J. Pharm. Pharmac., 1969, **21**, 302–308 *Received November* 22, 1968

The influence of ATP in brain extracts on the estimation of acetylcholine assayed on the frog rectus abdominis muscle

I. H. STOCKLEY

Pharmacy Department, University of Nottingham, Nottingham, England

Comparative studies of the sensitizing properties of creatine phosphate, ATP and a trichloroacetic acid brain extract on the acetylcholine-induced contractures of the frog rectus muscle indicate that alkaline boiling increases the sensitizations due to these substances threefold. This increase is reflected in a 25% reduction in the values for acetylcholine in brain extracts when assayed on the rectus muscle using Feldberg's method. Since tissue extracts made with acid-alcohol, formic acid-acetone, eserine-saline and sucrose-saline also contain sensitizing substances, the acetylcholine values obtained by this assay method may be in error.

Because substances present in brain extracts increase the contracture of the frog rectus muscle produced by acetylcholine,Feldberg (1945) introduced a modification of the assay of acetylcholine to allow for their presence. This involved boiling part of the brain extract briefly in alkali to destroy the acetylcholine present, and after neutralization, adding a known amount of acetylcholine. The untreated extract was then assayed against the boiled extract on the rectus muscle using a bracketing dose technique. The substances responsible for the increase in contracture appear to be adenosine triphosphate and creatine phosphate (Babskii, Koronevskaya & Minaev, 1945; Feldberg & Hebb, 1947; Babskii & Minaev, 1946a, b, c; 1947 a, b; Minaev, 1947; Golubtsova & Minaev, 1947).

In recent experiments made in this department, results have been obtained which suggest that Feldberg's modification introduces an error into the assay which is as big as that it was designed to obviate. The experimental evidence on which this observation is based forms the content of this report.

EXPERIMENTAL

Preparation of tissue extracts

Mice, guinea-pigs, rats and rabbits were killed either by submersion in liquid oxygen (or nitrogen), or by decapitation under sodium pentobarbitone anaesthesia. The anaesthetic was given by the intraperitoneal route in doses sufficient to cause deep anaesthesia as evidenced by the loss of all somatic reflexes. The brain tissue was removed immediately at death, weighed and extracted with either trichloroacetic acid, acid-alcohol, or formic acid-acetone, according to the methods of Crossland, Pappius & Elliott (1955), Stone (1955) and Toru & Aprison (1966) respectively.

Frozen brain tissue from animals killed by submersion in liquid oxygen was subjected to similar extraction procedures after first being powdered in a cooled steel anvil and homogenized with the extraction medium in a cooled glass mortar. The dissection was made quickly to minimize tissue autolysis. All extracts were

adjusted to pH 7 before assay, those being examined solely for brain tissue sensitizer were boiled for 3 min at pH 11 to destroy the acetylcholine present. Solutions (0·1N) of NaOH and HCl were used in conjunction with BDH Universal indicator paper for pH adjustment.

Unless otherwise stated, the expression "brain tissue sensitizer" refers to trichloroacetic acid extracts of brain which had been subjected to the alkaline boiling procedure.

The assay of ATP and creatine phosphate

The assay of ATP and creatine phosphate in brain extracts was according to Lepage (1957) and is based on a comparison of the extinctions at 680 nm of test and control solutions which had been treated with ammonium molybdate solution and Fiske and Subarrows reagent.

Bioassay of brain tissue sensitizer

The frog rectus muscle was suspended in a 3 ml bath in amphibian Ringer-Locke solution (NaCl 6·5, $CaCl_2$ 0·12, NaH_2PO_4 0·01, KCl 0·14 g/litre of water) containing neostigmine sulphate in a concentration of 1 : 1,000,000. Muscle contractures were recorded on a smoked drum using a gimbal lever with a tenfold magnification. Only muscles showing a similar sensitivity were employed for assay, namely those responding to 0·03 μg/ml acetylcholine with magnified contractions of approximately 1–2 cm. The per cent sensitization remains the same for a constant dose of sensitizer when estimated on acetylcholine-induced contractures which are less than 50% of maximal. Under these same conditions the dose-response relation of sensitizer is linear. The same dose of acetylcholine was administered to the muscle on a regular cycle (within the range 4–10 min depending on the relaxation time of the muscle) and kept in the bath for 1 min. Bioassay was by administering the acetylcholine and sensitizer simultaneously, the per cent sensitization being calculated by comparing the size of the sensitized contracture with the mean of the previous and subsequent unsensitized contractures. When for example a contraction of 2 cm is increased to 2·4 cm by the sensitizer, it is said to be a 20% sensitization. Aliquots of 1 ml of diluted extract containing the equivalent of approximately 100 mg of fresh tissue were used when assaying both for acetylcholine and for the substances causing sensitization.

