CLXVI. THE CHEMICAL CONSTITUTION OF SPERMINE. I. THE ISOLATION OF SPERMINE FROM ANIMAL TISSUES, AND THE PREPARATION OF ITS SALTS.

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(*Received November 1st, 1924.*)

An investigation of spermine has been proceeding for some time in these laboratories, and we propose to deal in this paper with the occurrence and isolation of this substance, and to describe some salts and derivatives which have enabled us to establish its chemical composition.

It has been pointed out in the preceding communication that, according to Schreiner [1878], the base is not specific to human semen, but is widely distributed in the animal organism. Schreiner's statement was discredited until one of us [M. C. Rosenheim, 1917] confirmed it and demonstrated the occurrence of spermine in the tissues of both male and female animals, and, further, succeeded in isolating it from yeast. It is clear, then, that we are dealing with a base of general biological interest, the presence of which has been overlooked for so long owing to the very small amounts occurring in the tissues. The yields of spermine phosphate which we have obtained from various tissues are as follows: testis (bull) 0·006 %, ovary (cow) 0·014 %, pancreas 0·025 %, brain 0·007 %, spleen 0·011 %, thymus 0·006 %, thyroid 0·003 %, and yeast 0·01 %. It is interesting to note that the highest yield was obtained from pancreas, and that a specimen of insulin "hydrochloride" prepared by the picric acid-acetone method yielded 7 %, the entire content in spermine of the pancreas from which the insulin was prepared[1]. We have failed to find it in defibrinated blood (14 l.), in serum and in cow's milk (16 l.).

Spermine is distinguished from all other naturally occurring bases hitherto described by the remarkable property of forming an extraordinarily insoluble phosphate. This salt is of the greatest historical importance. It is the most remarkable of all the salts of spermine, its characteristic crystalline form and great insolubility being the properties which led to its early detection (cf. the

[1] Mr H. P. Marks kindly tested the effect of spermine on the blood sugar of rabbits and found that it has no action.

preceding paper). In order to be certain that we were dealing with the same substance as that occurring in semen we considered it essential to isolate it in the form of this classical salt.

In spite of its great insolubility it is by no means easy to bring about the crystallisation of the phosphate unless special conditions are observed. These conditions were unfortunately not defined by Schreiner with the result that subsequent workers (Majert and Schmidt [1890] amongst others) failed to obtain the salt again and doubt has even been cast recently upon its existence. In fact, it seems unlikely that, with the exception of Poehl [1898], who for reasons of his own refrained from giving details, it had ever been prepared again until two of us (M. C. R. and O. R.), several years ago, determined the conditions necessary for its formation.

Ladenburg and Abel [1888] suggested that an essential constituent of the phosphate might be calcium, since they found this metal in a sample of the material which Schreiner sent them. They considered that the phosphate might possibly have the formula $(C_2H_6N)_4.Ca(PO_4)_2$, which agrees with Schreiner's analysis better than his own formula. We find, however, that the pure phosphate is free from calcium and that Schreiner's analysis of the phosphate is correct, although he misinterprets the results, assuming the formula $(C_2H_5N)_2.H_3PO_4.3H_2O$, which was supported by faulty analyses of the hydrochloride and chloroaurate.

There are several possible methods for isolating the material from tissues. That adopted in earlier work by two of us (M. C. R. and O. R.) consisted in the extraction of the tissue with hot acidulated water. From the filtrate, after treatment with lead acetate, a precipitate of phosphotungstates was obtained under the usual conditions. This precipitate was then extracted with acetone, which dissolves the bulk of the phosphotungstates, leaving a small insoluble fraction containing the whole of the spermine.

A considerable purification having been thus effected, the insoluble fraction was decomposed with baryta, and after removal of excess baryta from the filtrate it was neutralised with an appropriate quantity of phosphoric acid. The characteristic phosphate of spermine crystallised out, this process being hastened by the addition of alcohol.

Another method consisted in subjecting the tissue extract to Kossel's silver fractionation process, when spermine was found to pass into the lysine fraction, from which the phosphate was isolated as before.

