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SHORT COMMUNICATIONS

Identity with authentic acetylcholine of acetycholine-like activity in extracts of rat brain*

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HOSEIN *et al.*[1, 2] have expressed doubt that the material extracted from rat brain and bioassayed as total acetylcholine (ACh) is, in fact, ACh. These workers believe that the great bulk (75%) of this material is a mixture of esters of α-butyrobetaine, crotonobetaine, and carnitine with coenzyme A (CoA) ,which behave qualitatively like ACh on a preparation of frog rectus abdominis muscle.

TABLE 1. CHROMATOGRAPHIC CHARACTERISTICS AND BIOLOGICAL ACTIVITY OF AUTHENTIC ACETYLCHOLINE-$^{14}CH_3$ CO-CHROMATOGRAPHED WITH EXTRACTS OF RAT BRAIN

Expt. No.	Materials chromatographed	System*	R_f of peak radioactivity for ACh alone alone	R_f of peak radioactivity for co-chromatographed ACh	Range of R_f values covering biological activity†	Remarks
1	ACh alone	Descending	0·1	—	—	
2	Ach with trichloroacetic acid	Descending	0·1	—	—	Tail of radioactivity throughout chromatogram
3	ACh alone	Descending	0·08		0·2–0·60	77% recovery
	ACh with crude extract			0·54		
4	Reineckate of ACh alone	Descending	0·12		0·12–0·36	70% recovery
	Reineckates of crude extracts with ACh			0·30‡		
5	Crude extr. with with ACh	Ascending		0·72	0·66–0·84	63% recovery
6	ACh alone	Ascending	0·40			
	ACh with crude extr.			0·75	0·50–0·78	68% recovery
7	Reineckates of ACh with crude extr.	Ascending		0·76§	0·50–0·83	50% recovery

* Ascending system is that of Hosein *et al.*, 1961; descending system is that of Augustinsson and Grahn, 1954. Cf. text for details.
† Biological activity was determined by bioassay on the frog's rectus abdominis muscle preparation.
‡ The eluate from this peak has been rechromatographed in the ascending system: R_f 0·30.
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Hosein and Ara[3] have suggested that 90% of the increased ACh-activity observed in the brains of animals depressed with either pentobarbital or ether is to be accounted for by an increased concentration of the esters of CoA named above. In view of the interest, in this and other laboratories, in

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drug-induced changes in cerebral ACh, estimated by bioassay,[4, 5] it seemed essential to us to attempt to identify the material contributing to the ACh-like activity of brain extracts. Up to the present time, the most careful studies of such activity in tissues have been made with extracts of ox spleen[6, 7] and of the honeybee.[8]

Our experiments were conducted in the following general manner. Brains of unmedicated, adult male rats (Sprague-Dawley, 150 to 180 g) were homogenized and extracted with trichloroacetic acid