Bioassay of acetylcholine

Bioassays of acetylcholine were made on the isolated frog rectus muscle sensitized with neostigmine sulphate using a bracketing dose technique, the method of Feldberg (1945) being used to allow for the presence of sensitizing substances.

RESULTS

A comparative examination of ATP, creatine phosphate and brain tissue sensitizer

Brain tissue sensitizer potentiated (or sensitized) the contracture of the frog rectus abdominis muscle produced by acetylcholine but had no direct stimulant action of its own. The sensitization was immediate and maximal on the first acetylcholine-induced contracture (Fig. 1). This sensitization appears to be of a different type from that caused by cerebrospinal fluid, according to the reports of Bhattacharya, Feldberg & Vogt (1957) and Ramwell (1964). This latter sensitization was only

evident after a 60–90s latent period and became maximal on the second or third contraction.

Solutions of ATP sensitized the acetylcholine-induced contracture of the frog rectus muscle, thus confirming the observations of Babskii & others (1945). Similar experiments failed to confirm the reports of Torda & Wolff (1945, 1946) that creatine phosphate has a slight but definite sensitizing effect.

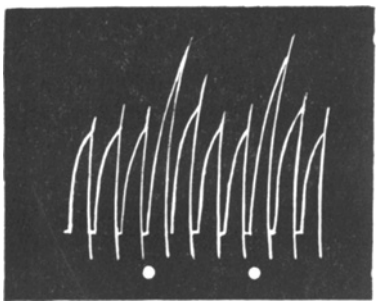


FIG. 1. Contractions of a frog rectus muscle to 0·03 μg/ml acetylcholine in the presence of neostigmine sulphate 1 : 1,000,000. The sensitizing effect of brain tissue sensitizer, the equivalent of 100 mg of fresh brain is shown at the dots.

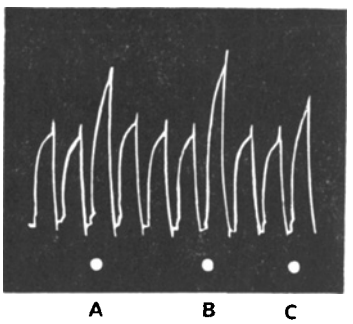
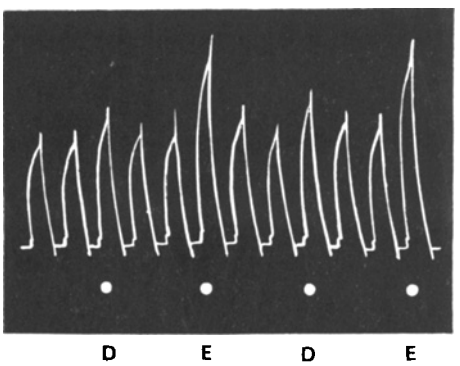


FIG. 2(a). The effects of 90 μg ATP (A), 90 μg ATP + 250 μg creatine phosphate (B), and 250 μg creatine phosphate (C) on the acetylcholine-induced frog rectus muscle contracture. All three solutions had been boiled in alkali for 3 min and reneutralized.



(b). The effects of 100 μg ATP (D) and 100 μg ATP which had been boiled for 3 min at pH 11 and reneutralized (E) on acetylcholine-induced frog rectus muscle contracture. The muscles were responding to 0·06 μg/ml acetylcholine in the presence of neostigmine sulphate 1 : 1,000,000.

Since the preparation of brain tissue sensitizer involves boiling the extract at pH 11 for 3 min to destroy the acetylcholine which is extracted by the trichloroacetic acid, further experiments were made on solutions of ATP and of creatine phosphate that had been submitted to the alkaline boiling procedure. Biological assay showed that creatine phosphate now caused slight sensitization, and the effect of ATP was much increased by alkaline boiling. Frog-Ringer solution subjected to the alkaline boiling procedure was found not to sensitize the contracture of the rectus. There was a marked similarity between the character of the sensitizations induced on the one hand by brain tissue sensitizer and on the other hand by ATP with creatine phosphate. The sensitizations were immediate with their maximal effect on the first acetylcholine-induced contraction, with diminishing effects on the subsequent contractions (Figs 2a, b). A small difference was that the sensitizing effects of the brain extract persisted longer than those of the ATP solutions.

Pooled rat brain tissue was assayed for ATP and creatine phosphate using the method of Lepage (1957). Based on the figures obtained, solutions were made containing equivalent amounts of ATP and creatine phosphate, and both these and the brain tissue extract were assayed for sensitization on the frog rectus muscle. The mean sensitization induced by a brain extract containing 100 μg ATP and 62 μg creatine phosphate was 60%, compared with a sensitization of 66% induced by a solution containing equivalent amounts of ATP and creatine phosphate.