The yield of spermine phosphate obtainable from tissues being so small, it was necessary, for the purpose of this research, to work up large quantities of material. In order to avoid the necessity of using such large amounts of phosphotungstic acid as would have been required in the processes just described, alternative methods of working up the concentrated extract were explored. A convenient process was found to consist in making this liquid alkaline and extracting continuously with butyl alcohol. Spermine passed into the alcohol and was precipitated by the addition of a solution of phospho-

tungstic acid in butyl alcohol. The precipitate was worked up by the same procedure as that employed in the case of the acetone-insoluble phosphotungstate described above. By these means a considerable saving of phosphotungstic acid was effected.

Spermine, which we have prepared in the free state, is a crystalline, odourless solid melting between 55° and 60°, and distilling *in vacuo* without decomposition. We find that it distils, somewhat slowly, with steam from strongly alkaline solution, and a very simple method of isolation has been based on this property. It is not only stable in hot concentrated alkali but also resists prolonged boiling with 30 % hydrochloric acid. The base, both as hydrochloride and in the free state, has been found to be optically inactive.

We have analysed the phosphate, hydrochloride, picrate, benzoyl derivative, chloroaurate and chloroplatinate. The entire series of analyses agree with the formula $C_5H_{14}N_2$, but the determination of the molecular weight of the benzoyl derivative by both Beckmann's and Rast's methods indicates that this formula should be approximately doubled. From these results, then, it becomes necessary to assign to spermine the molecular formula $C_{10}H_{26}N_4$.

Work on the constitution of spermine is being continued. The examination of the products of dry distillation of the hydrochloride, of exhaustive methylation of the base, of decomposition of the chloroaurate with magnesium has yielded valuable information in this connection which, along with other observations, will be reported in a later communication.

Experimental.

Preparation of spermine phosphate from pancreas.

(1) *Acetone method.* 5 kg. of minced fresh pancreas (ox) are poured into 10 litres of boiling water. The mixture is again heated to boiling and 30 cc. glacial acetic acid are added with vigorous stirring. After cooling the clear liquid is filtered off and as much fluid as possible is pressed out of the coagulated protein. Glacial acetic acid in the proportion of 5 cc. per litre of filtrate, and a saturated aqueous solution of lead acetate are added until no further precipitate is formed. The solution is then filtered and the excess of lead removed by cautious precipitation with sulphuric acid. After filtration the clear liquid is concentrated *in vacuo* (water bath at 45–50°) to about one-eighth of its original volume. Its content of sulphuric acid is brought up to 5 % and the bases are precipitated with phosphotungstic acid (25 % solution in 5 % sulphuric acid), about 500 g. being required for 5 kg. pancreas. The precipitate is filtered on a Buchner funnel, washed and pressed as dry as possible. It is then ground up in a mortar with acetone and transferred with more acetone to a flask, about 500 cc. acetone per kg. pancreas being used. A granular yellowish deposit settles rapidly in the dark brown solution. The precipitate is washed with acetone by decantation, filtered and dried. A yellowish powder, about 25 g.

from 1 kg. pancreas, is obtained. The phosphotungstate is decomposed in the usual way by grinding with saturated baryta water and solid baryta in a mortar. The filtrate is freed from baryta by CO_2 and subsequent warming to 70°.

The final filtrate, containing the base as carbonate, is accurately measured, one-third of it is withdrawn and titrated with 10 % phosphoric acid, using methyl orange as an indicator. The addition of the acid is continued carefully after the indicator has changed colour until the reaction is distinctly acid to sensitive Congo paper. The remaining two-thirds of the basic solution are then mixed with the acidified portion and the mixture will then be found to be amphoteric to litmus paper. Spermine phosphate crystallises out slowly in rosettes and single crystals on allowing the solution to stand for some hours.

In order to obtain the maximum yield, however, it is advisable to add one-third of the volume of alcohol when an immediate crystallisation of slender needles of spermine phosphate begins. The substance is recrystallised from 50–60 volumes of boiling water. Yield 0·9 g.

