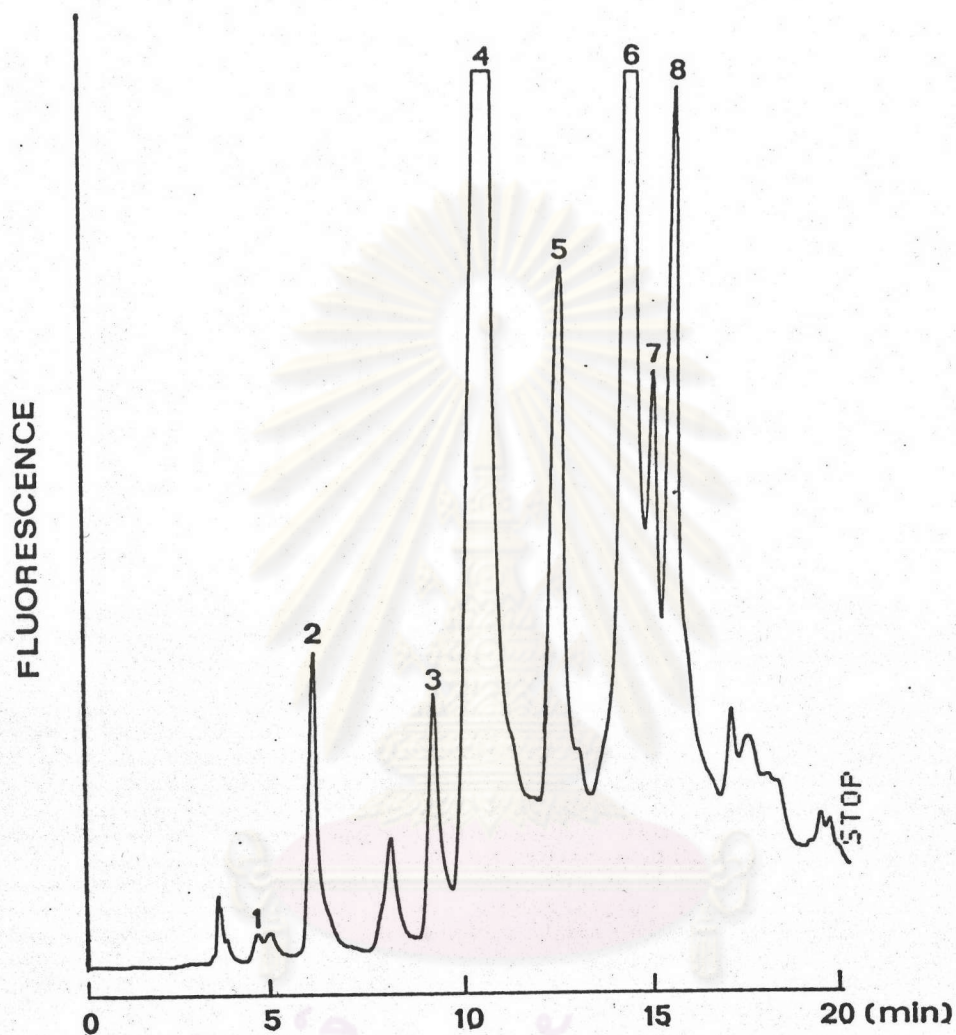
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perfusing media was dependent on the placement of the push-pull cannula. When measurable amounts of amino acids were obtained in chromatographic determination, post-experiment histological examination always revealed correct localization of the cannula tip within the limit of vestibular nuclei, as suggested by the tissue damage on the histological sections. Fig. 9 shows an example of chromatogram of perfusate obtained from a successful experiment, whose histological section illustrated in Fig. 10, showing the scar caused by the cannula tip in the vestibular nuclear mass. By contrast, incorrect placement of the cannula yielded less conspicuous amount of amino acid release. An example of such case is shown in chromatogram in Fig. 11, which was obtained from the experiment from which histological section in Fig. 12 was derived, showing the cannula tip site located outside the limit of the vestibular nuclei.

