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THE PRESENCE OF HISTAMINE AND ACETYLCHOLINE IN THE SPLEEN OF THE OX AND THE HORSE.

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I. Introductory.

The fact that extracts from different animal organs (liver, lung, voluntary muscle) exhibit physiological activities resembling those of histamine and choline, and contain those bases in quantities sufficient to account for these effects, has been demonstrated in previous communications from this Laboratory (1, 2). When due allowance is made for the inevitable losses in the rather difficult processes of separation, the quantities isolated and chemically identified have been sufficient, indeed, to warrant a more general presumption that, when activity of either of these types can be clearly demonstrated in an extract from any fresh animal organ, it is probably due to the presence of the corresponding simple basic principle. It appeared to us desirable, however, to apply the methods, successfully used in the cases already investigated, to the extract of one additional organ, namely the spleen. Among the many publications dealing with the activities of organ extracts there are several concerning those obtained from the spleen. Oliver and Schafer (3) mention a fall followed by a gradual rise of the arterial pressure of the dog, as the result of injecting such an extract intravenously. Zuelzer (4) claimed to have extracted from the gastric mucosa, taken during digestion, a hormone which stimulated peristalsis of the intestine. The proprietary preparation "hormonal," made at his direction, appears, however, to have been made from the spleen, on the supposition that this organ acted as a depot for the hormone produced by the stomach. The makers supplied it as a spleen extract to Berlin (5). Berlin's investigations were chiefly concerned, however, with an extract of fresh spleen prepared by himself, by boiling the minced tissue with water for an hour. From this, after purification, he precipitated the bases as phosphotungstates and fractionated them by Kutscher's method. Fraction A was found to have

an activity resembling that of choline, and the latter base was isolated from it as the chloroaurate, of which 17·5 g. (corresponding to 4·78 g. of choline) were obtained from 23 kg. of spleen substance. Fraction B had an activity resembling that of histamine, and this accounted for a large part of the total activity of the extract; but, since the active principle was not precipitated at the appropriate stage of the silver fractionation, Berlin concluded that the substance was not histamine itself, and that this base was, indeed, not present in the extract. From "hormonal" he separated two similar fractions, but without isolating the base in either case. Stern and Rothlin (6), who had compared the actions of a series of organ extracts on isolated arterial strips and other forms of plain muscle, found that the extract of spleen had a specially powerful stimulant action. From a watery decoction of spleen, purified by evaporation and solution in alcohol, they obtained a preparation with a relatively high activity. The active principle was soluble in alcohol, insoluble in ether, not precipitated by tannin, but completely by phosphotungstic acid. The physiological effects of the preparation were strongly reminiscent of those produced by histamine; but, since they found that the activity was destroyed by heating with alkali, and that hot chloroform would not extract it completely from the residue obtained by evaporation with sodium carbonate, Stern and Rothlin were of opinion that the principle was not histamine itself, and provisionally named it "lienine."

In other publications, many concerned with "hormonal," the suggestion can be found that the activity of spleen extracts is due to choline, or histamine, or similar active principles.

We knew from our own experience, previously recorded (1), that a principle, occurring in a complex mixture of tissue bases, may show apparent differences in its chemical behaviour from that of pure histamine, as great as those described by Berlin and by Stern and Rothlin, and yet be histamine itself. It seemed desirable to examine anew the question of the chemical identity of the histamine-like constituent of spleen extracts. The investigation undertaken with this object has led to additional results of unexpected interest.

II. Recognition of two vaso-dilator principles.

As in our work on other organs, we have used extracts prepared by mincing the fresh spleens directly into cold alcohol, in place of the watery decoctions with which earlier investigators have started. We have mentioned the fact that Berlin isolated choline from his extracts in

relatively high proportion; and, although his prolonged boiling of the tissue with water might conceivably have contributed to this result, we were prepared for the possibility that our alcoholic extracts might also show a relatively large "choline" component in their depressor action. We found, indeed, when the depressor action of the extract was measured, as usual, in comparison with that of a standard histamine solution on the arterial pressure of a cat under ether or urethane, that a surprisingly large proportion of the original effect of the extract was eliminated by administering atropine. This activity, in terms of choline, was determined by the following method. The histamine-like effect of the extract was first determined, in terms of pure histamine, on an atropinized cat under ether. On another cat, similarly anæsthetized, but without atropine, the same dose of spleen extract was again compared, as regards depressor action, with the same dose of histamine. The effect of the spleen extract being now much stronger, choline in varying doses was added to the constant dose of histamine, until this mixture again matched the dose of spleen extract in effect. The quantity of choline thus indicated as present in the spleen extract was unexpectedly and, indeed, almost incredibly great. Drs W. Bauer and Dickinson Richards, who were working with us at the time, carried out an assay of the type described on the extract from our first large batch of 50 kg. of ox spleen, from which, in due course, histamine was isolated. Their estimate showed a "histamine" activity corresponding to 0·42 g. of histamine, or about 8 mg. per g., and, in addition, a "choline" activity corresponding to 116 g. of choline, *i.e.*, more than 2 g. of choline per kg. of fresh spleen substance. Our immediate object, at that time, was the isolation of the histamine-like constituent; but we noted that the choline action was practically unchanged by the purification with basic lead acetate, being estimated as equivalent to a total of 112 g. of choline, while 95 p.c. of it disappeared in recovery of the mixed bases from their phosphotungstates by the use of baryta. The lost activity was not in the phosphotungstic acid filtrate, and the suggestion was obvious that we were dealing, not with choline itself, but with some much more active derivative of choline, having a high sensitiveness to treatment with alkali. This suggestion was readily confirmed by treatment of samples of the original extract with alkali. One sample was made alkaline to *p*H 10, and left at room temperature for 48 hours. On re-testing after this treatment it was found to have retained its histamine action unimpaired, while the "choline" action had practically disappeared; the volume of extract matching a given dose of pure histamine in depressor

action was now not significantly changed by administering atropine, whereas before the alkali treatment it had been more than doubled. Addition to the original extract of an equal volume of normal sodium hydroxide produced a similar destruction of the "choline" component of the depressor action, in ten minutes at room temperature.

The main bulk of this large-scale extract of ox spleen had already passed through the stage of purification at which this alkali-sensitive principle was almost entirely lost. We decided, accordingly, in the further investigation of this batch, to follow the histamine-like component only. In due course, by the procedure described in detail in a later section, we isolated this, and found it, as in other organ extracts, to be histamine itself. Our original objective was thus attained; but the evidence of an alkali-sensitive principle with a choline-like action raised possibilities of new and unexpected interest, requiring different methods of attack.

