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THE ALLEGED OCCURRENCE OF ACETYLCHOLINE IN OX BLOOD.

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In spite of the closest and friendliest cooperation between Prof. Kapfhammer and ourselves, we are still unable to reach agreement on the question of the alleged occurrence of physiologically important amounts of acetylcholine in normal blood.

In a long series of experiments we have consistently failed to detect physiologically more than the merest traces of activity resembling that of acetylcholine (equivalent to not more than 0·1 mg. of acetylcholine chloride per litre of blood) in blood extracts at any stage in the process of purification, while he and his collaborators describe the detection and chemical identification of amounts up to as much as 40 mg. per litre.

It appears to be desirable to report the results of our final attempt to clear up the discrepancy, since, having regard to the present state of the literature on this question, our silence might reasonably suggest that we no longer maintained our original position.

About a year ago, a full and friendly exchange of information and experiences having failed to disclose the reason for our contradictory findings, at the invitation of Prof. Kapfhammer I went to Freiburg in the hope of discovering, by collaboration with him in his own laboratory, the explanation of our discordant results.

I will state forthwith that I had no difficulty in isolating acetylcholine from the two samples of blood examined during my visit to Freiburg. Ox blood was collected on two occasions from the municipal slaughter-house. Dr C. Bischoff and I each worked up 1 litre of each specimen according to the Freiburg technique. This consists in precipitating the blood proteins with alcohol, and treating the extract, after concentration, with trichloroacetic acid to remove residual protein. Fat and excess of trichloroacetic acid are then removed by extraction with ether.

After appropriate concentration of the extract acetylcholine is precipitated with "Reinecke" acid, the "reineckate" is decomposed and the base converted into chloroaurate for identification.

At each stage of the process Dr Bischoff's and my results were identical in both experiments.

The two experiments were particularly remarkable in that the physiological behaviour of the acetylcholine associated with the first differed strangely from that of the acetylcholine associated with the second.

The first sample of blood was taken to the stage of purification with trichloroacetic acid before it was tested for physiological activity. The result of this test was quite concordant with our own experiments at Hampstead, for it displayed only a minute activity, equivalent to 0·1–0·5 mg. of acetylcholine chloride per litre of blood. Nevertheless, we proceeded to the next stage, namely precipitation with Reinecke acid, and most surprisingly the solution of chlorides derived from the precipitated "reineckate" possessed physiological activity equivalent to 40 mg. of acetylcholine chloride per litre of blood. There was no doubt that this activity was in fact due to acetylcholine, since the chloroaurate of the base was isolated and identified chemically.

The second sample of blood was precipitated with alcohol, and after 1 hour the alcoholic filtrate was tested physiologically, at Dr Bischoff's suggestion, before concentration on both the cat's blood-pressure and the rabbit's isolated intestine. Although the alcohol interfered to some extent in the second method, an unmistakable acetylcholine effect was observed in both, indicating approximately 22 mg. of acetylcholine chloride per litre of blood. Assayed subsequently at the trichloroacetic acid and "reineckate" stages, this extract gave equivalents of 27 and 25 mg. respectively, and acetylcholine was finally isolated and identified as the chloroaurate.

Thus acetylcholine was actually isolated from the two blood extracts and identified chemically. The appearance of the active base at the end of the fractionation of the first extract, which up to that point had given no indication that it contained more than a trace, was uncanny; particularly as the second extract displayed at every stage of purification the typical physiological activity in a degree corresponding to the amount of acetylcholine eventually isolated.

It is true that Bischoff, Grab and Kapfhammer [1931] have reported that alcoholic extracts of blood are sometimes obtained which give no physiological indication of the acetylcholine which they actually

contain until they have been treated with trichloroacetic acid, the presumption being that acetylcholine may lie hidden in such extracts as a physiologically inactive complex which is broken down by trichloroacetic acid treatment. In my first Freiburg experiment, however, this hypothetical complex apparently survived that stage, and only after precipitation with Reinecke acid did normal, physiologically active acetylcholine appear. Since a fundamental disagreement still exists between Kapfhammer's results and our own, it is superfluous to discuss this hypothetical complex. Apart from the chemical difficulties which it presents, its inconstancy in appearance and behaviour is perplexing; and in our investigations at Hampstead we have never encountered it.