The effect of alkaline boiling on the sensitization caused by ATP and brain tissue sensitizer

Solutions of authentic ATP were assayed for sensitizing activity both before and after brief alkaline boiling. Concurrent assays were also made using a brain tissue

Table 1. *Sensitizations of the frog rectus muscle contracture in the presence of either* 100 μg *ATP or an extract from* 100 *mg of brain tissue containing sensitizer*

Sensitizations due to ATP

	% sensitization		
Muscle No.	Before alkaline boiling	After alkaline boiling	Increase in sensitization
I	36	91	2·5
II	23	75	3·3
II	26	63	2·4
III	26	73	2·8
III	20	68	3·4
Mean values	26	74	2·8

Sensitizations due to brain tissue sensitizer

	% sensitization		
Muscle No.	Before alkaline boiling	After alkaline boiling	Increase in sensitization
I	33	90	2·7
I	31	107	3·4
II	38	93	2·4
II	28	89	3·1
III	25	65	2·6
Mean values	31	89	2·9

extract which contained too little acetylcholine to cause contraction of the rectus muscle when the equivalent of 100 mg brain was added to the organ bath. The results of the assays are in Table 1 from which it can be seen that alkaline boiling increased the sensitization in both cases by a factor of almost 3. This increase in sensitization is shown in Fig. 2b.

Feldberg (1945) does not state precisely the conditions for alkaline boiling but it was found that raising the temperature to 100° only momentarily was sufficient to cause a marked increase in sensitization.

The effect of the increase in sensitization due to the alkaline boiling on the assay of acetylcholine in brain tissue extracts

The results of the previous experiments indicated that the three-fold increase in the sensitizing activity of brain tissue extracts when boiling briefly in alkali would be reflected in a reduction in the values for acetylcholine obtained when using Feldberg's acetylcholine assay modification (1945). To confirm this experimentally, assays were made on a series of brain extracts (see Table 2) using Feldberg's method.

Table 2. *The results of acetylcholine assays made on the frog rectus muscle using the Feldberg* (1945) *method. The amounts of acetylcholine added to each extract and the amounts recovered are placed alongside one another*

Animals and method of killing	(i) Acetylcholine content of extract (μg/g brain)	(ii) Acetylcholine added to extract (μg/g brain)	(iii) Acetylcholine recovered (μg/g brain)	(iv) % recovery of added (col. ii) acetylcholine
Liquid nitrogen Rats 1 and 2 ..	0·75	1·68	1·16	69
Liquid nitrogen Rats 3 and 4 ..	1·23	1·11	0·95	86
Decapitation Rats 5 and 6 ..	1·80	0·77	0·57	74
Decapitation Rats 7 and 8 ..	1·38	1·45	1·02	70
				Mean: 75

Known amounts of acetylcholine were then added to the extracts and each was re-assayed by the same method. The amounts of added acetylcholine were not revealed to the assayist until the assays had been made. The mean percentage recovery of the added acetylcholine was 75%. This is in agreement with a theoretical expectation of an approximately 70% recovery which may be derived from a consideration of the probable effect of a threefold increase in sensitization due to alkaline boiling. For example, it is usual when assaying extracts for acetylcholine to add the equivalent of about 100 mg of fresh brain to the rectus muscle in a 3 ml organ bath. This amount of extract would contain about 0·05–0·2 μg of acetylcholine, and about 100 μg of ATP, sufficient to cause a 20–25% sensitization. The alkaline boiling would increase this to 60–70% approximately. Under these circumstances, 0·1 μg (say) of acetylcholine with unboiled sensitizer would cause the same sized rectus muscle contracture as would be obtained with approximately 0·07 μg of acetylcholine in the presence of the same amount of boiled sensitizer.

Preliminary investigations were made on the presence of ATP in extracts of brain tissue made with media other than trichloroacetic acid. Extracts of equivalent amounts of brain tissue made with acid-alcohol (Stone, 1955) and formic acid-acetone (Toru & Aprison, 1966) were found to induce sensitizations of the same order as those induced by TCA extracts.

DISCUSSION

The experiments described in this paper add experimental evidence to the suggestion made by Feldberg & Hebb (1947) that the sensitizing substances in brain extracts might be ATP and creatine phosphate. In addition they support the results of experiments of Minaev, Babskii, Koronevskaya and Golubtsova (Babskii & others, 1945; Babskii & Minaev, 1946a, b, c; 1947a, b; Minaev, 1947; Golubtsova & Minaev 1947).

The large increase in the sensitizing activities of brain tissue extracts and solutions of ATP after brief alkaline boiling seems to cast some doubt on the accuracy of those acetylcholine assays made on the frog rectus muscle using Feldberg's method (1945). This method appears to have been accepted uncritically and used widely even by the Russian workers who observed that '. . . exposure (of the rectus muscle) to acetylcholine with unboiled, but especially with boiled (brain tissue) emulsion caused greater contraction of the muscle than with acetylcholine alone'. (Golubtsova & Minaev, 1947). This is all the more surprising since Babskii & Minaev (1946) had at that time published a method for the assay of acetylcholine on the rectus muscle which allowed for the presence of sensitizing substances and which appears to be virtually identical with Feldberg's method (1945).