We satisfied ourselves, in the first place, that choline itself was not present, in our first large-scale extract, in quantities of the order required to produce the observed effects. The most obvious possibility was that we were dealing with an unstable ester of choline, having an activity so much greater, that hydrolysis would practically annul it. Among the esters of choline which had been examined, only acetylcholine was known to have the required characteristics of intense activity and extreme liability to hydrolysis in alkaline solution. If the principle were, indeed, an ester so unstable as this, the fact that its presence had not been detected by the earlier observers, who made their spleen extracts by prolonged boiling with water, needed no explanation. It seemed unlikely that our own extraction with alcohol, made without reference to the presence of so unstable a substance, would have preserved it all without decomposition. We accordingly made a series of experiments, in which we had valuable help from Mr H. P. Marks, in order to determine what additional precautions were desirable. These experiments were greatly facilitated by the opportunity of obtaining spleens, immediately after death, from horses killed at the Farm Laboratories of the National Institute, through the valuable co-operation of our colleague Major G. W. Dunkin. In a few experiments we froze the spleens immediately on removal, by immersing them in alcohol cooled to about $-60°$ C., and minced them while still frozen, returning the mince immediately to the cold alcohol, which rose slowly to the ordinary temperature as the extraction proceeded. We obtained good yields of the choline-derivative in this way, but not better than in other trials in

which such precautions were omitted. The one essential condition for a good yield was found to be the *immediate* mixing of the spleen substance with alcohol, actually during the disintegration. A spleen was cut into two portions. One of these was minced in cold alcohol, which was poured into the mincing machine with the spleen substance. After thorough extraction, and removal of the alcohol, the extract was tested against acetylcholine chloride, and found to contain the activity-equivalent of 13 mg. of that substance per kg. of spleen. The other portion was minced and weighed before alcohol was added, the procedure occupying a few minutes only. The extraction and preparation for testing were thereafter carried out in strict parallel with those of the first portion. The activity-equivalent was only 0·75 mg. of acetylcholine chloride per kg. of the same spleen substance.

The fact that nearly the whole of the active principle thus disappeared from the minced tissue in the few minutes preceding the addition of alcohol, made it unlikely that, even with the greatest precautions, we should extract and preserve the whole of what was originally present. One point, however, was made clear, namely that disintegration of the spleen substance was the main cause of loss. Spleens kept intact for hours in the laboratory, in one case over night, yielded, on an average, extracts as rich in the unstable principle as those worked up immediately after death, provided that mixture with alcohol and mincing were simultaneous in every case. We were unable, further, to detect any loss when once the mixture with alcohol had been effected; the mixture of alcohol and mince could be left for days in the cold store before filtration, without detectable loss of activity. The yields from individual spleens, all extracted under the best conditions indicated by these trials, showed fairly wide variations. Expressed in terms of acetylcholine, the activity might be as low as 5 or as high as 30 mg. per kg. of spleen. It should be noted that the same range of activities would approximately correspond to those of 5 to 30 grammes of choline, acetylcholine being about 1000 times as active. The weakest of these extracts, made with the precaution mentioned from individual spleens, therefore contained substantially more of this activity per kg. of spleen than our original large-scale extract from ox spleens, in which the equivalent was 2 g. (= about 2 mg. of acetylcholine) per kg.

These experiments, while providing indications for large-scale procedure, had given additional evidence as to the nature of the substance with which we were dealing. It was not only unstable in alkaline solution, but was destroyed even more rapidly when liberated from the

spleen cells which presumably contained it, and brought into contact with the cells and juices of the whole minced organ. The behaviour was so strongly suggestive of a choline ester, resembling acetylcholine in its properties, that we decided to make a more complete comparison of its physiological actions with those of that substance.

By this time our preliminary chemical experiments had so far advanced as to show that the unstable principle was precipitated by mercuric acetate, and was recoverable from the precipitate. In an extract made with suitable precautions from a spleen, and purified in this manner, the choline-like effect so greatly preponderated in the depressor action, that any histamine still present made no significant contribution. A dilution of this purified extract was prepared which, on being tested for depressant action on a cat under ether, against a standard solution of acetylcholine, gave an equivalent of 0·4 c.c. of diluted extract to 0·01 mg. of acetylcholine (Fig. 1). The same solutions were then compared with respect to their stimulating action on the tone and rhythm of an isolated loop of rabbit's intestine, suspended in 100 c.c. of warm oxygenated Tyrode's solution. This preparation, as shown by one of us in an earlier investigation (Dale(7)), is a very sensitive and specific reagent for acetylcholine. It is practically unaffected by histamine in the quantities here in question, and under favourable conditions it will discriminate between two submaximal doses of acetylcholine differing by not more than 5 p.c. We have used it constantly in the numerous determinations of acetylcholine required in the further course of these investigations. In this preliminary experiment it gave precisely the same equivalent as that obtained for the depressor effect in the cat, 0·4 c.c. of the same spleen extract producing a rise in tone of intestinal muscle identical with that produced by 0·01 mg. of acetylcholine (Fig. 2).

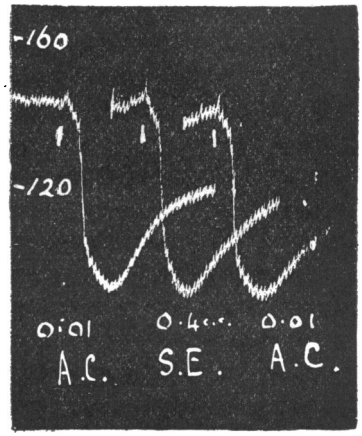

Fig. 1. Comparison of purified spleen extract and acetylcholine solution; blood-pressure of cat under ether.

The correspondence of the action with that of acetylcholine in these two directions was striking. Both these effects, however, are of a type which that ester shares with muscarine and other "parasympathetic" stimulants. There is another group of actions, however, which, among

such substances, the choline esters alone produce, and acetylcholine with a peculiar intensity. The easiest of these to observe and measure

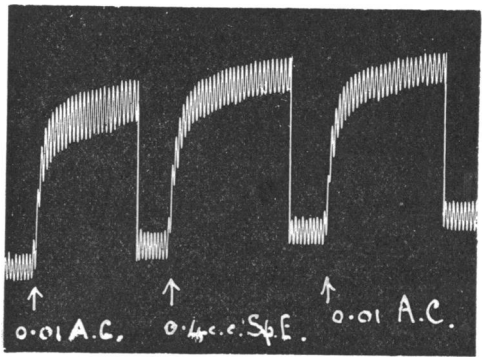

Fig. 2. Comparison of same two solutions on isolated jejunum of rabbit.

was the curious slow wave of contraction, produced in denervated mammalian voluntary muscle. (Cf. Frank, Nothmann and Hirsch-Kauffmann(8), Dale and Gasser(9).) The sciatic nerve of a cat was cut by one of us (H. H. D.), under ether anæsthesia and with aseptic precautions. Ten days later the cat was again anæsthetized with ether, and the gastrocnemius on the denervated side was dissected, the Achilles tendon being connected with an isometric lever, and the femur transfixed by a steel rod clamped to a rigid frame. The external iliac artery on the other side was isolated and clamped close to its aortic origin, and a cannula inserted, through which, on release of the clamp, injections could be made into the aorta near its bifurcation, so as to be carried directly

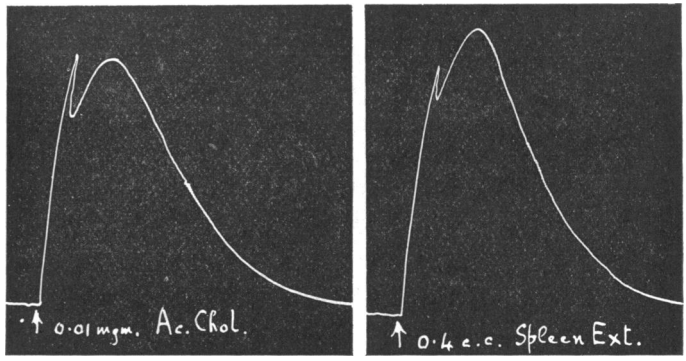

Fig. 3. Comparison of same two solutions, given by arterial injection into cat under ether, for effects in producing contraction-wave of denervated gastrocnemius.