3. Spontaneous Release of Endogeneous Amino Acids

When perfusion was performed with standard artificial CFS in a successful experiment, measurable amounts of various amino acids could be recovered in the perfusate throughout the period of perfusion (100 minutes). The spontaneous release of endogenous amino acid is shown in Fig. 13 and Table 2. The first period



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. Chromatogram of the perfusate sample from the rat vestibular nucleus. Amount of amino acids shown under the chromatogram were interpreted from standard curve.

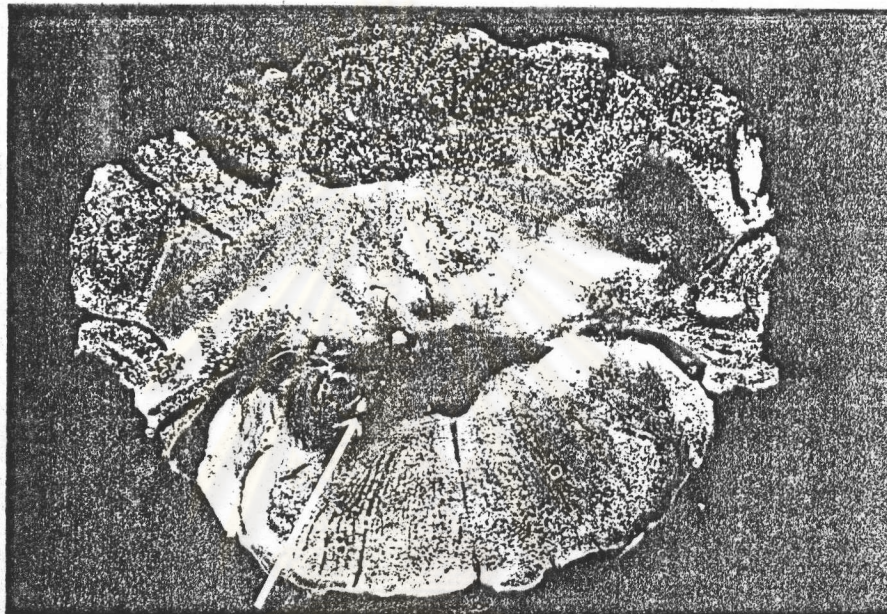


Figure 10. Histological section from a successful experiment whose chromatogram is shown in Fig. 9. Arrow shows the scar caused by the canula tip in the vestibular nucleus.

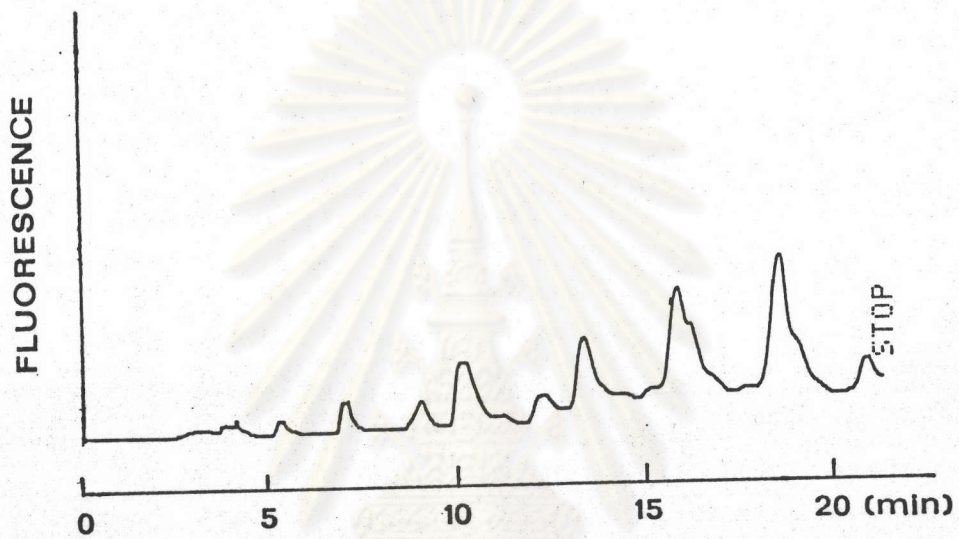


Figure 11. Chromatogram of the perfusate sample from incorrect placement of the push-pull canula.

