XV. VASODILATOR CONSTITUENTS OF TISSUE EXTRACTS.

ISOLATION OF HISTAMINE FROM MUSCLE.

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IN a previous communication [Best, Dale, Dudley and Thorpe, 1927] we were able to prove that the chief vasodilator constituent of both ox liver and lung was histamine (β -iminazolyethylamine) by the isolation of this substance in a state of chemical purity from alcoholic extracts of each organ under conditions which precluded its formation by autolysis or bacterial action during the process of isolation.

Now since extracts of almost all organs of the body have been shown to provide depressor substances, it would be of considerable physiological importance if more definite information as to the chemical nature of these could be obtained; for until our own investigations began no depressor substance other than choline had been isolated under conditions which are absolutely free from suspicion of contamination by bacterial action. The important work of Abel and Kubota in this connection has been considered in the paper quoted above. With a view to selecting the most suitable organ for examination it became necessary to review the distribution of the depressor substances; for although this field has been explored by a number of workers their extracts have been both prepared and tested by different methods. To collect such data and use them as a comparative survey of the distribution of the vasodilators would in all probability be very misleading. Thus it seemed that reliable information could only be obtained by preparing and estimating the activity of the extracts under standardised conditions.

For this purpose the alcohol method of extraction already described [Best, Dale, Dudley and Thorpe, 1927] seemed preferable since, while absolute sterility was ensured, this method would be least likely to permit of any hydrolytic changes taking place.

Extracts from a number of organs of horse and ox have been prepared by the alcohol method and the extracts thus obtained, after removal of alcohol and fat, were tested on the blood-pressure of the cat in comparison with pure histamine. Except in the case of spleen no attempt has been made to determine the influence of choline on the histamine-like activity. Since the values

were only intended as a guide for the selection of material for future chemical work, the additional labour of estimating the histamine and choline separately did not seem to be justified, for the depressor activity of 1 mg. of histamine is only matched by quantities of choline varying from 300 mg. to 3000 mg. according to the sensitiveness of the cat employed.

All the organs mentioned in Table I were worked up within an hour of the killing of the animal. In most cases the experiments are in duplicate and values from two different animals are given. For convenience in comparison, the values obtained for liver and lung, already published, are included. It is hardly necessary to point out that in the large scale experiments any slight error in the matching of the activity with that of histamine (the normal dose of histamine was of the order of 0.003 mg.) would be very greatly magnified in calculating the total activity of the extract. On the other hand the losses involved in making extracts of small organs weighing only a few grams would be proportionately much greater than those involved in preparing an extract from 500 g. or more material.

Table I. *Distribution of depressor substances in various organs.*

Organ	Depressor activity expressed as mg. histamine per kg. fresh tissue		Source	Remarks
Lung	44,	75	Ox	75 = L.S.
"		35	Horse	—
Ovary		9	Ox	K.
Plain muscle (bladder)	7,	8	Horse	—
Spleen	7.5,	5	"	Values from atropinised cat. C. (Values before atropine 9 and 17)
Parotid gland	5.3,	6.7	"	c.
Liver	5.4,	5.0	Ox	L.S. } C.
"	2.5,	6.6	Horse	—
Kidney	2.6,	3.3	"	—
Pancreas	1.6,	3.0	"	c.
Testis		1.8	Ox	K.
Striated muscle		1.0	Cat	—
"		1.0	Dog	After purification with basic lead acetate
"	1.1,	1.4	Horse	—
"		4.0	Ox	L.S.
Submaxillary gland		0.6	Horse	—
"		0.5	"	—
Thyroid gland		0.5	"	K.
Suprarenal gland		0.3	"	Purified with phospho- tungstic acid to re- move adrenaline

L.S. = Values obtained from extracts prepared on a large scale.

C. = Choline present in large quantity.

c. = Physiological tests suggested presence of choline.

K. = Extracts had to be kept some time before testing—values probably low.

Considering the nature of the experiments the above results show good agreement. The most serious discrepancies are seen with liver extracts. Here choline is known to be present in such large quantities that the depressor activity is greater than that which would be produced by the summation of the

separate effects of the histamine and choline actually present [Best, Dale, Dudley and Thorpe, 1927, p. 403]. Hence the low value (2.5), obtained with a cat not very sensitive to choline, is considered to be more nearly the correct value for the histamine content.