by the blood-stream to the denervated leg. The internal iliac arteries were tied. Fig. 3 shows the effects produced by the injection of 0·4 c.c. of the dilution of spleen extract used in the previous comparisons, and of 0·01 mg. of acetylcholine, in this manner. The contractions are as similar as those which two identical injections of acetylcholine, given with the same interval, would produce. Such evidence made it clear that, whatever its exact chemical nature might prove to be, we were dealing with a choline ester, and with one so similar to acetylcholine in all its characters that we might adopt the latter as our physiological standard for controlling the effects of our procedure, and might safely plan our chemical manipulations on the assumption that the substance which we were endeavouring to isolate was, indeed, acetylcholine itself. The result, as shown in a later section, proved that this assumption was correct.

III. Isolation of histamine from ox spleen.

The method adopted was that which had already been successfully applied by Best, Dale, Dudley and Thorpe(1) to liver and lung, and by Thorpe(2) to muscle.

Immediately after the killing of the animals, 50 kg. ox spleen were collected at the slaughter-house, brought to the laboratory and minced into 94 p.c. alcohol (1·5 litres per kg. tissue) without delay. The mixture was stirred at frequent intervals during the first three hours, and then allowed to stand over night. It was then filtered, and the mince was re-extracted with 2 litres of 60 p.c. alcohol per kg. fresh tissue. After standing over night, this extract was filtered, and the filtrate was mixed with the first extract. The combined fluid was then acidified with sulphuric acid, 0·36 c.c. 50 p.c. (vol.) acid being added for each litre of extract. After standing for four hours, the liquid was filtered through paper-pulp, and concentrated *in vacuo*; the temperature of the liquid did not rise above 25° during the distillation. Volume of concentrate = 5·8 litres.

A small sample of this concentrate was centrifuged, and diluted 16 times. 0·25 c.c. of the diluted liquid was found to be equivalent to 0·0025 mg. histamine in lowering the blood-pressure of the unatropinized cat, while 0·55 c.c. was equivalent to 0·0025 mg. histamine in the atropinized cat.

0·55 c.c. of the diluted liquid, tested on the unatropinized cat, matched a solution containing 0·0025 mg. histamine + 0·7 mg. choline chloride.

From these tests the histamine content of the concentrate was estimated to be 0·42 g., and the "choline" content 116 g.

The concentrate was treated with basic lead acetate until no more precipitate formed, when it was filtered. From the filtrate the main excess of lead was removed as sulphate. After filtration the small amount of lead remaining was removed with hydrogen sulphide. The lead-free filtrate was concentrated to 2 litres.

Physiological assay estimated the histamine and choline chloride contents of this at 0·56 g. and 112 g., respectively. (For measuring of the apparent gain of histamine, see below.)

To the 2 litres concentrate, 115 c.c. sulphuric acid were added. 88 g. of potassium sulphate were precipitated by this treatment; the liquid, after filtering, was only faintly acid to Congo red, and was concentrated *in vacuo* to 900 c.c. to remove some of the free acetic acid present. After making it up to 1 litre with water, 1 litre of 10 p.c. sulphuric acid was added. Phosphotungstic acid was now added, 13·2 litres 25 p.c. solution in 5 p.c. sulphuric acid being required for complete precipitation. The precipitate was filtered off, washed with 500 c.c. of 1 p.c. phosphotungstic acid in 5 p.c. sulphuric acid, and then repeatedly extracted with 75 p.c. acetone until very little more went into solution. The combined acetone extracts were distilled *in vacuo* until most of the acetone had been removed, and the acetone-soluble phosphotungstate was then decomposed in the usual way with baryta solution. Excess of barium was removed from the filtrate with carbon dioxide, and the solution was concentrated *in vacuo* to 2·6 litres.

The physiological assay, made as before, estimated the histamine and choline chloride contents of this at 0·261 g. and 5·23 g. respectively. It will be seen that about 38 p.c. of the histamine and 95 p.c. of the "choline" activity were lost at this stage.

To this solution were added 30 c.c. sulphuric acid, making it strongly acid to litmus, but hardly acid to Congo red, and then 750 c.c. of 40 p.c. silver nitrate solution (sufficient to produce an immediate brown precipitate on adding barium hydroxide solution to a drop withdrawn for testing). The precipitate thus formed ("purine" fraction) was removed, and to the filtrate was added hot, saturated barium hydroxide solution, until the "histidine" fraction (tested in the usual manner with ammoniacal silver nitrate) was precipitated. The filtrate from this was saturated with barium hydroxide to precipitate the "arginine" fraction.

The "histidine" and "arginine" fractions were suspended in dilute

sulphuric acid and decomposed with hydrogen sulphide. Hydrogen sulphide was removed by aeration, and excess of sulphuric acid by barium carbonate, from the filtrate. The physiological assay of these two solutions indicated that the "histidine" fraction contained 0·0141 g. histamine and the "arginine" fraction 0·250 g. The "arginine" fraction, which contained practically all the histamine-like activity, was refractionated by the silver method, and gave small "purine" and "histidine" fractions, which had negligible activities, whilst the activity recovered in the "arginine" fraction represented 0·20 g. histamine.

This "arginine" fraction was concentrated *in vacuo* to about 100 c.c. A small amount of crystalline material (not examined) was removed, and the solution again evaporated until the volume was about 25 c.c. 25 g. powdered barium hydroxide crystals were then added to the liquid, and the resulting paste was dried in a vacuum desiccator over sulphuric acid. The mass was then powdered, and extracted with absolute alcohol in a Soxhlet thimble for three hours. After regrinding the mass, which had set to a hard solid, it was extracted for five hours and then re-ground and extracted for a further five hours. In this way three alcoholic extracts were obtained, to which water was added. The solutions contained a small amount of barium, which was removed as sulphate on careful neutralization of the alkaline solutions with dilute sulphuric acid. The total activity of the combined alcoholic extracts represented 0·108 g. histamine, determined physiologically.

The addition of sodium picrate to this solution (120 c.c.) threw down 2·61 g. of a picrate, crystallizing in dark yellow prisms, melting at about 200°, and practically devoid of physiological activity. The mother-liquor was concentrated and treated with a further quantity of sodium picrate solution; 1·4 g. of picrate were obtained, which on re-crystallization from 25 c.c. water yielded 0·237 g. of pale yellow crystals, m.p. 232–4° with decomposition, while the solution was still warm. After these had been filtered off, on cooling, the liquor deposited 0·798 g. of darker prismatic crystals, m.p. 198–200°. After two re-crystallizations of the pale yellow crystals, 0·1997 g. of a picrate, melting at 236–8°, was obtained. This picrate was identical in crystalline form and physiological activity with an authentic specimen of histamine dipicrate, and a mixed melting point determination showed no depression.

Analysis: 0·0430 g. picrate gave 0·0817 g. nitron picrate.