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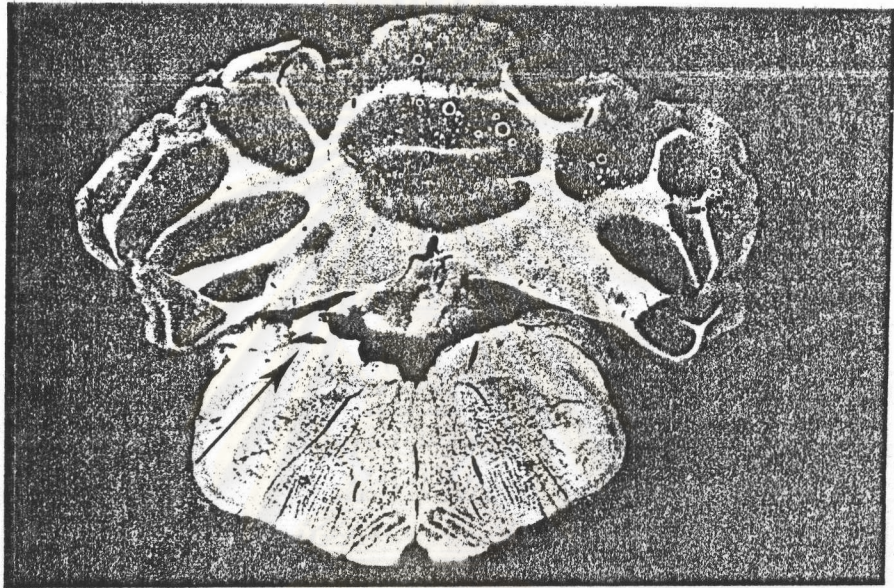


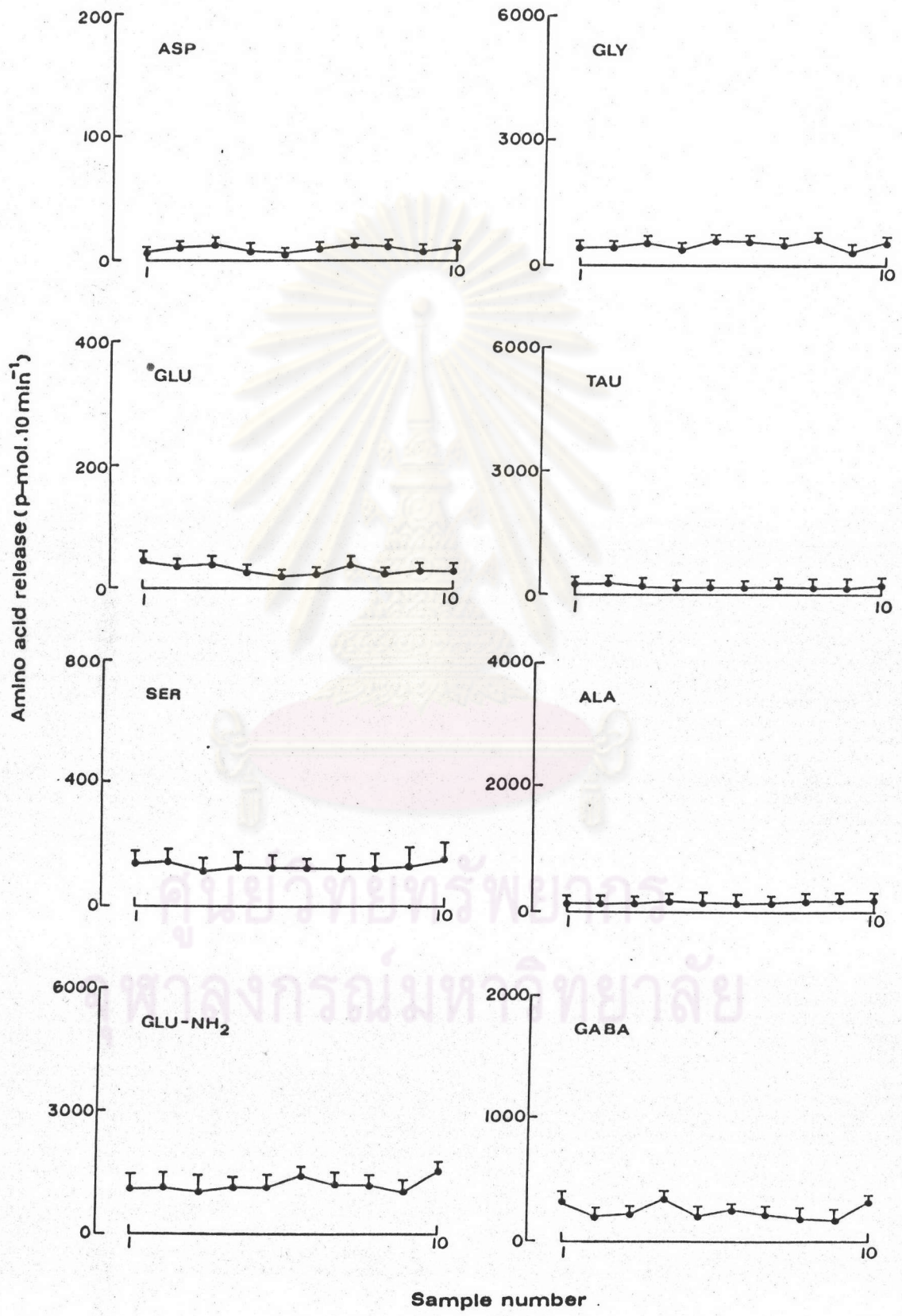
Figure 12. Histological section of the canula tip site located outside of the vestibular nuclei. Chromatogram of this experiment shown in Fig. 11.

