J. Physiol. (1960), **153**, *pp.* 423–432
Printed in Great Britain

THE STATE OF FACTOR I IN RAT BRAIN: THE EFFECTS OF METABOLIC CONDITIONS AND DRUGS

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(*Received* 29 *February* 1960)

The term Factor I refers to the substance or substances in brain which inhibit impulse generation in the stretch receptor neurones of crustaceans (Florey, 1954). Gamma-aminobutyric acid (GABA) shows strong Factor I activity, can account for most of the activity in whole brain, and exerts marked effects on the physiological activity of brain and other organs (see Elliott, 1958; Elliott & Jasper, 1959).

In previous publications (Elliott & Florey, 1956; Elliott & van Gelder, 1958) it was shown that much of the Factor I in brain is present in a bound or occult form from which it is released by heat, suspension in hypotonic medium, or mild acid or alkali. In the present studies an attempt has been made to ascertain the significance of the difference between free and occult Factor I, to determine whether drug-induced changes in the GABA content of brain can be correlated with changes in Factor I content, and to determine the effects of drugs on the proportions of the free and occult forms of the Factor.

No definite conclusion could be drawn about the intracellular localization of occult Factor I, since this factor is released when brain tissue is suspended in media which are commonly used for the separation of subcellular particles. Decreases in the GABA content of brain have been found after administration of hydrazide (Killam & Bain, 1957) and insulin (Cravioto, Massieu & Izquiero, 1951), and increases after hydroxylamine administration (Baxter & Roberts, 1959). Substances other than free GABA are apparently present in brain and exert Factor I activity (McLennan, 1957, 1959) and effects of a variety of other inhibitory substances have been described (see Elliott & Jasper, 1959) but the present work shows that the changes in chemically-determined GABA content are paralleled by changes in Factor I activity. It is also shown that hypoxia and iproniazid cause changes in Factor I activity. The agents which decrease Factor I usually affect mainly the occult form and those which increase it affect mainly the free form. A number of neurotropic drugs have no obvious effect on the amount and condition of Factor I.

METHODS

Hooded rats weighing about 200 g were used. Drugs were administered intraperitoneally, unless otherwise specified, to rats which had been deprived of food since the previous day. The animals were decapitated and their cerebral hemispheres studied. For routine determinations of free and occult Factor I the tissue was immediately homogenized, by means of a Potter–Elvehjem homogenizer, in 4 volumes of potassium-free crayfish saline containing 5 mM glutamate, and the suspension was centrifuged at about 16,000 *g*. The Factor I activity found in the supernatant fluid is referred to as 'free Factor I' and the activity found in the residue, after resuspension and heating for 15 min, is referred to as 'occult Factor I'. Variations from this initial procedure are noted below and in Table 1.

The method for assaying Factor I and the solutions used for making and diluting tissue suspensions are those described previously (Elliott & Florey, 1956; Elliott & van Gelder, 1958) except as noted later. 'Crayfish saline' (CS; van Harreveld, 1936) contains (mM): NaCl 205, KCl 5·4, $MgCl_2$ 2·6, $CaCl_2$ 13·3. Potassium-free crayfish saline was the same but with KCl omitted. When necessary, small volumes of concentrated salt solutions were added after centrifuging to the supernatant fluids and residues to make their salt concentrations equal to that of crayfish saline. Resuspension of residues and further dilutions of all extracts for assay were made with complete tris-maleate buffered (0·01 M, pH 6·5) crayfish saline containing 5 mM glutamate. Factor I activities are expressed in terms of the amounts of GABA (μg) which would show the same activity, per gram of brain. None of the drugs used were found to affect the sensitivity of stretch receptor preparations to GABA, even in concentrations considerably higher than were likely to be present in the brain extracts as diluted for assay.