The unique position of lung extracts in this list has already been discussed [Best, Dale, Dudley and Thorpe, 1927] and requires no further comment here.

The relatively high activity of spleen extract makes this organ suitable for examination. On physiological grounds also an examination of spleen would be interesting in view of the work of Stern and Rothlin [1919] and others. A preliminary experiment with horse spleen suggested that histamine was probably present, the active substance being concentrated with little loss into the fraction from which histamine was actually isolated in the experiments with lung. The presence of choline in spleen was confirmed.

At the same time a preliminary experiment was carried out with striated muscle, for, although the vasodilator activity of this tissue was relatively low, it was of considerable physiological importance to have decisive information as to the chemical nature of the substance or substances responsible for this activity. This communication, therefore, is mainly concerned with the isolation of a vasodilator substance from muscle which has been identified as histamine.

THE ISOLATION OF HISTAMINE FROM STRIATED MUSCLE.

Preliminary experiments made it obvious that the isolation of histamine from muscle extracts would be a difficult technical task. The activity of the extracts was poor, corresponding to only about 2 mg. histamine per kg. of fresh meat, and the extracts were much more complicated than those of liver and lung with respect to basic extractives. The amount of accompanying material was so great that only a small final yield of histamine could be expected.

In a preliminary exploratory experiment the procedure successfully employed for lung was observed. The activity was concentrated into the histidine-arginine fraction with little loss. When, however, the mass was dried with baryta only 14 % of the activity could be recovered by repeated extraction with alcohol. This was found to be due to the presence of large quantities of substances of an amino-acid or simple peptide nature. When the alcoholic extract was examined it was found to consist largely of methylguanidine (and small amounts of creatinine) which was precipitated, along with the active substance, by picric acid. The relative quantities of the two substances (2 g. methylguanidine to 3 mg. histamine picrate) made it obvious that no hope of separating the active substance could be entertained.

Two problems thus arose: (1) the removal of methylguanidine, and (2) the reduction of the quantities of amino-acid-like substances in the mass to be extracted with alcohol.

(1) It is now generally accepted that methylguanidine does not exist as such in fresh muscle, and that in those cases where it has been isolated it has

been formed from creatine by the action of the silver oxide during the silver fractionation. The methylguanidine actually found only represented a small part of the creatine originally present, for large quantities of the latter crystallised out at an early stage. An attempt to remove the creatine remaining (before proceeding to the silver fractionation) in the form of potassium creatinine picrate proved to be impracticable because the picrate carried down 50 % of the activity.

According to the literature methylguanidine comes down in the arginine and creatinine in the histidine fractions of the Kossel-Kutscher silver fractionation. When, however, the histidine and arginine fractions of the muscle extract were separated it was found that the reverse was the case, the greater part of the methylguanidine coming down in the histidine fraction.

The problem was finally solved by a very simple modification of the original method. In order to reduce the amount of basic material accompanying the active substance, the phosphotungstates were extracted with acetone in which a small part was insoluble. On working up the acetone-soluble phosphotungstates alone (the active substance goes into this fraction with little loss) it was found that the histidine fraction was not only free from methylguanidine, although a small quantity of creatine was present, but also contained nearly the whole of the activity. The phosphotungstates which had been left after extraction with acetone consisted mainly of potassium phosphotungstate, with a small amount of organic base which has not yet been examined. It would seem that the anomalous results obtained in the silver fractionations in the exploratory experiments were probably due to the presence of large amounts of potassium salts.

(2) The same method also served to solve the second problem, for the histidine fraction was mainly composed of carnosine nitrate, most of which could be easily removed by allowing it to crystallise out from an alcoholic solution. In this way sufficient material was removed to make possible the isolation of histamine from the mother-liquor.

