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OBSERVATIONS ON A VASOACTIVE MATERIAL IN BRAIN EXTRACTS

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The extraction and chromatographic purification of a vasoactive material in brain tissue are described. It is inactive on the guinea-pig isolated ileum and rat uterus. Its chemical identity is as yet unknown. It is insoluble in nonpolar organic solvents, is stable in watery solution, but is inactivated by oxidizing agents. It is probably neither a nucleotide, a known amine, nor an inorganic salt.

Several of the pharmacologically active substances in the brain are present in high concentration in the brain stem, whereas much lower concentrations, or none, have been found in some other parts of the brain, including the cerebellum. In the experiments described here extracts of the cerebellum and of other parts of the brain were examined for activity on some biological test preparations.

METHODS

Dogs and cats were anaesthetized with chloroform or ether and killed by bleeding. Tissues were dissected within 15 min after the death of the animal. Some experiments were done on tissue stored at −20° C for up to 10 days.

After preliminary mincing, the tissue (0.2 to 1.0 g) was homogenized in 0.5 N-perchloric acid (4 ml./g of tissue). The homogenate was centrifuged, the sediment discarded, and the supernatant fluid adjusted with 3 N-potassium carbonate solution to approximately *p*H 4. The sample was chilled in an ice:water mixture and centrifuged. The supernatant fluid was collected and evaporated to dryness *in vacuo*. The residue was stirred with a small volume of pure ethanol. After centrifugation the ethanol supernatant fluid was applied to paper for chromatography.

In some experiments the tissue was homogenized in acidified ethanol as described by Vogt (1953). This extraction procedure gave a slightly lower yield of the active material than that described above using perchloric acid.

Two solvent systems were used for ascending paper (Whatman No. 1) chromatography, namely phenol:hydrochloric acid (Vogt, 1953) and *n*-butanol:glacial acetic acid:water (4:1:1), both run for 15 hr at room temperature or at 26° C. Strips were cut out and eluted with water, and the eluates were evaporated and taken up in a small volume of 0.9% saline for bioassay. Sometimes the strips were eluted with 0.4% (w/v) sodium dihydrogen phosphate solution and the residue from the evaporated eluate was dissolved in water. Eluates of successive strips were routinely assayed on the blood pressure of the pithed rat (Muscholl & Vogt, 1957). The phenol:hydrochloric acid solvent was used in most experiments because

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it gave better separation of the vasoactive materials present in the extracts. In some experiments assays were done in parallel on the blood pressure of the pithed rat and on the chick nerve-muscle preparation. In these experiments chicks weighing 100 to 200 g were anaesthetized with pentobarbitone sodium (30 to 40 mg, intramuscularly). The Achilles tendon was attached to an isometric myograph lever and the sciatic nerve was stimulated electrically with shocks of supramaximal intensity delivered by a Grass SD 5 stimulator, at a frequency of 6 or 12 shocks/min and a duration of 1 to 5 msec. Drugs and eluates were also tested on the blood pressure of the anaesthetized rat and guinea-pig, the guinea-pig ileum, the rat uterus, the rabbit perfused ear and the goldfish isolated intestine suspended in a microbath (Gaddum & Szerb, 1961).

Drugs. Doses of noradrenaline refer to the base and those of the other compounds to the salts. The drugs used were (−)-noradrenaline bitartrate anhydrous (L. Light & Co.), (−)-adrenaline base (Burroughs Wellcome), (±)-isoprenaline sulphate (Boots), 5-hydroxytryptamine creatinine sulphate (Upjohn), histamine acid phosphate (B.D.H.), acetylcholine chloride (Roche), 1,1-dimethyl-4-phenylpiperazinium iodide (Parke, Davis & Co.), atropine sulphate (B.D.H.), mepyramine maleate (Anthisan, May & Baker), methysergide (Sandoz), phenoxybenzamine hydrochloride (Dibenyline, Smith, Kline & French), pronethalol (2-isopropylamino-1-[2-naphthyl]ethanol hydrochloride, I.C.I.), hexamethonium bromide (May & Baker), adenosine monophosphate (C.F. Boehringer & Soehne), uridine triphosphate (Pabst Laboratories), uridine diphosphate (Sigma), uridine monophosphate (Pabst Laboratories) and l-(+)-glutamic acid (Hopkin & Williams).

RESULTS

When the eluates of successive strips of a chromatogram of brain extract were tested on the blood pressure of the pithed rat, a rapid and brief pressor response was produced by strips cut out at the R_F value of approximately 0.7 (phenol: hydrochloric acid) or 0.3 (butanol: acetic acid). Fig. 1 shows the type of response to a portion of an eluate corresponding to 160 mg of fresh cerebral cortex: it is much more short-lived than that to an equipressor dose of noradrenaline, and similar to the first phase of the response to adrenaline. The dry weight of the whole of such an eluate was less than 1 mg, and for a single injection not more than one-fifth of the eluate was used.