RESULTS

Free and occult Factor I in brain dispersed in various media

The results given in Table 1 show that when brain is homogenized in saline medium (modified crayfish saline) the proportion of the total Factor I which is found occluded in the centrifuged residue is rather constant, in spite of quite wide variations in the total Factor I activity found in the brains of individual animals. The volume of saline medium used in the initial homogenization and the length of time of homogenization had no obvious effect, nor did the use of mammalian Ringer-type solution or normal saline for the initial dispersion. Further, after grinding the tissue thoroughly with sand about the same amount of occult Factor I was found as after ordinary homogenization. In sand-ground tissue there was some increase in free and total Factor I but this might be accounted for by production by the tissue of extra, free Factor I during the time required for grinding. It was usually noted that elevated Factor I activity, particularly in the free condition, was found when whole brain or brain suspensions were allowed to stand at room temperature before heating or centrifuging (cf. Elliott & Florey, 1956).

When brain was homogenized in sucrose solutions of various concentrations, cold or at room temperature, the proportion of the total Factor I activity found in the free condition was considerably increased. The presence

of 0·1 mM ethylenediamine tetraacetate (Versene) or 7·5% polyvinylpyrrolidone (PVP) did not prevent this liberation of Factor I. The presence of low concentrations of sodium, magnesium or calcium chloride in sucrose solution decreased the liberation appreciably.

Brain hemispheres were homogenized in cold 0·25 M sucrose + 0·1 mM Versene; the suspension, the pH of which was between 6·9 and 7·1, was subjected to differential centrifugation and the sediments were assayed

TABLE 1. Occult and free Factor I in rat brain dispersions

Initial homogenization medium, etc.*		Factor I activity, (μg γ-aminobutyric acid/g fresh brain) Occult	Free	Total	% free
CS + glutamate, no K† (averages ± S.D., 29 animals)		212 ± 27	108 ± 10	321 ± 31	34 ± 4
CS + glutamate, low K† (19 vol.)		186, 200, 213	130, 101, 123	316, 301, 336	41, 34, 37
CS + glutamate, low K†, 1–4° C		197	85	282	30
Sodium chloride (154 mM)		184	110	294	37
Mammalian Ringer-type solution‡		202, 223, 240	132, 123, 150	334, 346, 390	37, 36, 38
Ground with sand 15 min, then in CS + glutamate, no K		202, 256	160, 139	362, 395	45, 35
Stood, not ground, 15 min§	(Range of 7)	208 to 270	143 to 178	353 to 448	40 to 45
Sucrose 0·25 M	18,000 *g*	80, 83	201, 247	281, 330	71, 75
Sucrose 0·25 M, 1–4° C	18,000 *g*	91	211	302	70
Sucrose 0·25 M + Versene 0·1 mM, 1–4° C	18,000 *g*	58, 64	284, 217	342, 281	82, 77
Sucrose 0·25 M + PVP 7·5%, 1–4° C	18,000 *g*	84, 104	210, 169	294, 273	72, 62
Sucrose 0·3 M		65	220	285	77
Sucrose 0·3 M + NaCl 15 mM		136	255	391	65
Sucrose 0·3 M + $MgCl_2$ 2·6 mM		130	230	360	64
Sucrose 0·3 M + $MgCl_2$ 6·5 mM		140	220	360	61
Sucrose 0·3 M + $CaCl_2$ 3·0 mM		154	225	379	60
Sucrose 0·3 M + $CaCl_2$ 13·5 mM		165	170	335	51
Sucrose 0·4 M		88	174	262	66
Sucrose 0·4 M, 1–4° C	18,000 *g*	88	222	310	72
Sucrose 0·88 M, 1–4° C	18,000 *g*	31	268	299	90

* The suspensions were prepared in 4 volumes of medium at room temperature and centrifuged at about 16,000 *g* unless otherwise specified. † Crayfish saline containing 5 mM glutamate, K omitted or reduced in ititial homogenization to allow for K in tissue. ‡ Bicarbonate-buffered solution with composition of average spinal fluid. § The tissue was stood dry, or in medium, before homogenization, or homogenized and then stood; the results were about the same in all cases.