Picric acid:	found	80·40 p.c.
,,	calc. for $C_5H_9N_3$ $(C_6H_3O_7N_3)_2$	80·48 p.c.

Summary of yields of histamine at the successive stages of fractionation, expressed as mg. histamine.

Original extract	Basic lead acetate filtrate	Phospho-tungstic acid precipitate	Arginine fraction	Alkaline extraction	Pure histamine (isolated as dipicrate)
420	560	261	200	108	40

The results are very similar to those obtained in our examination of extracts from other organs. As we have earlier pointed out, the physiological estimates have not a high order of accuracy; they are, of necessity, made on very small samples, the results given by which are multiplied by very large factors. There are, however, discrepancies too large to be accounted for in this manner. The apparent gain of histamine in the treatment with basic lead acetate is seen also in some of our earlier records from other organ extracts. It has been explained by Bauer's and Richards's observation (30), that acetates have a definite depressor, vaso-dilator action, which intensifies that due to histamine. The acetates are again eliminated in the phosphotungstic precipitation, so that the yield at this stage should be compared with the estimate on the original extract, showing an apparent loss of about 38 p.c. Some of this is probably genuine, and is readily intelligible when the bulk of the precipitate to be handled at this stage is considered. It must further be remembered, however, that the total depressor effect of the original extract on the atropinized animal involves components due to potassium ions, and possibly other unspecific agents, which are eliminated in fractionating the phosphotungstates.

The loss in the silver fractionation is small (23 p.c.) and could probably have been almost entirely eliminated by including the "histidine" as well as the "arginine" fraction. This, however, would have carried on to the next stage, at which the most difficult separation occurs, a quantity of other substances out of proportion to the small additional amount of histamine. It will be noted that in this case, as with other organ extracts, it is in the arginine fraction that most of the histamine is precipitated. Berlin (5) expected, from Ackermann's (10) account of its isolation from a putrefactive mixture, that any histamine present in his spleen extracts would be precipitated in the histidine fraction; not finding any of the active substance there, he wrongly concluded that it was not histamine.

As with other organ extracts, we encountered a serious loss on the extraction with alcohol of the free bases from the arginine fraction (alkaline extraction). It is the fact that there is yet no method available by which histamine can be extracted quantitatively from such a mixture.

The method used, though the most effective which we have found, always involves a substantial loss. The final yield of pure histamine dipicrate, from the bases so extracted, could have been substantially improved by repeated re-working of the picrate mother-liquors. It seemed to us, however, that the time and labour would be ill spent. We had already an ample quantity of the pure salt for complete identification, and, when our evidence from other organ extracts is put alongside this from the spleen extract, a sufficient justification for concluding that this also owes its histamine-like action to the presence of histamine itself.

IV. Isolation of acetylcholine from horse spleen.

A. General description of process.

Horse spleens were chosen for this purpose on account of the greater ease of obtaining them in quantity at a known interval after death of the animal. We had evidence of a similarly acting unstable principle in the spleen of the ox, and there is no reason for supposing that the yield from it would have been less under similar conditions.

Since the unstable active principle of the crude extracts had been shown to be similar, in its physiological activities and chemical behaviour towards dilute alkali, to acetylcholine, the method adopted for the fractionation of the extract was framed on the general lines of that employed by Ewins(11) for the isolation of acetylcholine from ergot; but numerous modifications were introduced, which seemed likely to increase the ease of operation and to conserve the very labile compound which we were attempting to isolate.

As in Ewins's method, the first stage was to get rid of everything from the extract which was insoluble in absolute alcohol; and it was found advantageous to proceed to this point without first carrying out the preliminary purification of the extract with mercuric chloride, which he had applied to ergot extract. The subsequent treatment of the material soluble in absolute alcohol was rather different from that of Ewins. He precipitated it directly as the mercury salt by the addition of alcoholic mercuric chloride, and extracted the active material from this precipitate with boiling water, then setting it free from its mercury compound by means of hydrogen sulphide, which, of course, produced a solution very strongly acid with hydrochloric acid. As acetylcholine salts are fairly easily hydrolyzed by boiling water, and also by mineral acids, it was feared that the application of these steps to the material from spleen might reduce seriously the already small amount present.

A gentler treatment was therefore devised. The alcoholic solution was diluted with an equal volume of water and the alcohol was then removed by distillation *in vacuo*. To the aqueous solution was added aqueous mercuric chloride and the precipitate produced, which contained none of the active substance, was removed. To the filtrate solid sodium acetate and powdered mercuric chloride were added; a heavy precipitate was formed, which, in a small exploratory experiment, contained the whole of the active material, of which 70 p.c. was recovered on decomposition of the precipitate with hydrogen sulphide[1]. Unfortunately, when this process was applied in the large-scale experiment reported in this paper, the separation of the active material was not so sharp, and successive treatments with mercuric acetate were necessary to remove a fair proportion of it from the solution. The step was therefore not such an improvement as was anticipated, and it might, after all, have been simpler to precipitate the mercury salt from alcoholic solution as Ewins did. The useful observation was made, that if the mercuric acetate precipitate was shaken with cold water a relatively small proportion dissolved, which contained the active constituent. A valuable purification was thus affected. The constituents soluble in cold water were treated with hydrogen sulphide at 0°, and the consequent development of acidity was not great.

At this stage Ewins got rid of a part of the relatively large amount of choline, which inevitably accompanies the active material in the fractionation, by converting it into the acid tartrate in alcoholic solution. Having crystallized out as much choline acid tartrate as possible, he precipitated the alcoholic filtrate with an alcoholic solution of platinum chloride, thus obtaining a mixture of chloroplatinates containing all the acetylcholine, together with excess of choline and other impurities. In the fractionation of the material from spleen this step was omitted. The aqueous solution from the decomposition of the soluble mercury salts was taken to dryness, and the portion soluble in alcohol was extracted from the residue. The alcoholic solution was then directly precipitated with alcoholic platinum chloride. This precipitate contained, by physiological estimation, about one-twentieth of its weight of acetylcholine chloroplatinate, the main impurity in it being choline chloroplatinate. A chemical investigation[2] of the problem of separating

[1] Partial or complete hydrolysis of acetylcholine occurs when its gold, platinum or mercury salts are decomposed with hydrogen sulphide (see Nothnagel (12)). Steps involving such decomposition must, therefore, be avoided as far as possible.

[2] This investigation will be described by one of us (H. W. D.) in a paper in the *Biochemical Journal*.

acetylcholine chloroplatinate from mixtures containing a large excess of the choline compound, showed that, from such mixtures, not acetylcholine chloroplatinate itself, but a double salt crystallizing in isotropic octahedra and containing one molecule of each (choline-acetylcholine dichloroplatinate) separated. The solubility in water of this salt, by means of which the acetylcholine from spleen was ultimately identified, is very much lowered in the presence of excess of choline chloroplatinate, which is itself very much more soluble in water than the double chloroplatinate. We had omitted the partial separation of choline and acetylcholine as acid tartrates, employed by Ewins. The omission was, at the time, dictated by caution, since the procedure involves the liberation of the free bases in alkaline solution, with inevitable loss of some of the ester; and the separation is, in any case, so incomplete (see Ewins(11)) that we doubted the advantage of attempting it. In the end, our neglect thus to reduce the excess of choline in the mixture proved to be a positive advantage, since, when the mixed bases had been converted into chloroplatinates, its presence depressed the solubility of the choline-acetylcholine dichloroplatinate, and enabled a substantial proportion of the latter to be finally isolated in pure condition. It was identified by analysis, by comparison with a pure sample prepared synthetically, and by determination of the physiological activity of the chlorides released from it, in comparison with that of the chlorides from the synthetic dichloroplatinate, and of acetylcholine chloride from its pure chloroplatinate.