Returning from Freiburg to Hampstead, I again examined ox blood from four different animals, using the technique with which I had become familiar in Kapfhammer's laboratory. In none of the extracts was more than a trace of acetylcholine-like activity (equivalent to not more than 0·1 mg. of acetylcholine chloride per litre of blood) physiologically detectable at any stage in the fractionation, which in each experiment was carried to completion in spite of the apparent absence of acetylcholine. The gold salts finally obtained consisted mainly of choline chloroaurate.

In order to test my technique, the unconcentrated alcoholic extract of the fourth sample (2 litres) of blood was divided into two equal quantities, to one of which I added 40 mg. of acetylcholine chloride. The two portions were then worked up simultaneously. Without difficulty, and with relatively little loss, the added acetylcholine was recovered, and finally identified as chloroaurate.

For the complete failure of the experiments made in this laboratory to confirm the positive results of those performed under precisely the same technique in Freiburg, we can find no reasonable explanation.

It should be mentioned that, since this work was completed, a short note by Vogelfanger [1933] has appeared, stating that there is no difficulty in obtaining from ox blood, by Kapfhammer's method, acetylcholine in amounts similar to those which he obtained. The author notes that the amount of oxalic acid recommended by Kapfhammer is inadequate to ensure a proper acidity of the initial extract, and emphasizes the necessity of adjusting the amount to that end. It might be implied that the failure of others to find acetylcholine in ox blood was attributable to neglect of this point. This criticism does not apply to our earlier work, for we ourselves discovered this defect in Kapfhammer's original directions, when applied to English ox blood, and took the

necessary steps to correct it [Dale and Dudley, 1931]. Apart from this critical note on a point already recognized and dealt with by us, Vogelfanger's account is a bare record, and does not assist to an understanding of our negative findings. Our numerous and consistently negative results prove that acetylcholine cannot be an artefact, and the erratic appearance of the substance in physiologically detectable form at different stages of the fractionation in different experiments rules out the improbable suspicion that there might be occasional batches of alcohol current in Germany which were contaminated with acetylcholine.

Since the same technique gives me positive results, confirming Kapfhammer's, in Freiburg, and negative results, confirming all our earlier observations, in London, it is obviously desirable that the question should be tested by others as widely as possible. Already Wrede and Keil [1931] have described negative results and Vogelfanger positive.

I earnestly hope that the publication of this paper will stimulate others, in this country and abroad, to investigate the problem. The method of Kapfhammer and Bischoff is admirably suited to its purpose, and has transformed the process of isolation of acetylcholine from one of great labour and difficulty to one of ease and simplicity.

In the following paper Chang and Gaddum publish experiments made on extracts of other tissues by physiological methods. Only in one case, that of the human placenta, as earlier in the case of horse and ox spleen, do these further experiments detect quantities of acetylcholine comparable to those found by the Freiburg investigators. With all other tissues, the experience of this laboratory entirely fails to confirm the presence of still higher proportions (nearly 200 mg. per kg. in some cases) of acetylcholine found by Kapfhammer and his collaborators.

Experimental.

The method of Kapfhammer and Bischoff, employed in the experiments under discussion, was carried out in the following manner in Freiburg.

Ox blood was collected at the municipal slaughter-house, where oxalic acid (1·6 g. per litre of blood) in saturated aqueous solution was added. It was then brought to the laboratory, a journey of about 20 min. duration, where 1 litre was poured with stirring into 5 litres of 96 p.c. alcohol. The reaction of the mixture was tested with blue litmus paper. As no definite acid reaction was detectable, aqueous lactic acid was added until the solution produced a faint reddening of litmus.

After standing for 1½–2 hours the solution was filtered through paper and mixed with the liquid expressed in a tincture press from the blood coagulum. The blood cake was re-extracted with 2 litres of 96 p.c. alcohol, which, after 2 hours' contact, was filtered off. The combined extract was then evaporated *in vacuo* (water bath at 40°) to 100–150 c.c.

An equal volume of 20 p.c. trichloroacetic acid was added to this concentrate and the mixture was placed in an ice-chest (4°) for 2 hours. It was then shaken out with ether four times, and the solution, now faintly acid to Congo-red, was concentrated *in vacuo* to approximately 100 c.c. Saturated aqueous Reinecke acid was added in slight excess and the mixture was placed in the ice-chest for 12–16 hours.