after being heated. The fraction 'nuclei and debris', which sedimented in 10 min at 1100 *g*, showed no more activity than the equivalent volume of supernatant fluid. The 'mitochondrial fraction', which sedimented in 15 min at 12,500 *g*, and the 'microsomal fraction' obtained after 30 min at 18,000 *g* each contained 30–40 μg/g brain more than could be accounted for by the content of the fluid remaining in these sediments. The final supernatant fluid contained 220–280 μg/g brain.

Factor I in brains of drug-treated animals

Results of determinations of free and occult Factor I in saline suspensions of brain from treated animals are shown in Table 2. Cravioto *et al.* (1951) found that the GABA content, determined by the chromatographic–colorimetric method, of the brains of rats is reduced after treatment of the animals with insulin. In agreement with this we find that the total

TABLE 2. Occult and free Factor I in brains from treated rats

Treatment (dose/kg*)	No. of animals	Factor I activity (μg γ-aminobutyric acid/g fresh tissue) Occult*	Free*	Total*	Symptoms immediately before decapitation†
None	29	212 ± 27 (S.D.)	108 ± 10 (S.D.)	321 ± 31 (S.D.)	None
Insulin 100–185 u.	2	195, 205	111, 136	309, 341	Beginning to be unsteady
	4	175 160–216	125 106–140	305 268–333	Coma up to 5 min, occasional convulsions
	2	137, 145	112, 114	249, 260	Coma for 10 min, occasional convulsions
	3	120 114–132	90 84–98	210 198–230	Coma for 25–30 min, occasional convulsions
	3	115 101–122	90 80–105	205 190–222	Coma for 60–180 min, occasional convulsions
Hypoxia	2	237, 266	180, 177	417, 443	Comatose; some movement possibly convulsive (30 min)
Anoxia	2	213, 217	120, 138	333, 355	Excited, then comatose. Died at 10 min
Thiosemicarbazide					
20 mg	2	171, 190	100, 98	290, 269	Convulsions for 20 min
30 mg	2	135, 148	110, 100	245, 248	Convulsions for 20 min
Semicarbazide					
30 mg	2	180, 190	113, 100	293, 290	None (90 min)
30 mg	2	210, 272	116, 117	326, 389	None (5 hr)
100 mg	1	191	103	294	None (90 min)
100 mg	1	172	94	266	Slight convulsions (90 min)
250 mg	2	125, 138	54, 44	179, 182	Convulsions for 10, 20 min
Hydroxylamine 50 mg	4	251 226–286	193 126–336	443 350–622	Twitching and cyanotic, then lethargic and cyanosis gone (90 min)
Metrazol 50 mg, 100 mg	2	226, 260	114, 131	340, 390	Convulsions for 6, 7 min
Megimide 16–25 mg	6	225 200–255	147 140–170	372 355–399	Convulsions for 10–85 min
Picrotoxin 9 mg	2	194, 218	133, 157	327, 375	Convulsions for 10, 20 min
Pentothal 100 mg	4	202 173–240	87 78–105	289 250–345	Anaesthetized for 10–15 min
Pentobarbital 50, 100 mg	2	230, 265	100, 105	330, 365	Anaesthetized for 10 min
Ether inhalation	2	204, 216	103, 106	307, 322	Anaesthetized for 10 min
Diphenyl hydantoin					
50 mg intramusc.	2	196, 210	105, 110	301, 325	None (30 min)
Acetazoleamide 10 mg	2	197, 206	85, 93	290, 290	Sedated (120 min)
Reserpine					
5 mg	2	208, 266	119, 150	327, 416	Shivering, piloerection, lethargic (120 min)
2·5 mg × 5	4	220 210–240	125 124–135	345 (334–375)	
Chlorpromazine 5 mg	2	212, 219	105, 123	317, 342	None (120 min)
Iproniazid					
50 mg	2	193, 201	122, 147	315, 348	Quiet; no obvious symptoms (120 min)
50 or 100 mg × 2 to 5	12	225 196–226	165 120–240	390 341–450	
Iproniazid + Megimide					
50 mg × 3 ipro. + 22 mg Meg.	1	245	210	455	Brief convulsion (60 min)
Iproniazid + reserpine					
50 mg ipro. + 5 mg × 2 res.	2	215, 221	167, 126	347, 381	As with reserpine alone (120 min)
50 mg × 3 ipro. + 5 mg res.	3	205 192–214	180 148–215	380 362–422	Excited, stereotyped behaviour (120 min)
Iproniazid + thiosemicarbazide					
100 mg × 2 ipro. + 20 mg thiosem.	1	202	180	382	One brief convulsion, then normal (95 min)
	1	173	117	290	Convulsions 25 min
50 mg × 2 + 100 mg ipro. + 20 mg thiosem.	1	203	171	374	Intermittent brief convulsions then normal (15 min)
	2	163, 175	131, 130	294, 304	Convulsions 15, 33 min