In view of the experimental evidence already described, it is probable that all the acetylcholine assays which have been made using the rectus muscle on aqueous extracts of brain (and other tissues which contain significant amounts of ATP) may be considerably in error. Aqueous extraction media known to extract sensitizing substances from tissues include trichloroacetic acid, acid-alcohol, formic acid-acetone, eserine-saline (Slater, 1966) and eserine-sucrose (Slater, 1966). The sensitizing activity of creatine phosphate on the other hand is small compared with ATP and it would seem unlikely to cause an error of any magnitude.

ATP is relatively stable to alkaline but not to acid boiling. Hock & Huber (1956) reported that when ATP is boiled for 1 h at pH 10·5, only some 30% is destroyed. The breakdown products of alkaline hydrolysis are ADP, AMP and orthophosphate, none of which together or individually induce a sensitization greater than ATP. Acid hydrolysis is much more rapid and liberates 2 of the 3 phosphates as inorganic phosphate with the production of adenine and ribose-5-phosphate (Tsujimoto & Yamaba, 1956; Michelson, 1963) none of which induce sensitization (Torda & Wolff, 1946). With the experimental evidence at present available it is only possible to make the most tentative suggestions about the cause of the increased activity of ATP when boiled in alkali. For example, it might result from an osmotic effect due to the HCl and NaOH added to the extract, or an ionic imbalance which increases the uptake of ATP by the muscle. These and other possibilities are the basis of further investigations into the phenomenon of sensitization by ATP of the rectus muscle contracture.

Acknowledgement

My thanks are due to Dr. J. Crossland for his advice and help in this investigation.

REFERENCES

BABSKII, E. B., KORONEVSKAYA, O. G. & MINAEV, P. F. (1945). *Bull. biol. Med. exp. U.R.S.S.*, **20**, 60–62.

BABSKII, E. B. & MINAEV, P. F. (1946a). *Nature, Lond.*, **158**, 238.

BABSKII, E. B. & MINAEV, P. F. (1946b). *Ibid.*, **158**, 268.

BABSKII, E. B. & MINAEV, P. F. (1946c). *Bull. biol. Med. exp. U.R.S.S.*, **22**, 5–8.

BABSKII, E. B. & MINAEV, P. F. (1947a). *Biokhimiya*, **12**, 231–240.

BABSKII, E. B. & MINAEV, P. F. (1947b). *Bull. biol. Med. exp. U.R.S.S.*, **23**, 98–101.

BHATTACHARYA, B. K., FELDBERG, W. & VOGT, W. (1957). *J. Physiol. Lond.*, **137**, 460–472.

CROSSLAND, J., PAPPIUS, H. M. & ELLIOT, K. A. C. (1955). *Am. J. Physiol.*, **183**, 27–31.

FELDBERG, W. (1945). *J. Physiol. Lond.*, **103**, 367–401.

FELDBERG, W. & HEBB, C. (1947). *Ibid.*, **106**, 8–17.

GOLUBTSOVA, A. V. & MINAEV, P. F. (1947). *Bull. biol. Med. exp. U.R.S.S.*, **23**, 169–172.

HOCK, A. & HUBER, G. (1956). *Biochem. Z.*, **328**, 44–55.

LEPAGE, G. A. (1957). *Manometric Techniques and Tissue Metabolism*, 2nd edn p. 268–281. Editors: Umbreit, W. W., Burris, R. H. & Stauffer, J. J. Minneapolis: Burgess.

MICHELSON, A. M. (1963). *The Chemistry of Nucleosides and Nucleotides.* 1st edn p. 154. New York: Academic Press.

MINAEV, P. F. (1947). *Bull. biol. Med. exp. U.R.S.S.*, **24**, 290–292.

RAMWELL, P. W. (1964). *J. Physiol. Lond.*, **170**, 21–38.

SLATER, P. (1966). *Ph.D. thesis, Nottingham University*, p. 38.

STONE, W. E. (1955). *Archs Biochem. Biophys.*, **59**, 181–192.

TORDA, C. & WOLFF, H. G. (1945). *Proc. Soc. exp. Biol. Med.*, **58**, 29–31.

TORDA, C. & WOLFF, H. G. (1946). *Am. J. Physiol.*, **145**, 419–429.

TORU, M. & APRISON, M. H. (1966). *J. Neurochem.*, **13**, 1533–1544.

TSUJIMOTO, T. & YAMABA, T. (1956). *Wakayama. Med. rept.*, **3**, 77–83.