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Figure 13. Time course of spontaneous release of aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Glu-NH₂), glycine (Gly), taurine (Tau), alanine (Ala), and γ -aminobutyric acid (GABA) from the perfusate of the rat vestibular nucleus. Each point represent the mean release of amino acid in p-mole during 10 min collection in five experiments and the S.E. of the mean is shown by the vertical bar.

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

Table 2 Levels of the spontaneous release of endogeneous amino acids from the rat vestibular nucleus. (n = 5). The values in the Table represent the mean total p-mole of amino acid released per minute \pm S.E. of mean (p-mole/min). n = number of observations.

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corresponds to a washout of material from damaged cells and intact cell surface. The pattern of release was consistent in all experiments. There was no significant alteration in the release of any of the amino acids. The amino acids released, as identified by the corresponding peak numbers in the chromatogram were Asp, Glu, Ser, Glun, Gly, Tau, Ala and GABA.

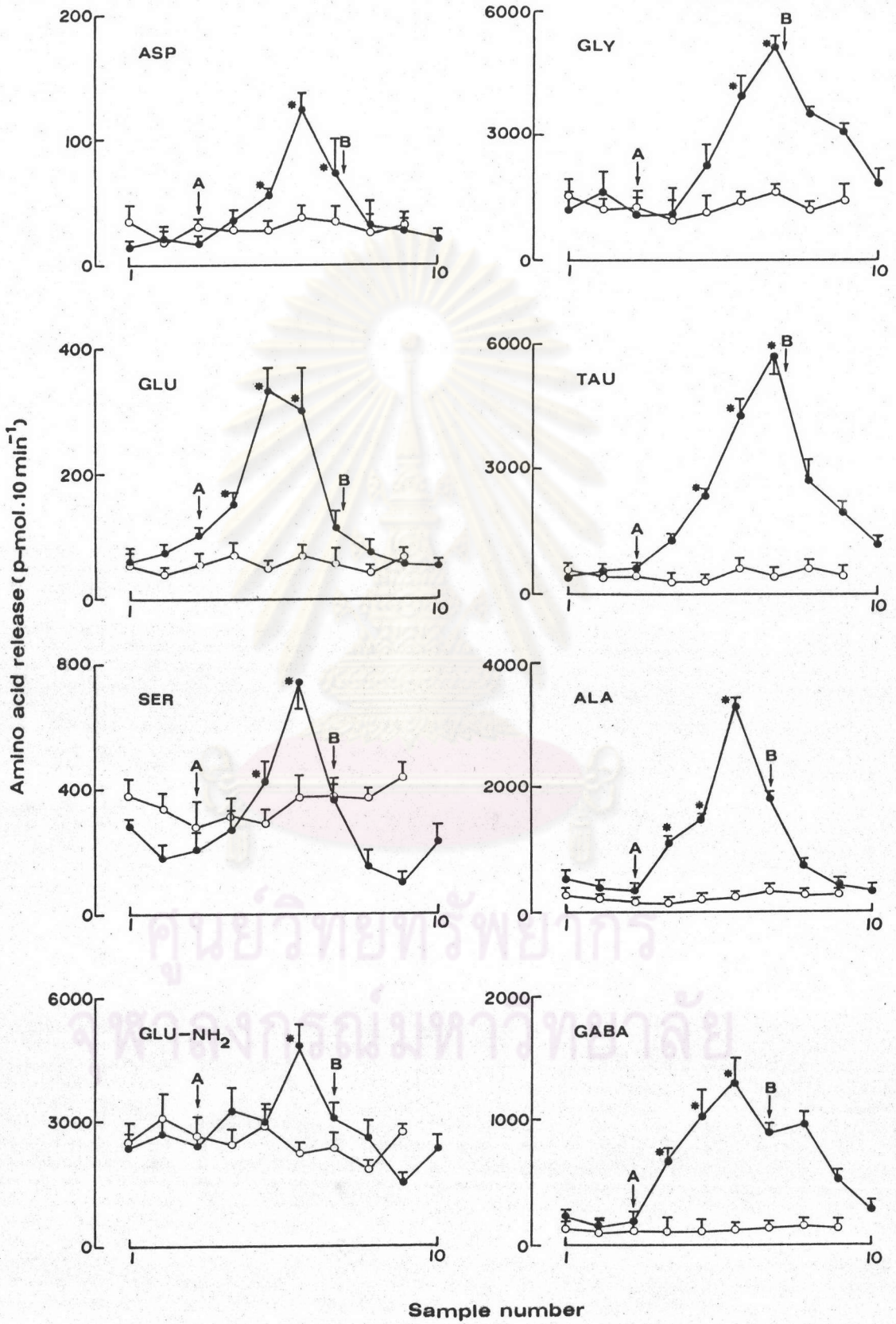
4. Evoked Release of Endogeneous Amino Acids

Once the base line release was established, usually after collection of three spontaneous release (filled symbols), the control artificial CSF was replaced with high K^+ (100 mM) - Ca^{2+} dependent for 30 min, after which the perfusion was returned to the control solution. Trial exposure to high K^+ (100mM) - Ca^{2+} dependent significantly increased the release of endogeneous amino acids. (Fig. 14 and Table 3)

5. Effect of Ca^{2+} on amino acid release