All tests for occurrence and chemical properties of this fast pressor activity were carried out on eluates of strips 4 to 6 cm wide centred around the R_F value of

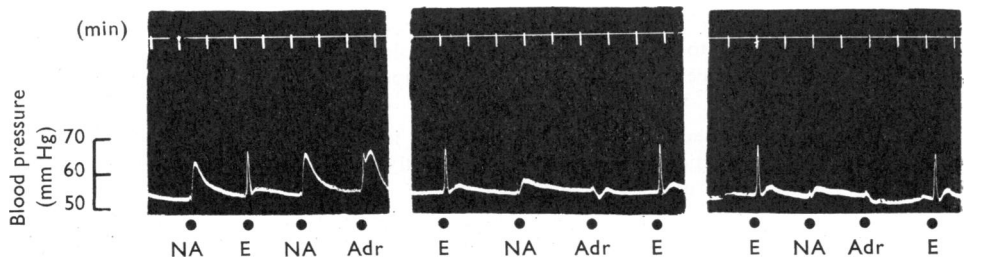

Fig. 1. Pithed rat, female, 175 g. Upper tracing, time in minutes. Lower tracing, arterial blood pressure. Black dots denote intravenous injections: NA, 10 ng of noradrenaline; Adr, 15 ng of adrenaline; E, 0.04 ml. of eluate (corresponding to 160 mg of fresh tissue) from a chromatogram of an extract of dog cerebral cortex. During the interval (12 min) between the first and second records 0.8 mg of phenoxybenzamine was injected intravenously. During the interval (11 min) between the second and third records 0.4 mg of phenoxybenzamine was injected intravenously.

0.7 in the phenol: hydrochloric acid solvent and tested on the blood pressure of the pithed rat. Substances of known R_F value which partially overlap with this activity are isoprenaline, which travels a little more slowly, and vasopressin, which travels faster (Vogt, 1959).

The fast pressor activity was present in all parts of the brain examined, including cerebellar cortex, cerebral cortex, nucleus caudatus, thalamus (massa intermedia), hypothalamus, mesencephalon, white matter of the hemispheres, corpus callosum and medulla oblongata. Cerebral cortex, neocerebellum and palaeocerebellum yielded approximately the same amount of activity ; the other regions were probably not very different in their content either, except for the mesencephalon which, in at least one instance, was only half as active as the cerebellar cortex. In the hypothalamus the fast pressor activity became apparent only after treatment of the eluate with thioglycollate. Without such treatment there was a large, slow pressor response, presumably due to vasopressin (Vogt, 1953).

Liver extracts gave variable results, indicating the presence in that tissue of little, or sometimes no, activity. A kidney extract contained, per gram, one-third of the activity of the cerebral hemispheres.

The active principle was readily soluble in water at neutral, alkaline, and acid *p*H values. It was poorly soluble in acetone and ethyl acetate. In partition experiments between water and diethyl ether, benzene, and *n*-butanol, carried out at acid *p*H (approximately 2.5) and alkaline *p*H (approximately 8.5), the activity remained in the water phase.

Heating the watery eluates for 25 min in a boiling water-bath at *p*H 2.5 or at *p*H 8.5 did not affect the activity. Heating the dry residue at 160° C for 1 hr decreased the activity by 30 to 50% and heating for 17 hr in 6 N-hydrochloric acid at 105° C abolished it. Incubation with carboxypeptidase (Fraenkel-Conrat, Harris & Levy, 1955) or thioglycollate (Vogt, 1953) did not inactivate the eluates. Heating for 30 min with two volumes of 6% (w/v) hydrogen peroxide solution in a boiling water-bath, or treatment with chlorine for 30 min at room temperature, both caused complete inactivation.

The eluates from a cut-up chromatogram developed in phenol: hydrochloric acid were examined for fluorescence at *p*H values of 4, 7 and 11 by scanning both exciting and emitted wavelengths in an Aminco-Bowman fluorimeter. Any fluorescence peaks seen did not coincide with the distribution of the fast pressor activity along the chromatogram. Nor did the absorption of ultra-violet light by the butanol: acetic acid chromatograms give any indication that the active material absorbed more light than adjacent, inactive substances.

The following antagonists were injected intravenously into the pithed rat and were found not to inhibit the fast pressor response to the eluates: atropine, methysergide, phenoxybenzamine, mepyramine and hexamethonium. These drugs were used in doses sufficient to abolish, or markedly reduce, the responses to acetylcholine, 5-hydroxytryptamine, noradrenaline and adrenaline, histamine and dimethylphenylpiperazinium respectively. Nor did pronethalol, in doses sufficient to abolish the depressor and increase the pressor response to adrenaline, alter the response to the eluate.

