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THE PRESENCE OF ACETYLCHOLINE IN *TRYPANOSOMA RHODESIENSE* AND ITS ABSENCE FROM *PLASMODIUM GALLINACEUM*

BY

EDITH BÜLBRING, E. M. LOURIE, AND URSULA PARDOE

From the Department of Pharmacology, Oxford University

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The finding in this laboratory (Burn and Vane, 1948) that acetylcholine is capable of restoring the spontaneous contractions of isolated rabbit auricles after they have been stopped by the antimalarial substance proguanil raised the question of the possible importance of acetylcholine for pathogenic protozoa in general. The further demonstration of a close relationship between the motor activity of auricular muscle and its ability to synthesize acetylcholine (Bülbring and Burn, 1949) suggested the possibility that this substance might be of particular importance to highly motile organisms such as trypanosomes.

I. Acetylcholine in *Trypanosoma Rhodesiense*

(*a*) *Identification of acetylcholine*

The trypanosome used was an old strain of *T. rhodesiense* which has been maintained at the Liverpool School of Tropical Medicine for the past 26 years. It causes a fulminating septicaemic type of infection in mice and rats, ending fatally three or four days after a light inoculation.

Preliminary experiments confirmed that washed rat blood cells neither contain, nor synthesize under the most favourable conditions, any measurable amount of acetylcholine.

For these experiments rats were bled into 1 per cent (w/v) sodium citrate in normal saline solution containing 1: 10,000 eserine, the blood cells then being centrifuged, resuspended in eserine-saline, and again centrifuged. The cells thus packed were present in concentrations ranging, in individual experiments, up to 17,800,000 per cu.mm. A mixture was made of equal parts of this packed blood cell material and a substance described by Feldberg and Mann (1946) as "activator," prepared by extracting an acetone-dried powder of rabbit brain with hot saline, and found by these workers to enhance the synthesis of acetylcholine by brain tissue. The mixture was then divided into three portions of 2 c.c. each. One of these was acidified with 1 c.c. N/3 HCl and boiled in order to destroy any possible choline acetylase present; the second portion received no preliminary treatment; while the third was converted into an acetone-dried powder by washing several times in acetone and drying in a vacuum desiccator, the powder being then suspended in 2 c.c. of "activator." The three specimens (2 c.c. each) were next incubated for 75 min. at 37° C. with the ingredients (and in the proportions) shown by Feldberg and Mann (1946) to be suitable for the synthesis of acetylcholine by brain tissue and used also by Bülbring and Burn (1949) for acetylcholine synthesis by rabbit auricles. The specimens, duly neutralized by the addition of N/3 NaOH, were then examined for acetylcholine by their effect on the frog rectus muscle. Any acetylcholine which might be shown to be present in the first specimen (acidified and boiled before incubation) must necessarily have been present from the beginning; any difference in content between the first and second specimens must represent synthesis that had taken place during the period of incubation; whilst acetylcholine shown to be present in the third specimen (the acetone-dried powder) must also have been synthesized during incubation. No acetylcholine could, however, be demonstrated in any of the specimens. Since these tests showed that rat blood cells neither contain nor synthesize acetylcholine, it was accordingly held to be unnecessary to separate blood cells from trypanosomes when preparing a suspension of trypanosomes for investigation of their possible acetylcholine content. The trypanosome material was therefore prepared in the following way.

Six rats were inoculated intraperitoneally (0.3 c.c. per rat) with heart's blood obtained from 2 very heavily infected mice. Two days later the blood of these rats, in turn, was swarming with parasites, and 36 c.c. were obtained by heart puncture and added to 108 c.c. of sodium citrate-saline solution (1 g. $Na_3C_6H_5O_7$, $2H_2O$/100 c.c.; 0.85 g. NaCl/100 c.c.). Eserine, 2.88 c.c. of a solution containing 0.5 g. eserine sulphate/100 c.c. was immediately included, giving a concentration of 1: 10,000 eserine in the trypanosome-blood mixture. This was then centrifuged at 5,500 r.p.m. for 2 min., the supernatant replaced by saline solution containing 1: 10,000 eserine, the mixture again centrifuged at 5,500 r.p.m. for 2 min., and the supernatant then discarded. The deposit, consisting of densely packed blood cells and trypanosomes, occupied 14.4 c.c. To this were added an equal volume of saline and 7.2 c.c. N/3 HCl, the mixture then being rapidly boiled and cooled, brought

to 72.0 c.c. in frog-Ringer solution, and stored in the ice-box for determination of acetylcholine the following day.