A series of experiments were carried out in order to ascertain the effect of Ca^{2+} on the release of the endogeneous amino acids. The time course of sample collection was divided into three phases, each lasting 30 min. From Fig. 14 and Table 4, after control

Figure 14. Effects of high concentration of K^+ -stimulated (100 mM) with Ca^{2+} -dependent (filled symbols) and K^+ -stimulated (100 mM) with Ca^{2+} -free (open symbols) on the release of endogeneous amino acids from perfusate of rat vestibular nucleus. The initial superfusion media was control artificial CSF ; after sample 3 was collected the medium is changed to high K^+ - Ca^{2+} -dependent and high K^+ - Ca^{2+} -free of each experiment (A). After sample 6 was collected the medium was changed to control artificial CSF (B). An asterisk adjacent to a point indicates a significant difference in statistical analysis (Student's t test, $p < 0.05$).





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

Table 3 Evoked release of amino acids from the rat 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 \pm S.E. of mean. n = number of observation = 6.

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

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

Table 4 The Ca²⁺ dependency of the K⁺-evoked release of amino acids from the rat vestibular nucleus.

The value in the Table represent the mean total p-mol of amino acid released per minute

\pm S.E. of mean (p-mole/min). n = number of obseration = 3.

spontaneous release had stabilized, the perfusate medium was changed to high K^+ (100 mM)- Ca^{2+} free solution containing 0.5 mM EDTA (open symbols). The release of the amino acid did not increase much above the baseline levels during this period. During the final phase the perfusing fluid was replaced again within in control solution (normal K^+ - Ca^{2+}).