TABLE 2 (*cont.*)

Treatment dose/kg*	No. of animals	Occult*	Free*	Total*	Symptoms immediately before decapitation
Iproniazid + semicarbazide 100 mg × 2 ipro. + 250 semicarb.	2	118, 145	97, 101	215, 246	Convulsions 20, 30 min
Harmine HCl 10 mg	2	245, 264	126, 122	371, 386	Spastic movements then quiet and unsteady (180 min)
Harmaline HCl 10 mg	2	218, 240	153, 135	371, 375	Spastic, then quiet and unsteady (180 min)
SKF-trans 385 10 mg	2	242, 252	126, 119	368, 371	Very fearful, salivating (90 min)

* Where more than two results were obtained the averages and ranges are shown (except for the controls). Repeated doses of iproniazid were usually given, two/day (10 a.m. and 5 p.m.). The notation '× 3', for instance, indicates 3 doses. When other drugs were given with iproniazid these were given 10 min after the last dose of iproniazid. † Times given between brackets are the times between the last dose of a drug and decapitation of the animal.

Factor I content decreases markedly, reaching about 65 % of the normal value. The decrease is most apparent in the occult Factor I.

Hypoxia (animal in a desiccator filled with 5 % O_2 in N_2) for 30 min causes a considerable increase in total Factor I, mostly accounted for by increase in the free form. Under anoxic conditions (5 % CO_2 in N_2) there was less change in the levels of Factor I but the animals died in a few minutes.

Killam & Bain (1957) have shown that administration of carbazides to rats causes a decrease in the chemically-determined GABA content of the brain and this decrease is associated with the development of seizures. Results given in Table 2 show marked reductions in total Factor I activity after administration of thiosemicarbazide and particularly after semicarbazide in the large dose required to produce convulsions. With thiosemicarbazide the decrease in Factor I occurred in the occult fraction; with the large dose of semicarbazide both fractions were decreased.

Baxter & Roberts (1959) have reported that administration of hydroxylamine causes an increase in the GABA level in various parts of the brains of rats. Figures in Table 2 show that hydroxylamine administration causes a variable increase in Factor I levels in cerebral hemispheres, the effect being most marked in the free Factor I. In our experiments no convulsions were observed, though Baxter & Roberts, with higher doses, observed convulsions for 10 min or less after administration of the drug.

The convulsant agents Metrazol (pentylenetetrazole), Megimide (methylethyl glutarimide) and picrotoxin caused no definite change from the normal Factor I levels, though there was a trend to a rise in the free and total Factor I levels. The anaesthetic agents pentothal, pentobarbital and ether caused no consistent changes in Factor I levels except that somewhat low levels occurred in two animals that received pentothal. Sodium diphenylhydantoin (Dillantin), acetazoleamide (Diamox) and chlorpromazine in the doses used had caused no obvious or consistent changes in Factor I

levels at the time when the animals were killed. Administration of reserpine, which is known to release bound serotonin, seemed to cause a slight elevation of free Factor I.