The physiological method provided another test of the identity of this compound. If it was, indeed, a double chloroplatinate of choline and acetylcholine, it should yield on decomposition a solution containing 1 molecule of choline chloride for each molecule of acetylcholine chloride. Since acetylcholine is about one thousand times as active as choline, the choline would make no measurable contribution to the activity of such a mixture. If the latter, accordingly, was completely acetylated, the activity of the resulting product should be just double that of the original mixture. The change in activity could not, on the other hand, have this exact ratio, if the chloroplatinate under investigation were anything other than the dichloroplatinate of choline and acetylcholine. The test on the rabbit's intestine gave us an accuracy well within 10 p.c., and the experiment was made, first on the synthetic dichloroplatinate, and then on the supposedly identical substance obtained from the spleen extract. In both cases the result verified the prediction; complete acetylation exactly doubled the activity of the chloride-mixture on the rabbit's intestine.

B. Experimental details.

The spleens were collected from the slaughterers throughout the day, in batches of six. Killing commenced at 7 a.m., and the first batch of six spleens arrived at the laboratory at 10 a.m. Further batches of six arrived at 1.15 p.m., 5.0 p.m. and 8.15 p.m.

When the spleens arrived at the laboratory each was weighed separately; the weights varied between 1 and 2 kg. 96 p.c. alcohol was taken in the proportion of 5 litres alcohol per kg. spleen for each organ. The spleen was cut into three or four strips and immediately passed through a powerful mincer, one-tenth of its ration of alcohol being poured into the mincer as the tissue was passing through it; the mince was delivered directly into a small enamelled pail containing the remaining nine-tenths of the alcohol, which was vigorously stirred as the tissue fell into it. The mincing of each spleen was completed in about one minute. The alcoholic suspensions of minced tissue were bulked in larger containers, stirred frequently, and allowed to stand over night.

The total weight of the spleens so extracted was 32·34 kg. On the following day the extract was filtered, first through cloth and then through paper-pulp. The filtrate was then concentrated *in vacuo*; the temperature of the water jacket was held at 45–50°, and the temperature of the extract never reached 20°. It was reduced to a volume of 5 litres in the course of six hours.

The concentrated extract contained much finely divided fat. On shaking with ether a stiff emulsion was formed which was broken by the cautious addition of alcohol. The ether layer was removed and the aqueous layer, after another extraction with ether, was evaporated *in vacuo* to 1120 c.c.

Physiological assay of this solution, made on the rabbit's intestine, showed it to have an activity equivalent to 334 mg. acetylcholine chloride.

In order to remove some of the water and to salt out impurities, the solution was warmed to 26°, and anhydrous sodium sulphate was stirred in until no more would dissolve. Alcohol was then added until a copious precipitate of hydrated sodium sulphate was produced, which was filtered off after standing in the cold room. The filtrate was concentrated *in vacuo* to 400 c.c. More sodium sulphate was precipitated on adding an equal volume of alcohol and the filtrate from this was concentrated *in vacuo* to 150 c.c. The addition of alcohol to this concentrate caused a syrupy layer to be formed. It was therefore put in a

bottle with 2 litres absolute alcohol and shaken violently for two hours. The alcohol was poured off from a very sticky residue remaining on the sides of the bottle, and the extract was concentrated *in vacuo* to 200 c.c.

Physiological assays of the alcoholic solution and of the sticky residue showed activities equivalent to 260 mg. and $< 10{\cdot}5$ mg., respectively, of acetylcholine. The residue was therefore discarded.

To the alcoholic solution 200 c.c. water were added, the alcohol was distilled off *in vacuo*, and to the residual solution (140 c.c.) 260 c.c. saturated mercuric chloride, and, finally, 30 c.c. of hot water containing 10 g. mercuric chloride were added. After standing in the cold room the precipitate was filtered off and washed with half-saturated mercuric chloride solution.

Physiological test of a solution prepared appropriately from the precipitate showed the latter to possess only a trace of activity.

To the filtrate were added 200 c.c. saturated aqueous mercuric chloride and then, cautiously, sodium hydroxide solution, since the filtrate was very strongly acid to litmus but not to Congo red. When the reaction to litmus was moderately acid the addition of sodium hydroxide was stopped and the precipitate which had formed was filtered off. This contained only a trace of activity. The filtrate was concentrated *in vacuo* to 170 c.c., and to it were then added solid sodium acetate and powdered mercuric chloride. The precipitate which formed was filtered off, and extracted repeatedly by shaking with successive quantities of about 200 c.c. water for 2-hour periods. The filtered solutions were immersed in ice-water and decomposed with hydrogen sulphide. After filtration from mercuric sulphide and removal of hydrogen sulphide by aeration, sodium hydroxide solution was added drop by drop until the reaction of the filtrates was faintly acid to litmus. They were then tested physiologically with the following results:

Water extract	Activity (as mg. acetylcholine chloride)
1	24
2	12·4
3	7·7
4	2·6

It was found that simple dilution of the solution, from which the mercury precipitate had been obtained, with saturated sodium acetate solution produced a further precipitate. An equal volume of the latter was, therefore, added; the precipitate produced was collected, extracted with water, and the extracts were decomposed and tested as before, with the following results:

Water extract	Activity (as mg. acetylcholine chloride)
1	31·4
2	7·2
3	0·5

The combined solutions, therefore, had a total activity representing 85·8 mg. acetylcholine chloride.

Physiological assay of the filtrate from which these two main mercury precipitates were separated detected an activity representing 36·8 mg. acetylcholine chloride, from which, by the addition of mercuric acetate and extraction of the precipitate with water as before, a solution of activity equal to that of 21·6 mg. acetylcholine chloride was obtained. This material was not added to the main bulk for further fractionation because it was heavily contaminated with sodium acetate.

The combined solutions of activity equal to 85·8 mg. acetylcholine chloride were concentrated to small bulk, the reaction was adjusted to faint acidity to litmus, alcohol was added, and precipitated salts were removed by filtration. The filtrate was then concentrated to small bulk and finally taken to dryness, over sulphuric acid, in a vacuum desiccator. The sticky residue was rubbed up with 24 c.c. absolute alcohol, and to the solution, after filtration from insoluble material, was added a solution of 3 g. platinum chloride in 15 c.c. absolute alcohol. The precipitated platinum salt was filtered off, washed with absolute alcohol and dried in a vacuum desiccator.

The weight of the platinum salt was 2·7183 g. A few milligrammes of this salt were decomposed by the potassium chloride method described by Ewins(11), and the activity, determined physiologically, referred to the total platinum salt, was equivalent to 88 mg. acetylcholine chloride. At this stage the platinum salt stood for 3½ months in a desiccator over calcium chloride, while the investigation of the properties of acetylcholine, already referred to, was being made.