6. Effect of perfusion with high K^+ solution

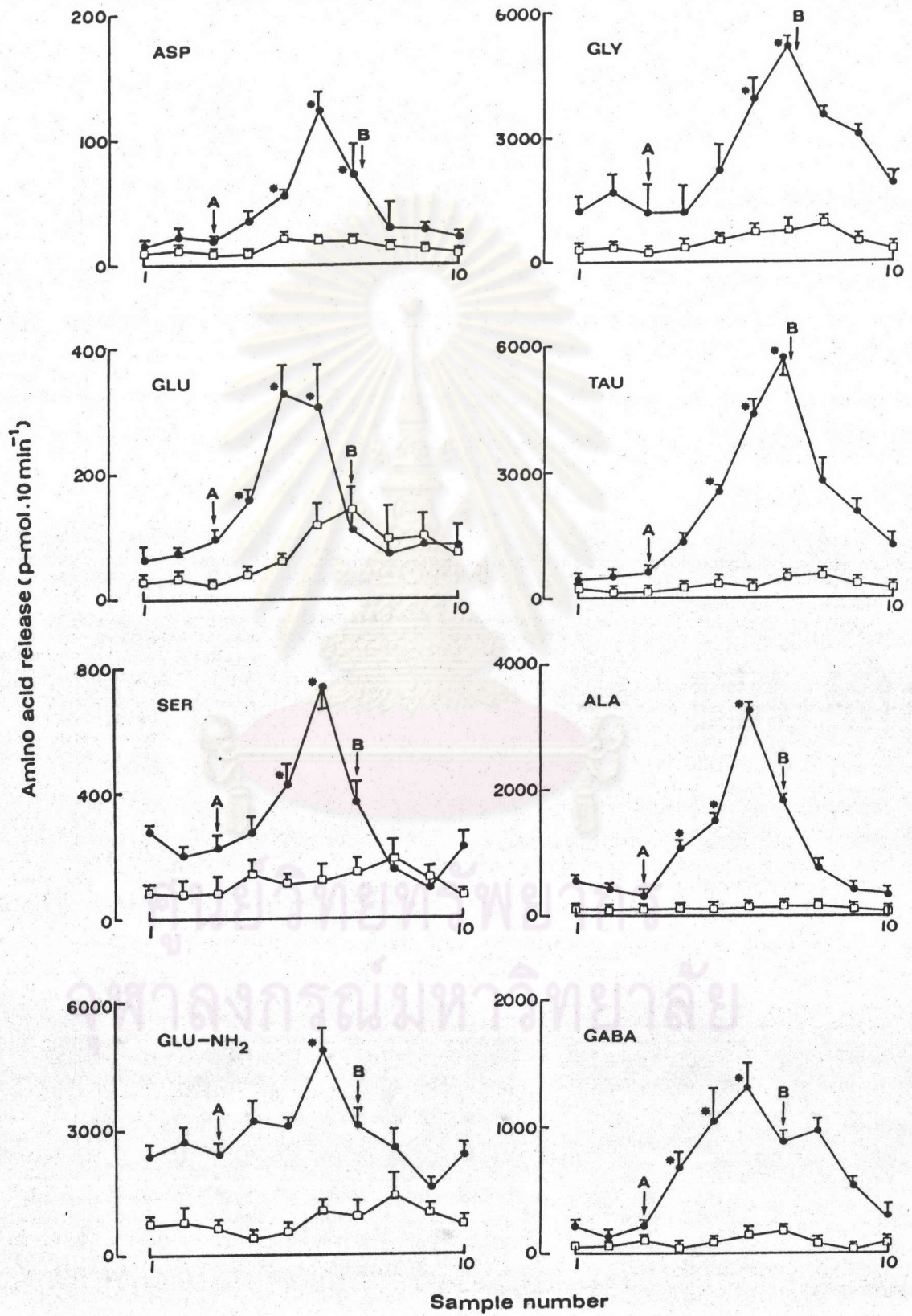
An attempt to induce enhanced release of the amino acid was carried out by replacing the artificial CSF with perfusing fluid containing high concentration of K^+ , in order to cause local depolarization of the excitable tissue within the vicinity of perfusion. Two concentrations of K^+ were use, i.e. 50 mM and 100 mM. Trial exposure to 100 mM K^+ significantly increased the release of all amino acids. In contrast exposure to 50 mM- K^+ non-significantly increase of all amino acid. (Fig 15, Table 3 and Table 5)

