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PRELIMINARY COMMUNICATION

Attempts to purify and identify an acetylcholine-releasing substance from neural tissue*

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RECENT reports [1,2] have described the pharmacological properties of a lipoidal material present in brain and peripheral nerve that causes a slow contraction of the isolated guinea pig ileum which can be blocked with atropine. Evidence has been presented[1,2] indicating that this material acts by releasing endogenous stores of acetylcholine in the ileum. We wish now to describe efforts at isolation and identification of this substance in the hope that others might desire to pursue the problem.

In the extraction and purification procedures, activity was followed by bioassay of the isolated guinea pig ileum;[1] we used as criterion a slow contraction that was totally blocked by atropine. The potency of the extract was estimated in terms of acetylcholine and expressed as units, one unit equaling the height of contraction produced by 1 ng acetylcholine.

The initial isolation procedure consisted of homogenizing neural tissue (either rabbit sciatic nerve or pig brain) in 20 volumes of cold acetone and stirring the homogenate at 0° for 60 min. After centrifugation, the acetone extract (which contained up to 300 units/g tissue) was evaporated under reduced pressure at room temperature, and the residue was taken up in chloroform : methanol (2:1 v/v). This was mixed with one fifth its volume of water, and the two-phase system was centrifuged. The lower chloroform phase was removed and evaporated under reduced pressure. The residue was then dissolved in a small quantity of petroleum ether (B.R. 30°–60°). Roughly 70 per cent of the activity in the initial acetone extract remained in the petroleum ether extract. This material was termed the "crude lipid extract."

TABLE 1. ELUTION PATTERN OF ACTIVE MATERIAL FROM SILICIC ACID COLUMN

Fraction	Eluting solvent	Volume (ml)	Biological activity (units/g fresh tissue)	Yield (% of activity added to column)
1	1% Diethyl ether in petroleum ether	250	0	
2a	2% Diethyl ether in petroleum ether	150	0	
b		75	0	
c		75	33	13
d		75	8	3
e		75	0	
3a	3% Diethyl ether in petroleum ether	75	0	
b		75	0	
c		150	0	
4	Methanol	300	0	

The crude lipid extract could be fractionated by column chromatography[3] on silicic acid (BioRad) at 4°. In a typical experiment (Table 1) the crude lipid extract from 35 g of frozen pig brain was added to a silicic acid column (48 g) which had been previously washed with petroleum ether. The column was eluted with 250 ml of 1% diethyl ether in petroleum ether, followed by successive elutions

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with 2% diethyl ether, which removed, reproducibly, the active material. Further elution with increasing concentrations of diethyl ether and with 100% methanol did not elute further active material. The yield from the column was low, averaging 20 per cent of that added (range, 7–28 per cent). The material eluted from the column, a small quantity of clear to yellow oil, was immiscible with water; for bioassay the material was suspended in 10% (by volume) propylene glycol in water. (One lot of silicic acid out of several lots tested was not satisfactory for this method; simultaneous chromatography on this lot in comparison with a second lot showed that the active material could be eluted from the second column but not from the first.)

Thin-layer chromatography was generally unsatisfactory. Attempts to purify eluates from the column by thin-layer chromatography on silica gel G with 5% diethyl ether in *n*-hexane[4] gave recoveries of active material of less than 10 per cent, all activity remaining at the origin. Somewhat better results were obtained when the chromatography was done in a nitrogen atmosphere. With benzene:ethyl acetate (2:1) two main zones of activity were found at R_f 0·1 and 0·6–0·7. Re-chromatography of each zone yielded active material mainly at R_f 0·1. Recovery under these conditions was frequently 50–100 per cent.

These results suggest that adsorption of the active material occurred and that this became more apparent with increasing purity. Thin-layer chromatography was useful, however, in detecting separation of the active principle from cholesterol, which was the most abundant impurity in acetone extracts of pig brains.

Because of these problems with chromatographic methods, solvent distribution techniques were tried on the crude lipid extract. Cholesterol was the most abundant impurity in crude extracts, and solvent systems to remove it were used. Methanol containing 10–30 per cent water vs. petroleum ether alone or containing small amounts of other solvents (e.g. 1% diethyl ether or 2% ethyl acetate) extracted over 90 per cent of the solids of the original acetone extract into the upper phase while the activity largely remained in the lower (methanol–water) phase, although the activity recovered was frequently only 10–30 per cent. Countercurrent distribution (mixtures of petroleum ether:methanol:water, 100:95:5; or petroleum ether:diethyl ether:methanol:water, 54:6:51:9) carried out at 4° confirmed the above simple extractions, but recovery of activity was also low.

Larger-scale operations, using up to 10 kg pig brain, were attempted, but greater losses occurred than with smaller-scale extractions, and the yields of active material were quite low.

The stability of the active material varied with the suspending medium and the storage temperature. Bubbling nitrogen through the solvents to be used was beneficial. Storage in chloroform at 4° resulted in complete loss of activity after several days, whereas the suspension in 10% propylene glycol was stable for several weeks in the refrigerator.

There was considerable difference in the units of active material obtained per gram of pig brain in our two laboratories. The yield obtained by the Yale group was 10 to 100 times as great as that obtained by the Abbott group. The cause of this discrepancy is not known, although it could lie in the source of material or in subtleties in the extraction or assay procedure.

Since attempts to isolate the active material in pure form were unsuccessful, determinations of lipid functional groups in the fractional elutions from the silicic acid column (Table 1) were undertaken. Measurements on successive elutions were made of cholesterol,[5] long-chain aldehydes,[6] and glycerol[7] both after saponification (representing the fatty acid glycerides) and after subsequent acid hydrolysis[8] (representing the alkenyl analogs of the glycerides). No correlation between the magnitude of any of these determinations and biological activity in the elutions was found. Although the activity of the material in the crude acetone extract was enhanced by heating at 100° for 5 min at pH 10,[2] refluxing in 1 N alcoholic KOH or HCl for 60 min destroyed activity. Paper chromatography of the hydrolysis products revealed no ninhydrin-positive spots, indicating that the active material was probably not a fatty acid amide of an organic amine.[9] Finally, when either the crude lipid extract or the active eluate resuspended in petroleum ether was extracted with aqueous K_2CO_3, essentially all the activity remained in the petroleum ether, indicating that the active material was not an acid.

In summary, no clear indication of the chemical nature of the active material was obtained, although many classes of lipids were tentatively eliminated. From the elution pattern on the silicic acid column,[3] the active material may be classified as a nonpolar neutral lipid; it is clearly not a phospholipid or sphingolipid. From its solvent distribution it did not appear to be an acid, distinguishing it from the group of biologically active long-chain acids such as the prostaglandins. Chemical analyses showed no correlation of the elution of the active material with cholesterol or cholesterol esters, fatty acid

glycerides or the ether analogs of the glycerides, or long-chain aldehydes, all of which were eluted chiefly in other fractions. Thus if the material belonged to one of these classes it was in insufficient quantity to be distinguished from the small quantities leading or trailing the major elution fractions of these compounds. Finally, chromatography of the hydrolysis products did not reveal the liberation of detectable quantities of any amine, arguing against a fatty acid amide in measureable quantities.

Although certain difficulties, such as the inability to secure sufficient quantities of active material and the instability of the active material to silicic acid chromatography, have hampered efforts to identify the substance, it is hoped that the unique and intriguing pharmacological properties of this lipid will encourage others to seek its identity.

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