On retesting after this period, its activity was found to correspond to 76·4 mg. acetylcholine chloride. A definite loss of activity had, therefore, occurred.

Isolation of choline-acetylcholine dichloroplatinate. The total weight of platinum salt used for the final stage of the isolation was 2·714 g. with an activity corresponding to 76·4 mg. acetylcholine chloride (equivalent to 0·1475 g. acetylcholine chloroplatinate or 0·2770 g. choline-acetylcholine dichloroplatinate).

The platinum salt was treated with 8 c.c. boiling water, which would have been sufficient to dissolve the material completely if it had been

simply a mixture of choline and acetylcholine chloroplatinates. As, however, complete solution was not effected, 50 c.c. cold water were added, and, after standing, 0·0486 g. (A) of an insoluble residue was removed by filtration. The solution was placed over sulphuric acid in a vacuum desiccator over night; the volume was thus reduced to 15 c.c., and octahedra similar in appearance to those of synthetic choline-acetylcholine dichloroplatinate, together with lighter coloured regular crystals, had been deposited. The solution was warmed, and, after the octahedra had gone into solution, decanted from the less soluble lighter material, 0·0244 g. (B). After cooling, the decanted liquid deposited octahedra which were filtered off; 0·378 g. (C). The filtrate was returned to the vacuum desiccator and reduced in volume to 7 c.c. It was warmed on the water-bath to dissolve the material which had separated, and, on cooling, deposited 0·070 g. of clean octahedra (D).

Weighed quantities of about 1 mg. each of (C) and (D) were decomposed by the potassium chloride method and tested physiologically against pure acetylcholine chloride. The result of the test was that, referred to choline-acetylcholine dichloroplatinate, both these fractions were about 50 p.c. pure.

(C) and (D) were, therefore, combined (0·4484 g.), and heated with 4 c.c. water on the water-bath. The whole of the material did not go into solution, as it would have done had it been pure choline-acetylcholine dichloroplatinate; 8 c.c. of cold water were therefore added, and, after 12 hours, the solution was filtered from a small amount of insoluble material (0·007 g.), and the solution was taken to dryness in a vacuum desiccator. The residue was again taken up in 4 c.c. hot water, but, as some material remained undissolved, the solution was held at 37° for 1½ hours, and the undissolved material was then filtered off; 0·138 g. (E). The filtrate, on cooling to room temperature, deposited octahedra, from which the solution was decanted after 5½ hours. The octahedra were washed with a little water, and weighed 0·0395 g. (F). The decanted liquid and washing were returned to the desiccator, and a further crop of octahedra removed when the volume was about 3 c.c.; 0·1030 g. (G). On concentrating to 1·5 c.c., as before, another crop of perfectly formed octahedra was obtained; 0·0914 g. (H). The final filtrate was taken to dryness, and, under the polarizing microscope, a small quantity of isotropic octahedra could be seen embedded in strongly anisotropic material (choline chloroplatinate).

The original solution from which (C) and (D) had been isolated was concentrated in a vacuum desiccator to about 3 c.c. The liquid was then

full of anisotropic prismatic needles (choline chloroplatinate). It was warmed until these needles were just dissolved, and then filtered to remove a small amount of minute regular crystals; 0·0220 g. (K). (A) and (B) were not examined, since their relative insolubility precluded the possibility of their being choline-acetylcholine dichloroplatinate.

Weighed quantities of (E), (F), (G), (H) and (K) were tested physiologically, after decomposition by the potassium chloride method, against pure acetylcholine chloride, with the following results:

	Taken (mg.)	Activity p.c.
(E)	2·5	2·45
(F)	0·95	52·2
(G)	0·8	48·5
(H)	1·15	55·1
(K)	0·8	7·1

The theoretical percentage activity of choline acetylcholine compared with that of acetylcholine, both weighed as chloroplatinates, is 53.

Within the limits of accuracy of the physiological method, the activities of the crops (F), (G) and (H) are, therefore, indistinguishable from that of pure choline-acetylcholine dichloroplatinate. The very small activities of (E) and (K) indicate that the fractional crystallization removed successfully a less soluble platinum salt, and that practically the whole of the acetylcholine was removed from the main solution in fractions (C) and (D).

Analysis: Two micro-analyses were made on material from crop (F).

	Found p.c.		Calculated p.c.
	1	2	
C	21·18	21·84	21·88
H	4·37	4·37	4·56
Pt	29·60	29·75	29·64

Melting points of crops (F), (G) and (H), synthetic choline-acetylcholine dichloroplatinate, and mixed melting points of each fraction with the synthetic salt were determined.

Material	Weight g.	Melting point °C.	Mixed melting point °C.
Synthetic choline-acetylcholine dichloroplatinate	—	260–1	—
(F)	0·0395	259–60	260–1
(G)	0·1030	259–60	260–1
(H)	0·0914	260–1	260–1
	0·2339		

The material isolated in crops (F), (G) and (H) is, therefore, identified as choline-acetylcholine dichloroplatinate. The total weight of the three crops was 0·2339 g. as compared with the physiological estimate of 0·2770 g. of the dichloroplatinate, as present in the crude mixture of chloroplatinates. It will be clear that a high proportion of the pure compound was separated from the crude mixture.

The amounts of acetylcholine chloride (in mg.) at each stage of the purification are set out in the following table. The estimates were made by the biological test, except in the last case, where the pure dichloroplatinate was weighed, and the corresponding amount of acetylcholine chloride calculated.

Original extract	Alcoholic solution	Hg acetate precipitate	Crude chloroplatinates	Crude chloroplatinates after standing 3½ months	Pure dichloroplatinate
334	260	85·8	88	76·4	64·5

It will be seen that the main losses occurred in the first two stages, the most serious loss being incurred in the precipitation with mercuric acetate and subsequent liberation of the substance from its mercury compound by means of hydrogen sulphide. Incomplete precipitation and unavoidable partial hydrolysis during decomposition of the mercury compound account for this fall in quantity. There was remarkably little loss after the mercuric acetate stage, and the yield of pure salt would undoubtedly have been higher if it had not been necessary to store the crude chloroplatinate for 3½ months while the chemistry of the final fractionation was being studied. Even so it is somewhat surprising, in view of the very unstable nature of acetylcholine, that about one-fifth of the amount estimated as present in the original extract was actually isolated as a chemically pure compound.