7. Amino acid acid release in 3-AP treated rat

Rats which received injection of 3-AP/harmaline/niacenamide (see Method) shows ataxic movement characterized by a mud-walking sign within 3 days. During this periods, histological study by

Figure 15. Effects of high concentration of K⁺ (50 mM, square symbols; 100 mM, filled symbols) on the release of endogeneous amino acid from the rat vestibular nucleus. The initial superfusion media was the control artificial CSF; after sample 3 was collected the medium was changed to high K⁺ -50 mM and 100 mM (A). After sample 6 was collected the medium was changed to the control artificial CSF (B).

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sample number amino acid	control			50 mM. - K ⁺							control		
	1	2	3	4	5	6	7	8	9	10			
Asp [^] a	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 [^] a	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			
Gln	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			
Tan	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 5 Evoked release of amino acids from the rat vestibular nucleua by stimulated with high K⁺ 50 mM.
The values in the Table represent the mean total p-mole of amino acid release per minute \pm S.E. of mean (p-mol/min). n = number of observation = 5.

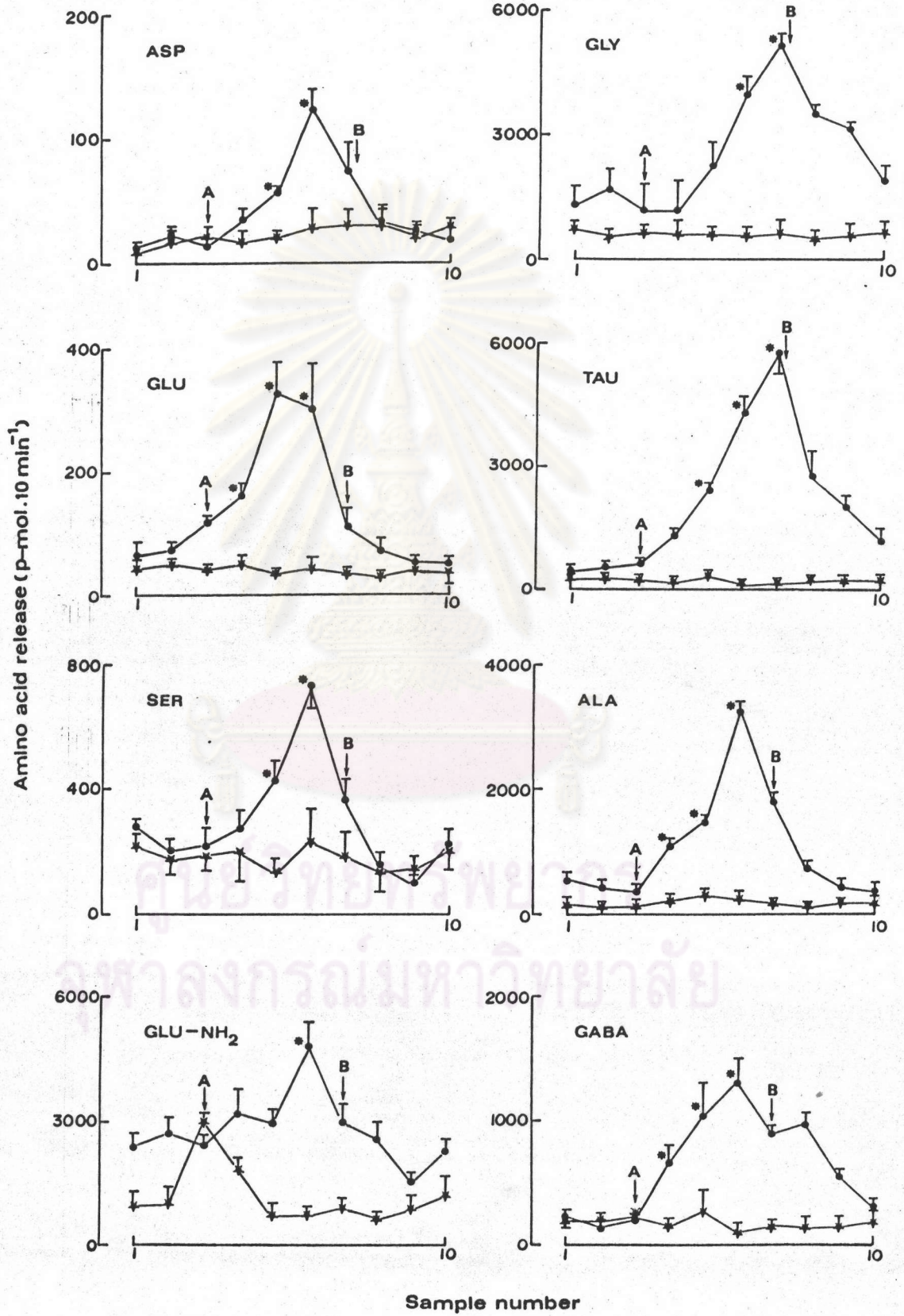
Desclin and Escubi (1974) revealed that neuropils in the inferior olive were totally destroyed. In the present experiments, rats which showed such ataxic movement were classified as olivary lesioned rats.