This mother-liquor, on drying with baryta and extracting with alcohol, gave a 33 % yield of the histamine. The alcoholic extract still contained a large amount of basic material, but fortunately only creatinine and histamine were thrown down by picric acid. By a long and difficult fractional crystallisation these two bases were finally partially separated and the vasodilator principle extracted from the fresh muscle was proved to be histamine.

EXPERIMENTAL.

35 kg. perfectly fresh ox muscle were minced and stirred into 79 l. 95 % alcohol. After 24 hours the meat was removed in a basket centrifuge and re-extracted with 72 l. 60 % alcohol for a further 24 hours. The combined extracts, after addition of sulphuric acid (0.36 cc. 50 % acid per l.), which produced a slight precipitate, were filtered through paper pulp. The clear filtrate was concentrated *in vacuo* below 30° to about 4 l. This was heated up to 60°

in a water-bath and then left over-night in the cold room. In this way most of the fat collected as a solid cake on the top of the liquid and could be removed by filtration. At the same time 38 g. creatine, which had crystallised out, were also removed. The last traces of fat were extracted from the filtrate by shaking with ether. The fat-free aqueous solution was then concentrated *in vacuo* to 800 cc. and, after removing 42 g. creatine which had crystallised out, neutralised with sodium hydroxide and treated with basic lead acetate until no further precipitate was produced. The filtrate and washings from this precipitate, after removal of lead as sulphide, were concentrated *in vacuo* to a thin syrup (700 cc.).

50 % sulphuric acid was added to the solution to produce a concentration of 5 % of this acid. This caused the separation of 76 g. potassium sulphate, which was filtered off and washed with a little 5 % sulphuric acid. It was then practically free from organic matter. The filtrate and washings were treated with phosphotungstic acid (25 % in 5 % sulphuric acid) until precipitation was complete. 7.7 l. were required. The precipitate was filtered off, washed with 5 % sulphuric acid and sucked as dry as possible. Without further drying, the damp precipitate was stirred up with 4 l. 75 % acetone (freshly distilled). After standing over-night the brown supernatant liquid was syphoned off and the residue centrifuged. The residue was thrice re-extracted with acetone. The residue consisted largely of potassium phosphotungstate, but also contained some organic bases which will not be described in the present communication.

The combined acetone extracts were concentrated *in vacuo* and decomposed with baryta in the usual way, excess of baryta being removed as sulphate. The solution of the bases was concentrated to 1300 cc. and divided into four fractions by the Kossel-Kutscher method. 400 g. silver nitrate in 1 l. water were added and the resulting precipitate, the "purine" fraction, filtered off (83 g.). Hot saturated baryta was then added cautiously until a precipitate was no longer formed on testing with ammoniacal silver nitrate. The granular precipitate, the "histidine" fraction, was filtered off and washed. The filtrate was saturated with baryta to precipitate the "arginine" fraction. The mother-liquor from this forms the "lysine" fraction.

Only one of the four fractions, the histidine fraction, showed appreciable depressor activity. The purine, arginine and lysine fractions were practically inactive and will not be discussed further in this paper.

The histidine fraction was suspended in sufficient dilute sulphuric acid to render the liquid just acid to Congo red and treated with hydrogen sulphide. The filtrate from the silver sulphide and barium sulphate was neutralised with baryta, and after removal of barium sulphate evaporated *in vacuo* to a mass of almost colourless needles. The mass was dissolved in 600 cc. boiling 80 % alcohol and allowed to cool, when a crop (35 g.) of large well-formed needles separated. These were collected and the mother-liquor treated with 1 l. absolute alcohol, when a further 7.4 g. separated in smaller needles. The mother-

liquor from these was evaporated *in vacuo* to a crystalline mass and the previous process repeated, giving a further 3.3 g. needles.