Acetylation of the mixed chlorides. 1·75 mg. of the dichloroplatinate from spleen was dissolved in 2 c.c. water; 0·2 c.c. of a solution containing 2 mg. potassium chloride was added, and the liquid was taken to dryness over sulphuric acid in a vacuum desiccator. The residue was extracted with four successive lots of absolute alcohol, each of 4 c.c., and the combined, filtered extract made up to 20 c.c. 10 c.c. of this solution were taken to dryness *in vacuo*, and the residue was dissolved in 10 c.c. water, giving a solution represented by *A* in the physiological records shown in Figs. 4 and 6. 5 c.c. of the solution were evaporated to dryness in a small test-tube immersed in a paraffin-bath at 95°. A tube drawn out to a capillary delivered the solution, a few drops at a time, at the bottom of the test-tube, so that the residue was deposited compactly. The evapora-

tion of the alcohol was facilitated by drawing air through a tube dipping about half way down the test-tube. 0·5 c.c. acetyl chloride was added to the dry residue; the tube was sealed and heated at 100° C. for five hours. The tube was then opened and placed in a flask. The excess of acetyl chloride was removed by vacuum distillation, 10 c.c. absolute alcohol were added, and, after standing for ten minutes to decompose traces of acetyl chloride, distilled off *in vacuo*. The residue was then dissolved in 10 c.c. water, giving a solution represented by *B* in Figs. 4 and 5. It will be noted that *B* represents only half the concentration of

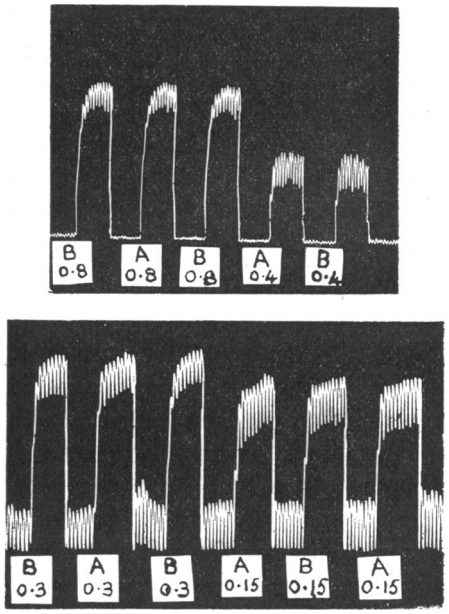

Fig. 4.

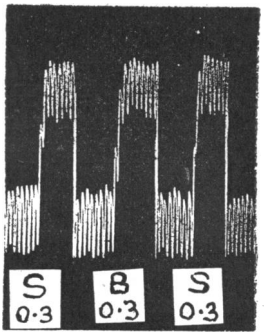

Fig. 5.

Fig. 4. Tests, on two preparations of rabbit's jejunum, of solutions *A* and *B* (see text) in four different, submaximal doses. The equivalence is in all cases exact. *B* being made up to one-half the concentration of *A*, the activity has been exactly doubled by complete acetylation.

Fig. 5. Comparison of *B*, in equal doses, with pure acetylcholine solution *S* (see text).

original chloroplatinate represented in *A*. A solution of acetylcholine chloride was prepared from pure acetylcholine chloroplatinate corresponding to that contained in 1·75 mg. of the dichloroplatinate, and was similarly made up to 20 c.c. with water. This solution is represented as *S* in Figs. 5 and 6, showing its comparison with *B* and *A* respectively. It will be seen that it exactly matched these in activity.

The results of the test showed that the activity of the material, liberated from the dichloroplatinate isolated from spleen, was exactly

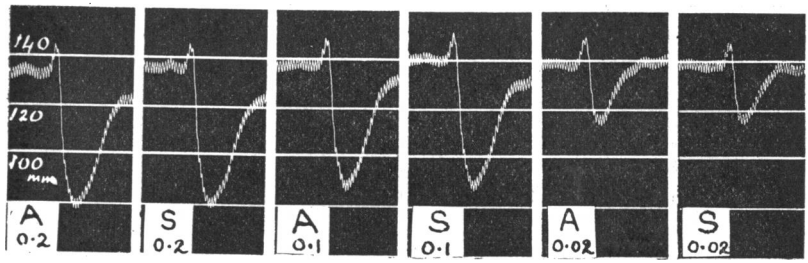

Fig. 6. Comparison of solutions *A* and *S*, in three different doses. Depressor effects on blood-pressure of completely pithed cat, maintained by slow, constant, intravenous infusion of adrenaline.

doubled by complete acetylation, and that the activity of the unacetylated material, in relation to that of pure acetylcholine chloride, had the theoretical value, viz. 53 p.c., on the basis of the acetylcholine content of choline-acetylcholine dichloroplatinate.

Discussion.

1. *Histamine.* The fact that histamine can be extracted by cold alcohol from the substance of the fresh spleen hardly needs separate discussion from that already given to similar findings in the case of other organs. In this, as in other cases, we regard the evidence as justifying the assumption that histamine is present in the cells of the living organ. With regard to its richness in this base, the spleen tissue seems to occupy an intermediate position. It contains less than the lung, and more than most other organs per unit of tissue. The physiological estimate on our large-scale extract showed about 8 mg. per kg. of spleen. This probably represents a higher concentration in the intrinsic spleen cells, since a substantial contribution to the weight of the whole organ must be made by the blood corpuscles, which contain very little histamine when they are in circulation. Our main concern was to show that there is no ground for suggesting that the histamine-like actions of spleen extracts are due to a special principle, or specific hormone. There is no real reason for attributing them to anything other than histamine itself.

2. *Acetylcholine.* The isolation of acetylcholine has an interest of a very different kind, this being the first occasion on which the substance

has been found to occur naturally in the animal body. It was first prepared artificially by Baeyer(13) in 1867, and had only a chemical interest until 1909, when Hunt and Taveau(14) observed that, though it resembled choline in the type of its depressor action, it was enormously more active. The interest in it of one of us (Dale(7)) was aroused by its identification by Ewins(11), then collaborating with him, as a natural constituent of certain ergot extracts. An examination of its action on various organs showed that it reproduced the effects of parasympathetic nerves, in an intense and evanescent form, with a fidelity rivalling that with which adrenaline reproduces effects of true sympathetic nerves. When these parasympathetic effects were annulled by atropine, acetylcholine, in somewhat larger doses, was found to possess another type of action, stimulating ganglion cells in a manner which ranged its action with that of nicotine. These two distinct types of activity could be traced, with varying intensities, in a series of other choline esters, other quarternary ammonium bases, and, as Hunt and Renshaw(15) have shown, also in phosphonium, arsonium, stibonium and sulphonium bases. Seen with peculiar intensity in the effects of acetylcholine, these "muscarine" and "nicotine" actions must be regarded as related to different portions or aspects of the structure of its molecule. One of its actions, to which much interest has attached in recent years, is its excitation of a peculiar, slow contraction, or wave of tonus, in certain normal muscles of frogs and birds, and in mammalian muscles deprived, by degenerative section, of their motor nerve-supply. (See Riesser(16), Frank, Nothmann and Hirsch-Kauffmann(8), Dale and Gasser(9).) There was a tendency to attribute this effect to the presence of a hypothetical, secondary, parasympathetic nerve-supply to skeletal muscle. Dale and Gasser, however, were able to show, by tracing the effect through a series of compounds, that it was essentially an aspect of the "nicotine," and not of the "muscarine" action.