The precipitate was then filtered off and washed with ice-cold water until the runnings were practically colourless. Ice-cold alcohol was then applied; the runnings were at first faintly coloured, but speedily became colourless. After a final washing with ether the residual precipitate was dried in a desiccator. It was then dissolved in 25–50 c.c. of dry acetone. The solution, after removal of a small amount of insoluble material by filtration, was mixed with dry benzene (10–15 vols.). This precipitated the "reineckate" quantitatively, and it was collected on a folded filter paper. After drying, it was dissolved in about 100 c.c. of 50 p.c. acetone, excess of aqueous silver sulphate solution was added, the precipitate of silver "reineckate" removed in the centrifuge and to the liquid a solution of barium chloride, equivalent to the silver sulphate used, was added. The solution, freed from silver chloride and barium sulphate in the centrifuge, was concentrated *in vacuo* to 5 c.c. A strong solution of gold chloride, neutralized to Congo-red with sodium hydrogen carbonate, was added to this solution. After standing in the ice-chest for about an hour the gold salt was filtered off and recrystallized from water.

The experiments made at Hampstead were carried out strictly in accordance with the technique just described. The amount of lactic acid added to each alcoholic filtrate was 1 c.c.

The "reineckate" was precipitated from acetone solution by means of benzene for the sake of strict conformity, but this step does not appear to serve any useful purpose and could be omitted.

Table I shows the amounts of acetylcholine determined physiologically at each stage of the fractionation of the two Freiburg specimens and of the fourth sample of English ox blood, which was worked up in two equal

TABLE I. Acetylcholine in ox blood (mg. acetylcholine chloride per litre).

	Freiburg experiments		Hampstead experiments	
	Blood I	Blood II	Blood IV a	Blood IV b
Alcoholic extract before conc.	—	22 (approx.)*†	—	—
Alcoholic extract after conc.	—	—	0·13†	40†
After trichloroacetic acid purification	0·1–0·5*	27†	0·13†	40†
After Reinecke acid precipitation	40†	25†	0·09† 0·05‡	33·3† 34·4‡
Weight of chloroaurate after recrystallization	50 mg. (M.pt. 162°; Au. 40·4 p.c.)	30 mg. (M.pt. 163°; Au. 40·4 p.c.)	40 mg. (M.pt. 258°; Au. 44·3 p.c.)	54 mg. (M.pt. 164°; Au. 40·5 p.c.)

Physiological determination: * Cat's blood-pressure; † Rabbit's intestine; ‡ Frog's eserinized rectus. Au. calc. for acetylcholine chloroaurate, 40·6 p.c.; for choline chloroaurate, 44·5 p.c.

portions, to one of which (IV b) 40 mg. of acetylcholine chloride had been added. The other three English ox bloods gave results similar to those of IV a. When such small amounts of acetylcholine as appear in blood IV a are under examination the frog's eserinized rectus gives a much more accurate assay than does the rabbit's intestine. With the latter a fraction of the total activity still persists after atropinization of the test tissue. The amounts of twice recrystallized chloroaurate, with melting points and analyses, are also presented. The crude chloroaurate obtained from blood extracts always contains the choline salt. When the acetylcholine salt is also present the volume of water used for recrystallization is such that the choline salt remains in the mother liquor, since acetylcholine chloroaurate is much less soluble than choline chloroaurate.

It is to be noted that the exhaustive washing of the "reineckate" results in a small loss (15 p.c.) of acetylcholine.

Summary.

Kapfhammer and his colleagues in Freiburg maintain that ox blood contains relatively large amounts of acetylcholine, whilst we at Hampstead have consistently failed to confirm their finding.

This fundamental discrepancy persists in spite of a collaborative attempt, described in the present communication, to discover a reason for our disagreement.

In conclusion I wish to record my appreciation of the friendliness with which Prof. Kapfhammer received me in his laboratory, and of the expert and willing advice and assistance afforded me by Dr Bischoff in making my experiments there.

I am indebted to Sir Henry Dale, Dr J. H. Gaddum and Dr H. C. Chang for their help in making the necessary physiological determinations.

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