In this way 45.7 g. needles, which proved to be carnosine nitrate¹, were removed from the histidine fraction with only slight loss of activity, the final mother-liquor containing 90 % of the depressor substance originally present in the histidine fraction. This mother-liquor was concentrated to a small volume and ground up with a large excess of barium hydroxide with the addition of a little plaster of Paris. This mass was thoroughly dried and ground to a fine powder which was extracted with absolute alcohol for 3 hours in a Soxhlet apparatus. The extract was diluted with water and neutralised with sulphuric acid which precipitated the small amount of baryta which had been extracted. After removal of the alcohol *in vacuo* the barium sulphate was filtered off. The filtrate was concentrated to a small volume when 0.72 g. of a relatively sparingly soluble crystalline substance separated. The mother-liquor was concentrated *in vacuo* to 6 cc. and treated with 30 cc. saturated picric acid. The crystalline precipitate (Crop 1) was collected and the mother-liquor treated with a further 90 cc., giving a second crop. The mother-liquor from Crop 2 was concentrated *in vacuo* to 40 cc. and 10 cc. picric acid added. On standing a crystalline precipitate (Crop 3) separated from the emulsion first formed. No further crystalline material could be separated from the mother-liquor, which was by now only feebly active.

The three crops weighed 0.85 g., 1.02 g. and 0.3 g. respectively, and melted (with decomposition) at 214°, 214°, and 202°. The first two, which appeared to be identical, were combined and recrystallised giving 1.67 g. needles which melted at 220° and were identified as creatinine picrate. The mother-liquors from the creatinine picrate and Crop 3 were subjected to a long and tedious fractional crystallisation. Since the separation of 44 different fractions was involved this process will not be described in detail. It will be sufficient to indicate the lines on which the fractionation was carried through.

In the early stages the course of the fractionation could not be guided by the melting points of the various crops, since it was only in the final stage that a picrate melting above 220° (the melting point of creatinine picrate) was obtained. Chief reliance had to be placed on experience of the appearances of the crystals under the microscope gained during the experiments with liver and lung extracts. The appearance of the crystals when a dilute solution of sodium hydroxide was allowed to flow up to them, when viewed through the microscope, was also of great value. The appearance of a fugitive bright red colour on the surface of the crystal indicated creatinine (Jaffé's reaction). A feeble reaction was given by impure histamine picrate crystals.

During the course of the fractionation mechanical separation of the crystals had to be resorted to at three stages, whilst at the end of the process a valuable separation was effected by removing a crop while crystallising from the warm solution. Crop (a), obtained from the warm solution, melted at

¹ The isolation of carnosine nitrate at this stage will be considered in a later communication.

to adsorption on the bulky precipitates or solubility in the large volumes. This leaves 46 % unaccounted for. During this stage 76 g. potassium sulphate were removed. This, of course, could not be tested for histamine physiologically, and it has to be decided whether the values obtained in the first two stages, *i.e.* 140 mg., are (a) the true values, in which case, presumably, 46 % of the histamine was carried down with the potassium sulphate, or (b) values enhanced by the presence of large quantities of potassium salts exerting a depressant action on the cat's blood-pressure. In the latter case the yield obtained from the phosphotungstates would be more reasonable. A combination of these circumstances would provide a justifiable and probable explanation of the relatively large loss at this stage. In any case there is no definite evidence that fresh muscle tissue contains any organic depressor substance other than histamine. It may be noted in this connection that neither chemical nor physiological examination revealed the presence of choline in the muscle extracts examined.

The yields of depressor material during the silver fractionation and separation of carnosine nitrate are sufficiently high to require no comment when the large amounts of accompanying material are taken into consideration.

The yield during the alcohol extraction was 33 % in one extraction. This too is reasonable when it is remembered that the baryta mass still contained relatively large amounts of substances of an amino-acid nature which, as was shown by the preliminary experiments, definitely hinder the extraction.

Of the 12 mg. obtained in the alcoholic extract, which contained approximately 7 g. of material, 10 mg. were precipitated as picrate. This can, under the circumstances, be regarded as a good yield. In the long fractionation involved in the separation of pure histamine picrate a greater yield than 33 % could hardly be expected.

When viewed in this light the 4 % yield of pure histamine actually obtained is much better than the actual figure would suggest, for this yield is the result of a number of small losses in a very large number of operations and not due to an unreasonably large loss at any particular stage.

Although it cannot be claimed that it has been decisively proved, it seems justifiable to conclude that the whole of the depressor activity of an alcoholic extract of muscle prepared and tested under these conditions is due to the well-known base histamine.

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