The discovery of activities of such interest, in a compound closely related to so general a tissue constituent as choline, has naturally provoked speculation as to the possibility that acetylcholine might occur in the body and be physiologically functional. Le Heux(17), taking as a basis Magnus's conception of choline as the normal stimulant of intestinal rhythm, observed that the action of choline on an isolated loop of intestine was enhanced by the presence of acetates in the surrounding saline medium, and speculated on the possible synthesis of acetylcholine as a preliminary to the effective action of choline. Hoet(18) observed that the atropine-sensitive rhythm of isolated intestinal muscle

was enhanced by strong, post-mortem faradization of the vagus nerves, before excision of the intestine. Hess(19), who has worked particularly on the slow contraction of frog's voluntary muscle in response to acetylcholine, detected in Ringer's solution, flowing from the perfused muscle during tetanic excitation, traces of a substance having activity suggesting that of acetylcholine. By far the most suggestive indications, however, of the natural function of a substance of this nature in the body have been afforded by the demonstration that the inhibition of the heart muscle, by stimulation of the vagus, is associated with, and presumably due to, the liberation of such a substance in intimate relation to the heart muscle fibres. Preliminary observations of this nature were made by Dixon(20) as long ago as 1906, before the activity of acetylcholine had been discovered. In a relatively recent series of investigations on the liberation of substances transmitting the effects of nerve stimulation in the frog's heart, Loewi and his coworkers(21) have produced an array of highly suggestive facts bearing on the nature of the "vagus substance." None of these facts has conflicted with the possibility of this substance being acetylcholine, and some of them clearly indicate a choline ester of similar properties.

The facts above mentioned, as to the dual nature of the action of acetylcholine, are also highly suggestive, when considered in relation to reactions of denervated voluntary muscle. Vulpian and Philippeaux(22), in 1863, showed that, after degenerative section of the hypoglossal nerve, the voluntary muscles of the tongue acquired a new type of sensitiveness, the organ responding with a slow type of contraction to peripheral stimulation of the chordalingual nerve, or of the separated *chorda tympani*, which normally produces only the well-known parasympathetic vaso-dilatation and secretory activity. Heidenhain(23), who later studied this phenomenon and termed it a "pseudomotor" reaction, endeavouring to explain it as due to turgescence with lymph, added the observation that a similar reaction of the denervated tongue was produced by nicotine. The phenomenon has received further study from v. Rijnberk(24) and others. More recently Frank, Nothmann and Hirsch-Kauffmann(8) observed that a closely similar reaction of the denervated tongue was produced by intravascular injection of acetylcholine. We have, then, this curious association of facts, that the stimulation of a parasympathetic nerve and the injection of acetylcholine, the action of which in the normal organ closely simulates the effect of such stimulation, both produce in the denervated organ a new reaction of the voluntary muscle fibres. If we suppose, on the analogy

of Loewi's experiments on the heart vagus, that parasympathetic nerves produce their effects by peripheral liberation of a chemical stimulant, we must also credit this substance with a stimulant action on denervated voluntary muscle; it must, therefore, be a substance having the two types of activity which are exhibited by the choline esters, and by acetylcholine with unique intensity.

We meet a similar relation, between normal vaso-dilator action and abnormal stimulant action on denervated voluntary muscle, in the phenomenon described by Sherrington(25) in 1894. He found that, when the muscles of the hind limb had been deprived of their motor innervation, by degeneration following spinal root section, stimulation of the sciatic nerve caused an abnormal, slow, weak contraction of the leg-muscles. The presumption was that the effect was produced by antidromic stimulation of the sensory nerve fibres. The only other possibility was an action of the sympathetic fibres, and this was excluded by v. Rijnberk(26), who produced Sherrington's phenomenon after preliminary degeneration of these. Hinsey and Gasser(27) have given further precision to the identification, by showing that the abnormal contraction of the voluntary muscles, and the normal antidromic vaso-dilatation, are alike produced by the sensory fibres of small diameter in the sciatic. In this case, again, we have a concurrent acquisition, by the muscle fibres deprived of their motor innervation, of a new sensitiveness to acetylcholine on the one hand, and to the stimulation of nerve fibres which normally cause dilatation of arterioles on the other. Hunt (28) has shown that the distribution of the vaso-dilator action of acetylcholine in the hind limb is closely similar to that of the antidromic vaso-dilatation.

We have again two sets of facts, which are at once brought into an intelligible relationship, if we make for the antidromic vaso-dilator effects the same supposition as for parasympathetic effects, namely that they are mediated by the peripheral liberation of a substance which dilates normal arteries and stimulates denervated voluntary muscle—of a substance which has the dual action of acetylcholine.

The suggestion, that these paradoxical responses of denervated voluntary muscle might find their explanation in the intervention of such a chemical mechanism, has been made by Bremer and Rylant(29), and more recently, and in more definite terms, by Hinsey and Gasser(27). But there has been a natural and proper reluctance to assume, in default of chemical evidence, that the chemical agent concerned in these effects, or in the humoral transmission of vagus action, was a substance known,

hitherto, only as a synthetic curiosity, or as an occasional constituent of certain plant extracts. Many things could be explained if the liberation of acetylcholine could be postulated; but the minuteness of the quantities required to produce the effects in question, and the extreme instability of the substance, while enhancing its theoretical fitness for the suggested functions, precluded any hope of its chemical identification at the sites of its possible liberation. There are certain discrepancies between the effects of atropine on the vaso-dilator effects produced by acetylcholine and by stimulation of parasympathetic and sensory nerves, and also between the antagonisms of adrenaline to these two methods of exciting the contraction of denervated muscle; but discussion of these may, with advantage, be deferred to a later communication, directly dealing with some of them.

It appears to us that the case for acetylcholine as a physiological agent is now materially strengthened by the fact that we have been able to isolate it from an animal organ and thus to show that it is a natural constituent of the body. We have yet no conception of the meaning of its presence in the spleens of these large ungulates. In a few quite preliminary experiments we have failed to find evidence of its presence in the spleens of the animals ordinarily available for the supply of fresh post-mortem material in the laboratory—dog, cat, monkey, or rabbit. In those of the ox and the horse, after our first accidental observation of its presence, we found it regularly, and in such quantities as to make the attempt at its isolation a reasonable and, in the event, a successful enterprise. The peculiar difficulties of dealing with a substance so labile make it very unlikely that we can extend the attempt to a range of other organs, as we have in the case of histamine. We feel, however, that its definite isolation from one organ has so far altered the position that, when an extract from, or a fluid in contact with the cells of, an animal organ can be shown to contain a principle having the actions, and the peculiar instability, of acetylcholine, it will be reasonable in future to assume the identification. On such lines a physiological survey of its distribution should be practicable. Similarly, when there is evidence associating some physiological event with the liberation of a substance indistinguishable from acetylcholine by its action, the presumption that it is, indeed, that ester will be strengthened by the knowledge that acetylcholine occurs in the normal body. That is the only significance which we can, as yet, attribute to its identification in the horse's spleen, though further evidence may associate its occurrence there with some function yet unknown.

SUMMARY.

1. The substance in alcoholic extracts of spleen, resembling histamine in its action, has been isolated and identified as histamine.

2. Treatment of the fresh spleen substance of the horse or ox, with alcohol applied simultaneously with the disintegration of the organ, extracts an intensely active, unstable ester of choline, which has been isolated from horse's spleen and identified as acetylcholine.

3. The bearing of the latter identification on certain physiological phenomena is discussed.

At various stages of this investigation we have been greatly indebted for help, especially in making the numerous physiological estimates involved, to Drs Bauer, Richards and Gaddum, and to Mr Collison of the laboratory staff. To Mr Marks, in addition to co-operation already acknowledged, we are indebted for the analyses by micro-combustion, and for much valuable assistance in the large-scale extractions.

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