After repeated doses of iproniazid (Marsalid) the total Factor I was always moderately high; most of this increase was due to an increase in the level of free Factor I. The averages for total and free Factor I were highly significantly different from normal ($P < 0{\cdot}01$).

High values for free and total Factor I were obtained also when iproniazid was administered before Megimide or reserpine. When iproniazid was administered before thiosemicarbazide or semicarbazide there seemed to be the tendency of the hydrazides to lower the Factor I content and the tendency of iproniazid to raise the free Factor I. As a consequence, when both thiosemicarbazide and iproniazid had been administered the total Factor I content was at about the normal level. In spite of this the animals suffered convulsions. Though the total Factor I was not below normal in these cases, the occult Factor I *was* usually below normal, but was accompanied by a rather high amount of the free factor.

Three other monoamine oxidase inhibitors, harmaline and harmine (Sjoerdsma, Gillespie & Udenfriend, 1959; Tabachnik & Rubin, 1959) and tranylcypromine (SKF-trans 385; Tedeschi, Tedeschi, Ames, Cook, Mattis & Fellows, 1959) were tested in single doses (10 mg/kg). These tended to raise the free, occult and total Factor I levels.

DISCUSSION

The amount of occult Factor I found in brains from untreated animals was not obviously affected by varying the length of time of homogenization or the volume of saline medium, or even by grinding the tissue with sand. This seems to indicate that the free and occult fractions represent different fractions which actually exist in brain; the division into these two fractions does not seem to be an artifact resulting from the mechanical disruption of the tissue. Further evidence for this is the fact that incubation of brain suspensions with added GABA does not produce an increase in occult Factor I, whereas with brain slices such an increase does occur (Elliott & van Gelder, 1958). It is probable, however, that the free Factor I fraction found does not represent truly free, extracellular Factor I present in brain *in vivo*. This is evident from the fact that, after incubating slices of cerebral cortex aerobically in saline medium, no detectable Factor I is found in the medium, even though the slices continue to contain about 100 μg of 'free' Factor I per gram (plus about 200 μg of occult Factor I per gram; Elliott & van Gelder, 1958). Further, electron microscope studies by a number of authors indicate that there is virtually no extracellular space in the central nervous system. Free Factor I can apparently

diffuse out of brain to a slight extent, since Florey & McLennan (1955) detected Factor I in fluids which had been in contact with the brains of living cats and traces of GABA have been detected in human cerebrospinal fluid (Knauff, 1958; Logothetis, 1958).

In attempts to localize the occult Factor I in subcellular particles, by suspension in sucrose solution and differential centrifugation, it was found that salt-free sugar solutions caused release of Factor I from its occult form. The mechanism by which Factor I is occluded is apparently different from those that apply to acetylcholine, adrenaline, noradrenaline and serotonin, since all these remain to a large extent in the bound forms during separation of subcellular particles in sucrose media (e.g. Hebb & Whittaker, 1958; Eade, 1958; Walaszek & Abood, 1959).

Dawson (1950, 1953) and Cravioto *et al.* (1951) have shown that the glutamate content of brain decreases during insulin hypoglycaemia. This decrease in the level of the substance from which GABA is formed would explain adequately the fall in Factor I level following insulin administration. The increase in the levels of Factor I, especially of the free Factor, during hypoxia might be due to decrease in the level of α-ketoglutarate in the brain with consequent decrease in the rate of removal of GABA by transamination.