The procedure outlined in the last stage of the method has been evolved after many failures, which taught the necessity of adjusting the final reaction to neutrality, the slightest excess of acid or alkali preventing crystallisation. A colorimetric determination of the p_H of solutions of pure spermine phosphate demonstrated the fact that crystallisation only takes place within the narrow limits of p_H 6·8 to p_H 7·2, a fact which may be of physiological significance.

(2) *Butyl alcohol method.* An extract of 5 kg. pancreas is prepared in the same way as under (1), except that the lead treatment may be omitted. The concentrated extract is made alkaline by adding 25 cc. of 50 % NaOH solution per 100 cc. and the solution is extracted continuously with butyl alcohol after the method of Dakin for about 24 hours. To the butyl alcohol extract is added a 25 % solution of phosphotungstic acid in butyl alcohol until no further precipitate appears. The precipitate is allowed to settle and washed four times with acetone, either by decantation or by the centrifuge. After filtration and drying *in vacuo* the phosphotungstate is obtained as a white voluminous powder (about 10 g. from 1 kg. pancreas). Spermine phosphate is prepared from it in exactly the same manner as described under (1). Yield 1·0 g.

Of the alternative methods for the recovery of the base from its butyl alcoholic solution, which have been successfully applied, the following may be mentioned: (1) neutralisation with phosphoric acid under certain conditions, the phosphate being precipitated in an amorphous state, (2) precipitation of the picrate with a butyl alcoholic picric acid solution, and (3) extraction with dilute mineral acid, followed by precipitation with phosphotungstic acid.

(3) *Steam distillation.* 5 kg. of the tissue are treated with boiling water and acetic acid as in the first method. The filtered extract is evaporated *in vacuo* to as small a bulk as possible. Solid NaOH (50 g. per 100 cc. fluid) is added and the solution steam-distilled in the usual way. Distillation is continued until the distillate no longer gives a precipitate with Dragendorff's reagent, which indicates that all the spermine has been removed. The distillate

is then treated with phosphoric acid in the same manner as the baryta-free solution of the base in the preceding sections, and evaporated to about 100 cc. per kg. of pancreas taken. Alcohol is added to produce a concentration of 25 % when spermine phosphate crystallises out[1]. Yield 1·5 g.

The above methods have been applied with slight modifications to other tissues. Spermine phosphate has also been obtained from Lebedeff's and distiller's yeast, but not from baker's yeast. The commercial yeast extract "Marmite" contains about 0·04 %. From Liebig's Extract 0·01 % spermine phosphate was isolated. It was found that when the crystallisation of spermine phosphate is completed, the filtrates contain the soluble phosphate of another base which has not yet been identified. The amount is only one-tenth of that of spermine (about 2 g. from 100 kg. pancreas), its phosphotungstate is insoluble in acetone and it yields a well crystallised picrate (M.P. 210°). The soluble phosphate from yeast has been identified with putrescine (picrate, M.P. 264°; phenylisocyanate, M.P. 240°; benzoyl compound, M.P. 178°).

Properties and solubility of spermine phosphate.

On recrystallisation from water the substance is obtained in colourless crystals, insoluble in alcohol, ether and organic solvents, easily soluble in dilute acids and alkalies. The crystals slowly lose water of crystallisation over sulphuric acid, rapidly at 100°, becoming opaque. Their typical form is seen in the photomicrograph given in the preceding paper. When rapidly formed by the addition of alcohol, they appear as slender long needles. Occasionally they occur under these conditions as anisotropic spherocrystals, which quickly change into the usual form. The solubility of the pure salt was found to be 1 part in 100 parts of boiling water. At 20° its solubility in water is 0·037 %.

Behaviour of spermine phosphate in melting point determination.

The salt begins to soften at 227°, froths up the tube with evolution of gas and melts between 230–234°. At 240° the evolution of gas has stopped and a white deposit lines the tube. At 260–262° this deposit runs together with a slight evolution of gas. There is no discoloration during these changes.

Analysis. Water of crystallisation.