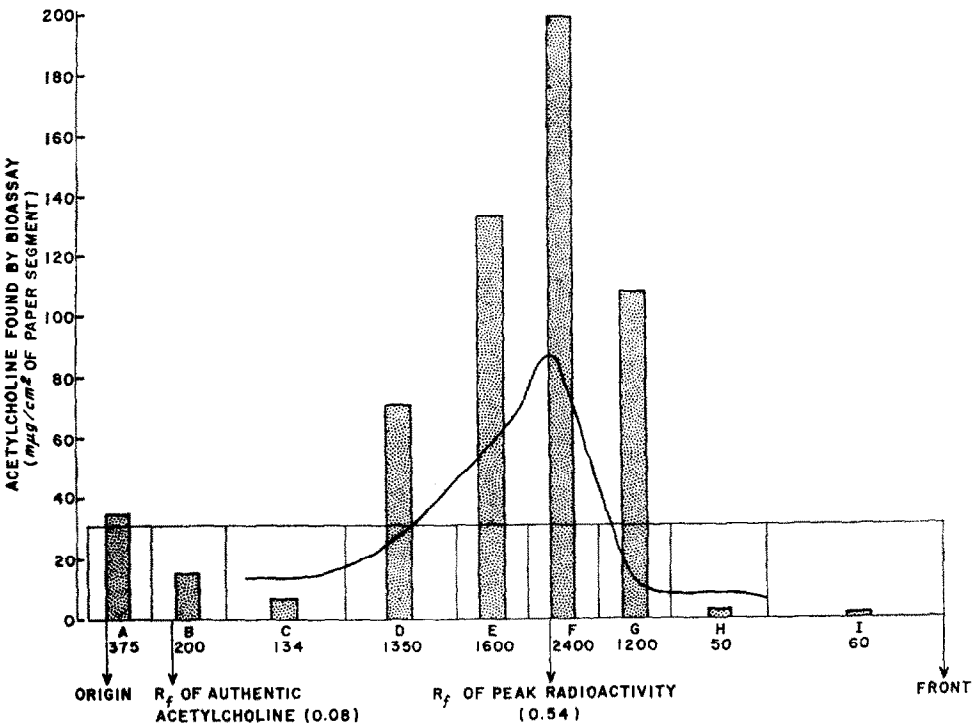

FIG. 1. Facsimile of the chromatogram derived from an extract of rat brain with co-chromatographed ACh-$^{14}CH_3$, developed in the descending system of Hosein *et al.*[2] (experiment 3 in Table 1). The continuous curve represents a scan of the radioactivity (obtained on a Vanguard automatic chromatogram scanner). The vertical bars show the amount of acetylcholine-activity estimated by bioassay and expressed in terms of mμg/cm² of paper segment because these segments differed in size. The numbers on the abscissa (beneath the letters) refer to the total amount of acetylcholine (in mμg) found in each paper segment. The R_f of authentic ACh was taken from another chromatogram developed in the same system.

(TCA), essentially by the method of Smallman and Fisher.[9] Ether was used to remove excess TCA from the crude extracts. Some of the extracts were treated with Reinecke salt at acidic pH* (after Gardiner and Whittaker[7]). Final residues of material obtained by evaporation *in vacuo* were

* Preparation of the Reineckates was as follows: the crude extract was treated three times with anhydrous ether (two volumes) and then allowed to stand with 2 ml Reinecke salt (saturated aqueous solution, ca. pH 5) for 1 hr at 5°. The mixture was centrifuged at 30,000 × *g* for 30 min and the precipitate washed with a minimal amount of water. After recentrifugation and decanting, the precipitate was taken up in the minimal volume of acetone necessary to dissolve it. Aqueous silver chlorate solution (2 M) was then added in drops until the solution contained a slight excess of silver. This was centrifuged and the supernatant fraction treated with saturated sodium chloride solution until no more precipitate of silver chloride formed. The precipitate was removed by centrifugation and the supernatant fraction was evaporated to dryness *in vacuo*. The dried residue was dissolved in ethanol prior to chromatography.

dissolved in ethanol and chromatographed in one of two different systems on Whatman 1 paper: (1) a descending system (according to Hosein *et al.*[2]), consisting of water-saturated *n*-butanol and development of the chromatogram for 17 hr at room temperature; (2) an ascending system (according to Augustinsson and Grahn[8]), consisting of butanol:ethanol:acetic acid:water (8:2:1:3) and development of the chromatogram for at least 24 hr at room temperature (minimum, 30-cm run).

In most experiments, acetylcholine-$^{14}CH_3$ chloride (10 μg; specific activity, 0·02 mc/μg; ca. 200,000 counts/min), added at the time of homogenization of the brain, was co-chromatographed with the crude extracts. Similar chromatograms, without added ACh-$^{14}CH_3$, were run under identical conditions and cut into 2- to 4-cm strips, each of which was eluted with frog-Ringer's solution and assayed for ACh on the frog's rectus abdominis muscle preparation (suspended in aerated frog-Ringer's solution containing physostigmine, 10 mg/l).