The material was neutralized and then assayed by four separate methods, two depending on motor and two on inhibitor effects characteristic of acetylcholine. The motor tests were on the isolated frog rectus muscle and the isolated guinea-pig ileum, and the inhibitor tests on the perfused isolated frog heart and the blood pressure of the spinal cat.

Results were as follows:

(i) *Contraction of the isolated frog rectus muscle* (see Fig. 1). In order to deal with the possible complication of non-specific contractions produced by the trypanosome material, a portion of the 72 c.c. obtained as above was treated with N/3 NaOH and boiled to destroy any acetylcholine that might be present. After cooling and neutralizing with N/3 HCl, this control specimen (S.BA.) failed to cause any contraction of the rectus muscle. By adding known amounts of acetylcholine to portions of the control specimen, and matching the contractions which these produced against the contractions produced by untreated portions of the test fluid, 1 c.c. of this fluid (S.) was found to correspond to 0.3 μg. acetylcholine.

(ii) *Contraction of the isolated guinea-pig ileum* (see Fig. 2). The contractions produced by 0.1 c.c. of the trypanosome material were found to be equal to those produced by 0.03 μg. acetylcholine, thus agreeing with the result obtained by the frog rectus method above. That it was acetylcholine which was responsible for the contractions produced by the trypanosome material is supported by the fact that the contractions could be prevented by atropine.

(iii) *Perfused isolated frog heart beat* (see Fig. 3). The diminution which 0.05 c.c. of the test specimen produced in the amplitude of the heart beat was the same as that produced by 0.015 μg. acetylcholine, thus agreeing quantitatively with the results obtained by the two motor tests above. Again the effect could be prevented by atropine.

(iv) *Blood pressure of spinal cat* (see Fig. 4). The blood pressure was reduced to the same extent by 1 c.c. of the test specimen as by 0.3 μg. acetylcholine, thus again agreeing quantitatively with the results obtained by the other methods. No fall in blood pressure was produced by the specimen after it had been boiled with alkali (S.BA.). The fall was increased by prior injection of eserine and abolished by atropine.

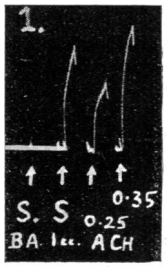

FIG. 1.—Contractions of the frog rectus muscle. S.BA. = specimen of trypanosome material boiled in alkali, cooled, and neutralized. S. = specimen not so treated. 1.0 c.c. of S. equivalent to 0.3 μg. acetylcholine (ACH).

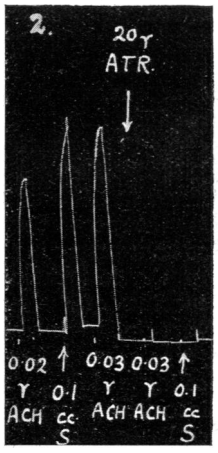

FIG. 2.—Contractions of guinea-pig ileum. 0.1 c.c. of trypanosome material (S.) equivalent to 0.03 μg. acetylcholine (ACH). Effect abolished by 20 μg. atropine (ATR).

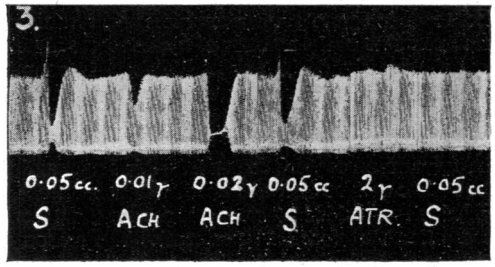

FIG. 3.—Perfused isolated frog heart beat. 0.05 c.c. of trypanosome material (S.) equivalent to 0.015 μg. acetylcholine (ACH). Effect abolished by 2 μg. atropine (ATR).