As has been clearly indicated by the work of Killam & Bain (1957) and Roberts, Rothstein & Baxter (1958) on the metabolism of GABA, the fall in Factor I level after carbazide administration can be accounted for by inhibition of glutamate decarboxylase through combination of the carbazide with the co-enzyme pyridoxal phosphate. Baxter & Roberts (1959) pointed out that hydroxylamine is a more effective inhibitor of GABA-α-ketoglutarate transaminase than of glutamate decarboxylase, and this might account for the elevation of brain GABA after hydroxylamine administration. The trend toward a rise in Factor I after administration of Metrazol, Megimide and picrotoxin might be connected with hypoxic states produced by these convulsants.

As far as we are aware the possibility that GABA can serve as a substrate for monoamine oxidase has not been tested. The increase in free Factor I in the brain following repeated doses of iproniazid suggests that monoamine oxidase may be to some extent involved in the metabolism of GABA. Another possibility is that accumulation of some other amine may interfere with the metabolism of Factor I.

Interference with production of Factor I, as in insulin hypoglycaemia and hydrazide intoxication, results predominantly in a decrease in the amount of the occult form. Interference with further metabolism, as perhaps during hypoxia or after iproniazid or hydroxylamine treatment, results predominantly in an increase of the free form. These observations

suggest that Factor I is initially produced in the occult form and that it is mainly the free Factor I, released from the occult form, which undergoes further metabolism.

When thiosemicarbazide and iproniazid are present together, the level of occult Factor I in the brain tends to fall and free Factor I tends to accumulate and the total Factor I content can be in the normal range though convulsions still occur. The evidence provided by Killam & Bain (1957) and Killam (1957), that the convulsive effects of the hydrazides result from their effect on the GABA content of the brain, is strong. From the present results with the hydrazides alone and with iproniazid it seems that it is mainly the level of the occult fraction of the Factor I which is concerned in convulsive effects. Insulin hypoglycaemia is accompanied also by a decrease in the occult form and there may be a relation between this decrease and the convulsions that occur. There is no doubt that solutions of free GABA applied directly to the brains of animals *in vivo* exert inhibitory actions and oppose the action of the hydrazides (Purpura, Girado & Grundfest, 1957; Iwama & Jasper, 1957; Killam & Killam, 1958; Dasgupta, Killam & Killam, 1958). It is, however, reasonable to suppose that much of this GABA is absorbed into the occult condition by the tissue, just as GABA is absorbed by brain slices (Elliott & van Gelder, 1958), so that the level of occult GABA is raised. On this hypothesis we would not expect the elevation of free GABA, as in hypoxia or after hydroxylamine administration, to affect the susceptibility of animals to convulsions. Eidelberg, Baxter, Roberts, Saldias & French (1959), however, have produced evidence that the raised levels of GABA produced in cerebral cortex by hydroxylamine administration are associated with raised electrographic seizure thresholds. Either the increase in free Factor I *is* effective or the associated smaller increase in occult Factor I is the significant factor.

No consistent effects on free or occult Factor I were found to be associated with most of the other drugs tested. It seems that the main effects of these drugs are not mediated through changes in Factor I unless such changes occur only in restricted specific regions of the brain.

SUMMARY

1. The proportion of 'free' (extractable with saline medium without heat) to 'occult' (liberated by heat) Factor I in normal rat brains is fairly constant. The occult Factor I is not released by grinding the tissue with sand.

2. When brain tissue is suspended in salt-free sucrose solutions most of the occult Factor I becomes free.

3. The total Factor I content is decreased in brains from rats suffering from coma and convulsions after insulin treatment and from rats suffering from convulsions induced by hydrazides. The decrease after insulin and thiosemicarbazide is mainly in the occult fraction.

4. The total Factor I content is increased by hypoxia, hydroxylamine administration and by repeated doses of iproniazid. These increases are most marked in the free fraction.

5. No obvious changes in Factor I content were observed after administration of various other convulsant, anaesthetic or psychotropic drugs.

This work was aided by grants from the Banting Research Foundation and the Wellcome Trust.

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