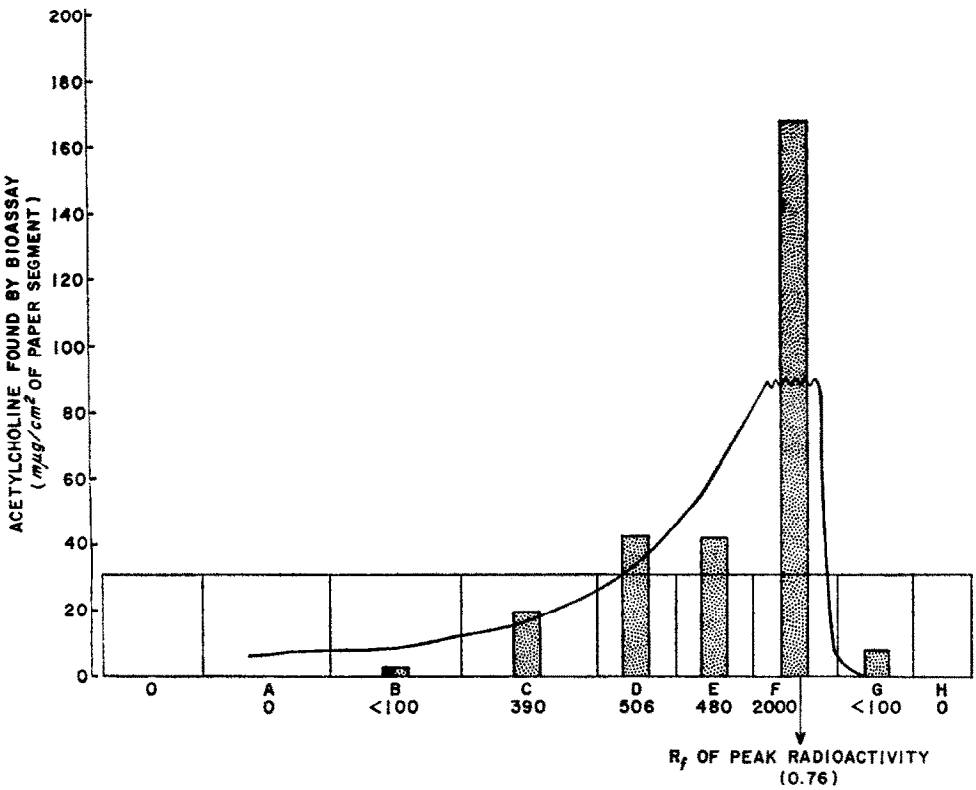

FIG. 2. Facsimile of the chromatogram derived from the Reineckates of a rat brain extract, with co-chromatographed ACh-$^{14}CH_3$, developed in the ascending system of Augustinsson and Grahn[8] (experiment 7 in Table 1). The meaning of the continuous curve and vertical bars is as indicated in Fig. 1. The R_f of authentic ACh taken from another chromatogram developed in the same system was 0·4.

With some experiments an independent strip bearing 10 μg of ACh-$^{14}CH_3$ was run; under the same experimental conditions a recovery of about 80% of this amount was achieved. Recoveries of ACh added to the crude extracts ranged from 63 to 77%, and of the Reineckates of ACh, 50 to 70%.

Some of the results are summarized in Table 1. These data show clearly that extracts of brain prepared under our conditions greatly alter the mobility of authentic ACh in both systems studied. The data in Table 1 also make clear that the great bulk of the biological activity is found in the region of the altered R_f of the co-chromatographed ACh-$^{14}CH_3$. Further confirmation of this point may be seen in Fig. 1 which depicts the biological activity and monitored radioactivity of a typical chromatogram of a brain extract co-chromatographed with 10 μg of ACh-$^{14}CH_3$. Similar relationships with

Reineckates of ACh and components of the crude extract are shown in Fig. 2. It should be pointed out that, although some of the biological activity seems to lie outside the peak of radioactivity, this may be more apparent than real, because it is possible that all the "discrepant" biological activity is sharply localized along the inside edge of the rather broad strips at each end of the radioactivity-peak.

We believe that these findings cast doubt on the validity of the contention of Hosein and Ara,[8] especially in view of the fact that the alteration in the R_f of ACh (0·54) caused by tissue extractives, as found in our studies with Hosein's system, falls within the R_f quoted by these authors for the betaine CoA esters (0·5 to 0·7). While the existence of such esters is not disproved by this work, it is apparent from our data that ACh is responsible for the bulk of the biological activity in these extracts.

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REFERENCES

1. E. A. HOSEIN in *Protides of Biological Fluids*, H. Peters, Ed., vol. 7, p. 275. Elsevier, Amsterdam (1960).
2. E. A. HOSEIN, P. PROULX and R. ARA, *Biochem. J.* **83**, 341 (1961).
3. E. A. HOSEIN and R. ARA, *J. Pharmacol. exp. Ther.* **135**, 230 (1962).
4. D. RICHTER and J. CROSSLAND, *Amer. J. Physiol.* **159**, 247 (1949).
5. N. J. GIARMAN and G. PEPEU, *Brit. J. Pharmacol.* **19**, 226 (1962).
6. J. BANISTER, V. P. WHITTAKER and S. WIJESUNDERA, *J. Physiol., Lond.* **121**, 55 (1953).
7. J. E. GARDINER and V. P. WHITTAKER, *Biochem. J.* **58**, 24 (1954).
8. K. B. AUGUSTINSSON and M. GRAHN, *Acta physiol. scand.* **32**, 174 (1954).
9. B. N. SMALLMAN and R. W. FISHER, *Canad. J. Biochem.* **36**, 575 (1958).

A folic acid-active compound strongly bound by DEAE-cellulose*

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POLYGLUTAMYL conjugates of folic acid (pteroylglutamic acid) and of a number of tetrahydrofolates are widely distributed in plant and animal tissues;[1] they cannot be measured by the usual microbiological assay techniques without prior enzymatic cleavage to folic acid, 5-formyltetrahydrofolic acid, or closely related compounds. *Lactobacillus casei* cannot utilize pteroylglutamates containing more than three glutamic acid residues per molecule,[2] and *Streptococcus faecalis* cannot utilize compounds more complex than pteroyl-γ-glutamylglutamate.[3] Accordingly, a compound that permits growth of *L. casei* or *S. faecalis* maintained in a folic acid-deficient medium is generally regarded as closely related in structure to pteroylglutamate or tetrahydropteroylglutamate. All the known compounds of this group migrate readily on DEAE- or TEAE-cellulose with phosphate buffers of neutral pH; the pteroylpolyglutamates tend to migrate more slowly than do the monoglutamates.[4, 5]

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