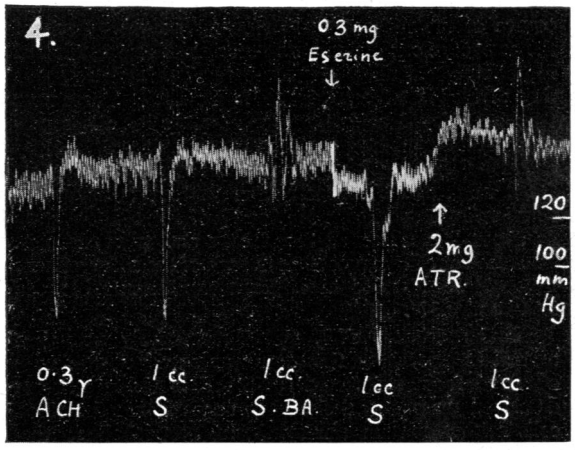

FIG. 4.—Blood pressure of spinal cat. 1.0 c.c. of trypanosome material (S.) equivalent to 0.3 μg. acetylcholine (ACH). No effect produced by 1.0 c.c. trypanosome material previously boiled in alkali, cooled, and neutralized (S.BA.). Effect of 1 c.c. S. enhanced by 0.3 mg. eserine and abolished by 2 mg. atropine (ATR).

The fact that the trypanosome material produced effects characteristic of acetylcholine in each of these four tests, and that, moreover, its acetylcholine content was in perfect agreement (0.3 μg. per c.c.) for each assay method, leaves no room for doubt that it was indeed acetylcholine and not some unidentified substance which was responsible for the reactions observed.

(b) Synthesis of acetylcholine

The possibility seems remote that the trypanosomes absorb preformed acetylcholine from some organ or body fluid of the host. It would be much more likely that they acquire the substance by their own powers of synthesis, and this we have been able to demonstrate. After a few orientating experiments, the following routine procedure was adopted in the preparation of trypanosome material for this further work.

Four rats were inoculated intraperitoneally with heavily infected mouse blood on which a trypanosome count had previously been carried out, so that the amount injected per rat could be adjusted to contain about 2×10^8 trypanosomes. Two days later the rats were sufficiently heavily infected for the required purpose. It was found advisable to use rats at levels of infection thus obtained rather than rats that are even more densely infected, since trypanosomes obtained from the latter tended to disintegrate soon after bleeding, resulting in an underestimate of the amount of trypanosome material present if there should be any undue delay in counting the trypanosomes. The rats were bled by heart-puncture and 30 c.c. of blood added to 90 c.c. of 1 per cent sodium citrate-saline solution.

The trypanosomes were counted at this stage, and the total of 120 c.c. then divided into two equal portions, to one of which was added 1.2 c.c. 1/200 eserine (final eserine concentration therefore 0.01 per cent). The reason for treating one portion with eserine was to preserve any acetylcholine that might otherwise be destroyed in the course of the subsequent *in vitro* operations. Each of the two 60 c.c. portions, non-eserinized and eserinized respectively (labelled *N* and *E* for convenience), was then divided into 3 equal lots of 20 c.c. each. These were then centrifuged at 5,500 r.p.m. for 2 min., the supernatant discarded and replaced by saline for *N* and by eserine-saline for *E*, centrifuged once more at 5,500 r.p.m. for 2 min., and the supernatant fluid again discarded, thus leaving 3 specimens of *N* and 3 of *E*, each consisting of about 2 c.c. packed blood cells and trypanosomes. The number of trypanosomes in each of the six specimens thus obtained varied in different experiments between 3.04^9 and 6.37^9.

The 3 specimens of *N* and *E* respectively were then treated in essentially the same way as the 3 specimens obtained from uninfected rat blood in the experiment described on p. 290 designed to confirm that the blood cells neither contain nor synthesize acetylcholine; that is, one specimen was acidified with 1 c.c. N/3 HCl and boiled, one received no such preliminary treatment, and one was converted into an acetone-dried powder.

The specimens were then incubated at 37° C. with the ingredients and in the proportions described by Feldberg and Mann (1946) and assays again carried out on the frog rectus preparation. Standard acetylcholine solutions for matching against the samples of unknown content were made up in the same ingredients as those of the latter samples, except that rat blood cells were not always included. Tests showed that the omission of this particular component did not affect the result. Errors due to contraction of the frog rectus by factors other than acetylcholine were eliminated also by boiling a portion of the unknown solution in the presence of N/3 NaOH, neutralizing, and making up with known amounts of acetylcholine for matching against the unknown. Since the blood cells neither contain nor synthesize acetylcholine, any of this substance discovered must have been derived from the trypanosomes, and, again, the difference in acetylcholine content between the specimen acidified and boiled before incubation and the specimen not treated in this way must represent the amount synthesized during the course of incubation. Any acetylcholine present in the specimen prepared from the acetone-dried powder must also represent synthesis.