An attempt to measure amino acid release was performed in six cases. The results are shown in Fig 16 and Table 6. Spontaneous release of all amino acids did not show any significant difference from those measured in the control animals, also some deviation towards the lesser release amount was observed. When an attempt to evoke the release with 100 mM K⁺ was performed, it was observed that no significant change in the amino acid contents in the perfusate was observed for all amino acids throughout the exposure period and thereafter (100 min.), as compared with the spontaneous release. Such results indicate that in olivary lesioned rat, K⁺ - depolarizing solution (with at lease 100 mM) failed to evoke amino acid release in the vestibular nuclei.



Figure 16. Amino acid release in 3-AP treated rats (star symbols) compared the result obtained in normal rats. The initial superfusion media was the control artificial CSF ; after sample 3 was collected the medium was replaced with high K⁺ (100 mM) of each experiment (A). After sample 6 was collected the medium was changed to control artificial CSF (B).

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sample number amino acid	control			100 mM - K ⁺						control		
	1	2	3	4	5	6	7	8	9	10		
Asp \hat{a}	15 \pm 4	20 \pm 6	21 \pm 7	19 \pm 7	20 \pm 3	28 \pm 14	29 \pm 10	33 \pm 13	24 \pm 7	26 \pm 5		
Glu \hat{a}	41 \pm 14	46 \pm 10	39 \pm 7	48 \pm 11	33 \pm 4	42 \pm 14	33 \pm 9	30 \pm 7	38 \pm 10	37 \pm 14		
Ser	217 \pm 33	179 \pm 41	187 \pm 37	198 \pm 77	145 \pm 34	236 \pm 100	185 \pm 71	153 \pm 55	145 \pm 40	206 \pm 42		
Glun	996 \pm 272	1047 \pm 287	3034 \pm 152	1873 \pm 256	765 \pm 253	759 \pm 200	855 \pm 233	610 \pm 124	826 \pm 309	1198 \pm 468		
Gly	731 \pm 168	534 \pm 134	623 \pm 128	586 \pm 310	595 \pm 136	543 \pm 137	653 \pm 205	494 \pm 140	604 \pm 233	639 \pm 200		
Tau	267 \pm 85	278 \pm 78	246 \pm 74	204 \pm 40	286 \pm 65	173 \pm 33	152 \pm 22	178 \pm 34	245 \pm 72	176 \pm 42		
Ala	150 \pm 40	130 \pm 25	137 \pm 56	223 \pm 38	258 \pm 55	242 \pm 98	135 \pm 27	85 \pm 23	157 \pm 70	169 \pm 65		
GABA	239 \pm 60	202 \pm 54	221 \pm 56	139 \pm 32	290 \pm 126	124 \pm 28	148 \pm 30	141 \pm 39	147 \pm 51	166 \pm 66		

Table 6 The release of amino acids from the rat which lesion of Inferior olivary nucleus, with 3-AP. The vestibular nucleus stimulated with K⁺ 100 mM. The values in the Table represent the mean total p-mol of amino acid released per minuted \pm S.E. of mean (p-mole/min). n = number of observation = 6.