0·3110 g. four times recrystallised salt lost 0·0671 g. at 103°, whence $H_2O = 21{\cdot}57$ %.

Calculated for $C_{10}H_{26}N_4.2H_3PO_4.6H_2O$: 21·34 %.

0·2423 g. dry salt gave NH_3 (Kjeldahl) = 23·98 cc. $N/10$ H_2SO_4;

0·2239 g. „ „ 0·1250 g. $Mg_2P_2O_7$.

Found: N = 13·86 %, P = 15·56 %;

Calculated for $C_{10}H_{26}N_4.2H_3PO_4$: N = 14·07 %, P = 15·56 %.

[1] Ladenburg and Abel [1888] subjected a small sample of spermine, prepared by Schreiner himself, to steam distillation and assumed that the base is volatile. Since they were, however, unable to regenerate the phosphate from the distillate, their evidence is inconclusive.

Several preparations from testis and pancreas were analysed and gave identical results.

Spermine picrate.

2·78 g. spermine phosphate were dissolved in 13 cc. of cold 2*N* NaOH. The solution was filtered and diluted to 75 cc. with cold water. Saturated aqueous picric acid was then added until no further precipitate was formed. The picrate was filtered off and recrystallised from 1200 cc. boiling water. It separates in slender yellow needles often about 2 cm. in length. Yield 5·72 g.

It becomes black at about 242° and melts sharply with decomposition at 248–250°, contains no water of crystallisation and was analysed for picric acid by means of nitron.

0·0576 g. spermine picrate gave 0·1113 g. nitron picrate, whence picric acid = 81·78 %.

Calculated for $C_{10}H_{26}N_4.4C_6H_3O_7N_3$: 81·98 %.

Spermine hydrochloride.

5·7 g. spermine picrate were suspended in 60 cc. of a mixture of 75 cc. alcohol and 25 cc. 14 % aqueous HCl. The suspension was heated on a boiling water-bath for 10 minutes. The picrate did not go entirely into solution but was nevertheless decomposed and the liquid was now filled with a suspension of the white hydrochloride. This was poured into 500 cc. of dry acetone, the hydrochloride being thereby precipitated as a granular crystalline powder. This salt was filtered off and washed with acetone and finally with ether, until free from picric acid. The funnel containing the salt was transferred quickly to a vacuum desiccator and the adherent acetone and ether were removed by cautious evacuation in the presence of sulphuric acid. After about half-an-hour the spermine hydrochloride was obtained as a dry, white, non-hygroscopic powder. Yield 1·8 g.[1]

For recrystallisation it was dissolved in 12 cc. 10 % HCl and, after heating the solution on the water-bath, 40 cc. of boiling alcohol were added. The salt crystallised from this solution in short prismatic needles.

It is extremely soluble in water though not hygroscopic, very slightly soluble in hot methyl and ethyl alcohols and insoluble in acetone, ether and chloroform.

In the melting point tube it turns brown at 300–302°, and at 310° the mass liquefies and a slow evolution of gas is observed.

[1] This method of preparing hydrochlorides from picrates is quicker and less laborious than the usual process in which the picrate is decomposed with HCl in aqueous solution and the picric acid removed by shaking with ether. It also yields the hydrochloride in solid form instead of in solution with a large excess of HCl. With suitable modifications of the amounts of alcohol and aqueous HCl it can be applied in many cases to the preparation of hydrochlorides from picrates and probably from other salts such as picrolonates.

Analysis. The salt contains no water of crystallisation.

0·1028 g. gave 0·1675 g. AgCl: Cl = 40·60 %.

0·1146 g. „ 0·1451 g. CO_2; 0·0881 g. H_2O: C = 34·53 %; H = 8·54 %.

0·1159 g. „ NH_3 = 13·19 cc. $N/10$ H_2SO_4 (Kjeldahl): N = 15·93 %.

Calculated for $C_{10}H_{26}N_4.4HCl$:

C = 34·48 %; H = 8·62 %; N = 16·10 %; Cl = 40·80 %.

Spermine picrolonate.