Eluates giving the fast pressor response in the pithed rat, injected intravenously into chicks in doses corresponding to as much as 0.4 g of brain tissue, had no significant action on the chick nerve-muscle preparation. The guinea-pig isolated ileum and the rat isolated uterus did not contract when the eluate from 0.4 g of fresh brain tissue was added to a 2 ml. organ-bath. In none of the three preparations were the responses to acetylcholine modified by the eluate. A preparation of the goldfish isolated intestine which responded to 1 ng of uridine diphosphate did not contract in response to a dose of eluate corresponding to 0.2 g of fresh brain tissue. When the eluate was injected into the perfusion fluid of a rabbit isolated ear the responses were variable; often a brief vasoconstriction was produced, usually followed by a more prolonged vasodilatation; sometimes a prolonged vasodilatation was the only effect. These effects were resistant to doses of phenoxybenzamine sufficient to block vasoconstriction produced by noradrenaline. In the anaesthetized rat and guinea-pig, in which the blood pressure was two- to three-times higher than that of the pithed rat, doses of eluates that produced the rapid pressor responses in the pithed rat were mainly depressor.

The effects on the blood pressure of the pithed rat of some other substances known to be constituents of brain tissue were examined, including four nucleotides. Of these, adenosine monophosphate gave a depressor response; and uridine mono- and diphosphate, in doses of 10 to 50 μg/kg and higher, gave pressor responses which were slower and more prolonged than those to noradrenaline and to the eluate. It was also found that uridine diphosphate travelled very slowly in a chromatogram run in the phenol: hydrochloric acid solvent, the R_F value being 0.1 or less. Uridine triphosphate (250 μg/kg) gave a diphasic response, consisting of a rapid initial depressor phase followed by a more prolonged pressor phase.

Three basic proteins, one of them prepared from ox cerebral cortex by Dr. L. S. Wolfe, were tested in amounts exceeding the total weight of vasoactive material when given as a single injection in the present work. They were an arginine-poor histone from ox brain (0.9 mg), salmine sulphate (2.5 mg) and clupein sulphate (2 mg); no effect on the pithed rat's blood pressure was seen. An arginine-rich histone from ox brain (1 mg) was also tested and found to be inactive, but it was poorly soluble and the quantity injected is therefore unknown. Sodium glutamate was inactive in doses up to 14 mg/kg, and so was potassium chloride in doses up to 2.5 mg. "Reagent blanks" carried through the whole chemical procedure, including chromatography, were inactive.

DISCUSSION

Although the chemical identity of the fast pressor activity on the pithed rat remains to be established, its properties make it distinguishable from many known constituents of brain tissue. The material was eluted from a region of the chromatogram to which vasopressin and isoprenaline might migrate, if present in the extract. However, in contrast to the fast pressor material, vasopressin is inactivated by thioglycollate and isoprenaline gives a depressor response on the blood pressure of the pithed rat. Other substances which occur in the brain, such as the amines acetylcholine, histamine, 5-hydroxytryptamine, noradrenaline and adrenaline, are

excluded, some by the nature of their effects on the blood pressure of the pithed rat, others by their R_F values. However, the fact that atropine, mepyramine, methysergide and phenoxybenzamine did not alter the fast pressor response of the pithed rat also excludes substances which would react with the receptors of the known naturally occurring amines, but, because of some chemical modification, have another R_F value. Glutamic acid, a powerful neurone depolarizing agent (Curtis, Phillis & Watkins, 1960), was inactive on the blood pressure of the pithed rat.

Special attention was paid to the question whether the pharmacological activity of the eluates could be due to uridine phosphates, in view of the recent finding by Gaddum & Smith (1963) that uridine diphosphate, when present in a tissue extract, can interfere with the bioassay of substance P, if steps are not taken to separate the two. Uridine diphoshate cannot be the pharmacologically active material in the eluates, because uridine diphosphate travelled much more slowly than the vasoactive material in the phenol: hydrochloric acid chromatograms, and because the eluates did not contract the goldfish intestine, which is very sensitive to uridine diphosphate (Gaddum & Smith, 1963). In addition, the blood pressure effect of uridine nucleotides on the pithed rat is quite different in shape from that of the eluates. Similarly, adenosine monophosphate, which also occurs in tissue extracts, is, in contrast to the substance in the eluates, very active on isolated tissues (Laszlo, 1963) and produces blood pressure effects of a different shape. The destruction of the vasoactive material by oxidative agents and its inactivation by acid hydrolysis, suggest that the active material is an organic compound. Considering its solubility characteristics, it seems unlikely that it is a lipid, but it might be a peptide or a basic protein. Among these, bradykinin is excluded since that substance is oxytocic, and depressor in the pithed rat. The basic proteins tried lacked pressor activity.

The amount of the fast pressor material needed to produce the characteristic response on the blood pressure of the pithed rat is unknown, but it is less than 200 μg. It is noteworthy that the material, in contrast to several other pharmacologically active substances and in spite of its distinct activity on blood vessels, is inactive on isolated smooth muscle preparations such as the guinea-pig ileum and the rat uterus. It might also be mentioned that the cerebellar factor (Crossland & Mitchell, 1956), which is present in the cerebellum and in other parts of the brain, is destroyed by brief boiling at *p*H 5.5 or below and is therefore probably not identical with the active material described here.

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