The accompanying Table shows the results of a number of consecutive experiments of this type. The amounts of acetylcholine have been calculated per 10^{10} trypanosomes because that number of packed trypanosomes weighs approximately 1 g.; the average of three determinations (in good agreement) of the wet weight of trypanosomes separated from blood cells and packed by centrifugation at 5,500 r.p.m. for 3 min. was, in fact, found to be 0.957 g. The variations of the results shown in the Table are due not merely to experimental error but to the fact that findings are included of the initial orientating experiments of the series before the procedure had been finally standardized as described above. Thus, the absence of acetylcholine in the specimen without eserine (*N*) of exp. 5 may have been due to the fact that there was an excessive concentration of trypanosomes in the specimen, resulting in their disintegration and the destruction of any acetylcholine that may have been present. Moreover, in these earlier experiments, considerable time was taken up by alternately freezing and thawing the specimens, a procedure which was discarded as unnecessary in the later experiments of the series. In the last three experiments, the acetylcholine contents of the preparations without eserine (*N*) were no longer significantly lower than those of the preparations with eserine (*E*). The fact that the use of eserine in preparing the specimens for incubation increased the apparent yield of acetylcholine in the earlier experiments affords additional support for

TABLE

ACETYLCHOLINE IN *Trypanosoma rhodesiense*, IN μG. PER 10^{10} TRYPANOSOMES (0.96 G. WET WEIGHT)

Experiment	Trypanosomes not exposed to eserine before incubation (*N*)			Trypanosomes exposed to eserine before incubation (*E*)		
	Content before incubation	Synthesized during incubation		Content before incubation	Synthesized during incubation	
		Trypanosomes not converted to acetone-dried powder	Trypanosomes converted to acetone-dried powder		Trypanosomes not converted to acetone-dried powder	Trypanosomes converted to acetone-dried powder
1*				2.67	0	0.60
2*a*				2.41	0.21	0.90
2*b**				2.28	0	0.28
3	3.58	1.80	0	4.54	0	0
4	1.86	0.94	0.28	5.08	1.58	0
5	0	0.71	0	8.08	1.60	1.15
6	10.3	5.58	1.24	8.23	4.23	0.74
7	2.71	2.41		3.08	2.56	
8	5.72	2.18		6.80	1.36	

* Trypanosomes separated from blood cells before incubation and assay.

believing that it is indeed acetylcholine and not some unknown substance with which we are dealing.

In two of the earlier experiments (Nos. 1 and 2*b*) the red cells had been carefully separated from the trypanosomes by differential centrifugation before incubation and assay of the latter, but the results as a whole appear to show that there is no advantage in this precaution.

Confining our attention now to the eserinized series alone, the mean acetylcholine content before incubation was found to be 4.79 μg. (range 2.28 to 8.23 μg.) per 10^{10} trypanosomes, or 5.00 μg. (range 2.38 to 8.60 μg.) per g. wet weight. Synthesis ranged between 0 and 4.23 μg. per 10^{10} trypanosomes in 75 min. In order to compare these figures for synthesis by trypanosomes with those found by Bülbring and Burn (1949) for rabbit auricles we have determined that an acetone-dried powder of 10^{10} trypanosomes weighs about 0.078 g. The amounts synthesized by trypanosomes were therefore 0 to 54.2 μg. per g. powder (71.5 μg. in one result of series *N*) as compared with 40.2 ± 12.7 μg. quoted by Bülbring and Burn for rabbit auricles. It is interesting that the figures for trypanosomes and for rabbit auricles should be in such close agreement. It might be emphasized, though, that we have not explored the optimum conditions for acetylcholine formation by trypanosomes. We have merely used those which Feldberg and Mann (1946) found to be optimal for brain tissue; under other circumstances, as yet unknown, it may well be that much higher figures could be obtained for the activity of trypanosome material in this respect. The Table shows that no particular advantage was gained by preparing an acetone-dried powder of the trypanosome material, and this procedure was therefore omitted in the last two experiments of the series.

II. LACK OF ACETYLCHOLINE IN *Plasmodium Gallinaceum*

In order to discover whether the blood stages of *Plasmodium gallinaceum* contain or are capable of synthesizing acetylcholine, the procedure adopted was the same as for *Trypanosoma rhodesiense*, except for minor differences necessitated by differences in the two types of infection. For example, in order to arrive at an estimate of the number of parasites in each specimen, trypanosomes were counted directly in a haemacytometer chamber (after suitable dilution of the specimen), whereas for malaria parasites the total number of red cells was first estimated in the usual way in a counting chamber, the number of parasites then being derived from the ratio of parasites to red cells as determined in stained blood films.