0·010 g. spermine hydrochloride was dissolved in 1·5 cc. water, 1 cc. $N/10$ Na_2CO_3 solution was added and finally 2·5 cc. of a 2 % alcoholic solution of picrolonic acid. The picrolonate separated as a lemon-yellow crystalline powder: 0·036 g. was obtained. This was recrystallised from boiling water in which it is only sparingly soluble, 140 cc. being required. It crystallised in microscopic short prismatic needles of a dull, pale yellow colour. It melts with decomposition at 288–289°. Its solubility in water at 5° is approximately 1 part in 56,500. On account of its great insolubility this salt may prove useful for quantitative estimations.

Spermine chloroaurate.

To a 5 % solution of the hydrochloride in water, or of the phosphate in just sufficient hydrochloric acid to bring it into solution, 20 % gold chloride solution is added until no further precipitate appears. It can be recrystallised readily from 5 % aqueous hydrochloric acid, and is obtained in golden yellow, lustrous leaflets. It melts with decomposition at 225°.

Analysis. 0·1601 g. lost 0·0023 g. H_2O at 103°, and the dry salt gave 0·0796 g. Au on ignition, whence H_2O = 1·4 % (calculated for 1 mol. H_2O, 1·14 %), and Au = 50·44 %.

0·1699 g. dry salt gave 0·2500 g. AgCl: Cl = 36·40 %.

Calculated for $C_{10}H_{26}N_4.4HCl.4AuCl_3$: Au = 50·40 %; Cl = 36·38 %.

Spermine chloroplatinate.

0·25 g. of the phosphate is dissolved in 5 cc. water by the addition of 0·2 cc. 30 % HCl and 7 cc. 10 % chloroplatinic acid solution are added. Crystallisation takes place more slowly than in the case of the gold salt. The orange-yellow salt can be recrystallised easily from 5 % aqueous hydrochloric acid, and is obtained in large, well-formed crystals, which are crystallographically described in the preceding paper. The substance melts with decomposition at 242–245°.

Analysis. 0·1594 g. lost 0·0069 g. at 103°, and the dry salt gave 0·0577 g. Pt on ignition, whence H_2O = 4·33 %, and Pt = 37·91 %.

Calculated for $C_{10}H_{26}N_4.2H_2PtCl_6$: Pt = 38·20 %.

Benzoyl spermine.

1 g. spermine phosphate is dissolved in 40 cc. 8 % NaOH solution and shaken in a stoppered bottle with 6 cc. benzoyl chloride. A granular white deposit is rapidly formed. After half-an-hour 5 cc. of 8 % NaOH solution and 1 cc. benzoyl chloride are added and the mixture again shaken. The liquid is then poured off and the benzoyl compound is washed several times with water. It is then dissolved in 20 cc. warm alcohol and poured into 200 cc. water. A white sticky precipitate, which solidifies on standing, is deposited from the milky fluid. The water is decanted and the precipitate in the flask dried *in vacuo*. It is then boiled out several times with ligroin to remove benzoic acid and ethyl benzoate, dissolved in 70 cc. hot acetone, and ligroin added until a permanent turbidity is produced. It crystallises in woolly balls of fine needles. It may also be recrystallised from acetone or alcohol, diluted with an equal volume of water. Yield 0·98 g. The substance melts at 155°.

Analysis. 7·545 mg. gave 20·505 mg. CO_2 and 4·300 mg. H_2O[1],
4·232 mg. ,, 0·36 cc. N at 17° and 716 mm.,
0·1508 g. ,, NH_3 = 9·63 cc. $N/10$ H_2SO_4 (Kjeldahl):
C = 74·12 %; H = 6·38 %; N = 9·43 % and 8·94 %.

Calculated for $C_{10}H_{22}N_4(CO.C_6H_5)_4$:
C = 73·78 %; H = 6·79 %; N = 9·06 %.

Molecular weight determination.

(*a*) By Beckmann's method:

Solvent: 10·52 g. glacial acetic acid.