Several experiments were carried out with *P. gallinaceum*, the following being a typical example. A massive infection was produced in an eight-week-old chicken by inoculating it intravenously with 10 c.c. blood from another heavily infected chicken. Two days after inoculation 22 c.c. blood was obtained by heart puncture and immediately added to 66 c.c. sodium citrate-saline solution containing 1 : 10,000 eserine. The mixture was then divided

into 3 equal portions, centrifuged, the supernatant replaced by eserine-saline, and the mixture again centrifuged. The three deposits (containing 9.72^9 parasites each) were then treated exactly as described above for the investigation of rat blood and trypanosomes; that is, one specimen was acidified with 1 c.c. N/3 HCl and boiled, one received no such preliminary treatment, and one was converted to an acetone-dried powder. The specimens were then incubated in the manner described above, and tested on the frog rectus preparation. No evidence of acetylcholine could be detected in any of the specimens tested.

Discussion

Since the isolation by Ewins (1914) of acetylcholine from a liquid extract of ergot, implying its production by *Claviceps purpurea*, very little attention has been directed toward the possibilities of acetylcholine formation by other micro-organisms. A recent and convincing contribution in this field has been the demonstration by Stephenson and Rowatt (1947) of acetylcholine production by a strain of *Lactobacillus plantarum* from sauerkraut. Bayer and Wense (1936) showed acetylcholine to be present in a species of the free-living protozoon Paramoecium, but there seems to have been no investigation till now of the production of acetylcholine in pathogenic protozoa.

It is not possible at the present stage to do more than speculate, in seeking for an interpretation of our finding that trypanosomes synthesize and presumably utilize acetylcholine while malaria parasites do not. It may, however, be relevant that acetylcholine is well known to be intimately concerned in a variety of motor mechanisms among higher organisms ranging from some of the invertebrates (e.g., the leech) up to the highest forms of vertebrate life. Its importance for motor processes has recently been suggested even in a location where it was formerly regarded as essentially an inhibitor substance, namely, in heart muscle; for the work of Bülbring and Burn (1949) provided evidence for believing that the activity of auricular muscle is inseparably linked with its synthesis of acetylcholine. One is therefore tempted to correlate the presence of acetylcholine in trypanosomes and its absence from malaria parasites with the fact that the former are highly active motile organisms while the latter are relatively immobile. This reference to the lack of motility on the part of malaria parasites should, of course, be qualified by recognizing, firstly, that it is only the blood stages and not the mosquito phases with which we are here concerned; and, secondly, that slight motility, varying in degree among different species of malaria parasite, is in fact exercised during the trophozoite (or amoeboid) stage of development. Indeed, the specific name *Plasmodium vivax* testifies to the prominence of this particular feature in the parasite of benign tertian malaria, in comparison with other species of malaria parasite. The motility is, however, relatively sluggish, being essentially amoeboid in type and quite unlike that of trypanosomes with their highly specialized organs of locomotion. The demonstration by Bayer and Wense (1936) of acetylcholine in Paramoecium may be significant in this connexion since Paramoecium is, of course, also characterized by morphological differentiation providing for a high degree of motility.

It need hardly be added that we do not attempt to assign to acetylcholine some role in connexion with high motility as its sole possible function among micro-organisms. Its presence in *Claviceps purpurea* and *Lactobacillus plantarum* is alone sufficient to indicate that this cannot be the case. Likewise, we do not exclude the possibility that a high degree of motility may occur in some species of protozoa or related organisms without acetylcholine being involved. Among the lower orders of metazoa Bacq (1947) points out that coelenterates and tunicates, which are far from devoid of motor properties, contain either no acetylcholine or only inappreciable amounts of that substance.

Summary

1. *Trypanosoma rhodesiense* has been found to contain acetylcholine to the extent of 2.28 to 8.23 μg. per 10^{10} trypanosomes, or 2.38 to 8.60 μg. per g. wet weight. The formation of acetylcholine has been demonstrated *in vitro* in amounts up to 5.50 μg. per 10^{10} trypanosomes, or 71.5 μg. per g. acetone-dried powder, in 75 min. at 37° C.

2. *Plasmodium gallinaceum* has been found neither to contain nor to synthesize acetylcholine.

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