Weight of substance introduced:	(1) 0·0489 g.	(2) 0·0984 g.
Depression of freezing point:	0·030°.	0·060°.
Molecular weight:	604.	608.

(*b*) By Rast's method:

(1) 5·0 mg. in 50·6 mg. camphor. Depression: 6·5°. M.W. 608.
(2) 10·4 mg. in 100·8 mg. camphor. Depression: 6·5°. M.W. 634.

Calculated for $C_{10}H_{22}N_4.(CO.C_6H_5)_4$: M.W. = 618.

Phenylisocyanate derivative of spermine.

0·055 g. spermine hydrochloride is dissolved in 2 cc. water, to which is added 0·2 cc. $2N$ NaOH. After the addition of 0·2 cc. phenylisocyanate the liquid is stirred continuously and a sticky white precipitate is deposited. When the reaction of the liquid has become only faintly alkaline a further addition of 0·2 cc. $2N$ NaOH is made. After the reaction is over the sticky precipitate hardens and becomes brittle. It is rubbed up with a glass rod, filtered and washed with cold water. The solid is taken up in absolute alcohol, water is

[1] The micro-analyses were made ten years ago, when only small quantities of the product were available.

added until a permanent turbidity is produced; the liquid is then warmed and set aside to crystallise.

The phenylisocyanate derivative crystallises from alcohol in colourless clusters of needles. After four recrystallisations it melted at 179–180°.

Optical inactivity of spermine.

(*a*) As hydrochloride: 2 g. dissolved in 16 cc. water were examined in a 2 dm. tube and found to be optically inactive.

(*b*) As free base: 2·8 g. of spermine phosphate were dissolved in 15 cc. 2*N* NaOH, examined in a 2 dm. tube and found to be optically inactive.

Spermine itself is obtained by adding 50 % potash to a concentrated solution of the hydrochloride or to a suspension of the phosphate in water. The base is extracted with chloroform and remains, after the removal of the solvent *in vacuo*, as a colourless oil which rapidly solidifies to an aggregate of needle-shaped crystals, melting at 55–60°. On distillation *in vacuo* it boils without decomposition at about 150° under 5 mm. pressure. The base is possibly obtained under these conditions as a hydrate, a point which will be investigated later. It is easily soluble in water, ethyl and butyl alcohol, insoluble in ether, benzene and ligroin. On exposure to air it rapidly absorbs carbon dioxide and liquefies.

Beside the above-described derivatives we have also prepared the arsenate, which resembles closely the phosphate in crystalline form and solubility. The oxalate crystallises in leaflets, M.P. 225°. The sulphate, nitrate, acetate and carbonate crystallise well, but are very hygroscopic.

SUMMARY.

1. The base spermine has been isolated from the following animal organs: testis, ovary, pancreas, muscle, liver, brain, spleen, thymus and thyroid. It has also been obtained from yeast. The methods of isolation are described.

2. The free base is a solid which can be distilled *in vacuo* and is volatile with steam. It is optically inactive.

3. The conditions necessary for the preparation of its most characteristic salt, the phosphate, are laid down.

4. The phosphate, hydrochloride, picrate, chloroaurate, chloroplatinate and benzoyl derivative have been analysed. The results agree with an elementary composition of $C_5H_{14}N_2$. Other salts and derivatives are described.

5. Molecular weight determinations of the benzoyl derivative indicate a molecular weight approximately twice that given above. On this account the formula $C_{10}H_{26}N_4$ has been adopted.

This investigation was begun in 1910 by two of us (M. C. R. and O. R.) in the Physiological Laboratory, King's College, London, with the help of a

grant from the Government Grant Committee of the Royal Society. Resources then available only permitted work on a relatively small scale.

In the preparation of large quantities of spermine for the purposes of the research here presented, we have been greatly assisted by the British Drug Houses, Ltd., who have carried out large scale extractions for us. To them and to Mr F. H. Carr of their directorate our best thanks are due.

We are greatly indebted to Mr W. W. Starling for valuable and skilled assistance in connection with this work.

For references see preceding paper by O. Rosenheim, p 1253.