CCII. SPERMINE IN HUMAN TISSUES.

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ALTHOUGH the structure of spermine has been elucidated by the work of Dudley, Rosenheim and their colleagues [1924, 1925, 1926], the physiological significance of this interesting base remains completely obscure. Originally observed in, and suspected as being specific to, human semen, subsequent investigation showed it to be a widely distributed constituent of mammalian tissues, both male and female.

That it is not essential to the process of fertilisation was made extremely probable by the failure of Dudley and Rosenheim [1925, 2] to detect it in the semen of the bull. An explanation of this striking difference from human semen is advanced in the present communication, which is concerned mainly with the distribution of the base in human tissues; it was considered desirable to make this survey in order to compare the results with those of Dudley and Rosenheim [1925, 2] for other mammalian tissues, and in the hope that the information obtained might eventually contribute to an understanding of the biological significance of the base.

Method of isolation.

The base was separated by the steam-distillation method [Dudley, Rosenheim and Rosenheim, 1924]. A number of minor modifications were introduced so that a description may be of value.

The organs, which were obtained from the *post mortem* room, were the seat of various pathological lesions. By working up one gland at a time it was hoped originally that variations due to different diseases might be revealed, at any rate in the case of the larger glands. In nearly all cases (for exceptions, see below), however, the yield of spermine was so low that it was necessary to use 5 kg. of minced organ to work quantitatively; for this reason the attempt to isolate spermine from a single organ had to be abandoned.

With the exceptions noted below, the following procedure was finally adopted. The organs were cleaned by dissection, minced, and stored under absolute alcohol, until 5 kg. or more of a particular organ had been collected. The fluid was then strained off, the alcohol was removed by distillation and the residue was added to the mince. The weight of the mince *plus* residue was determined, and the final yield was expressed as mg. of spermine phosphate per 100 g. of this material. These were obviously not the yields per 100 g. of fresh

organ (they were a little higher in most cases), but were satisfactory for comparative purposes. In the case of liver, however, all the observations were made on organs as observed at autopsy; in the case of lung and brain also it was sometimes possible to obtain enough minced organs to avoid storage under alcohol.

The 5 kg. of minced organ were added to 10 litres of boiling tap-water, and thoroughly mixed. After the addition of 30 cc. of glacial acetic acid and further stirring, the mixture was again boiled and kept simmering for half an hour. The coagulated protein was allowed to settle, and the supernatant fluid was syphoned off and filtered on a large Büchner funnel. The bulky mass of coagulated protein was transferred to a press, and as much fluid as possible was squeezed out and then passed through the Büchner funnel. The combined filtrates were then evaporated to about 500 cc. The contents of the pan were transferred, without filtration, to a glass vessel, together with a little wash-water. After introducing 25 cc. of glacial acetic acid, a saturated aqueous solution of normal lead acetate was added till there was no further immediate precipitate. The mixture was either filtered on a Büchner funnel or (more conveniently) passed through a Sharples centrifuge. The excess of lead was removed from the filtrate by cautious precipitation with 50 % *v/v* sulphuric acid. The lead sulphate was removed by filtration or centrifuging. The filtrate was made slightly alkaline to litmus by adding flake sodium hydroxide, and concentrated to about 400 cc. Ammonia was thus largely removed, but no spermine was lost.

The concentrate was transferred, with sufficient wash-water to make a total volume of 500 cc., to a 3 litre round-bottom flask of resistance glass, and 50 g. of flake sodium hydroxide were added. A grease-mark was made on the flask to indicate the level of the fluid which was maintained at constant volume. The mixture was steam-distilled in the usual way, a Kjeldahl trap being inserted in the exit tube to prevent loss of the solution by frothing or spitting. Frothing was often troublesome, and it was generally essential to regulate the distillation carefully for a few minutes, and sometimes to add a little vaselin.

Distillation was continued until there was no longer any smell of ammonia, and until the distillate was only slightly alkaline to litmus (usually 2 to 6 litres of distillate). In some cases the fluid which came over gave a positive Dragendorff's reaction, but it was shown that this was due to other bases and never to spermine, provided that the contents of the distilling flask were not allowed to become concentrated. (To make certain of this point, this "10 % distillate" was on many occasions worked up separately, but no spermine was recovered.) When a positive Dragendorff's reaction was obtained, distillation was continued until it became negative. This preliminary distillation from 10 % sodium hydroxide was a valuable means of getting rid of most of the ammonia (formed by the action of the alkali), and of some of the "lower bases."

The contents of the distilling flask were allowed to cool a little, and then a further 200 g. of flake sodium hydroxide were added. The steam-distillation was again started, and continued vigorously until samples of the distillate no

longer gave a positive Dragendorff's reaction. Care had to be taken that the contents of the flask did not become diluted by condensed steam. To ensure that the whole of the base had been distilled over, a further 50 g. of sodium hydroxide were added and the distillation was continued. This was repeated if necessary until Dragendorff's reaction was negative. As a rule some 5 to 15 litres of distillate were collected. This was made acid with concentrated hydrochloric acid and taken to complete dryness by distillation *in vacuo* or by evaporation on the water-bath. (Completion of the drying and the volatilisation of the excess of hydrochloric acid were facilitated by adding absolute alcohol in the last stages of evaporation.)

The very crude spermine hydrochloride thus obtained was dissolved in the minimum of distilled water and filtered with suction through a large filter-paper to remove a small amount of greasy pigmented matter, which was washed with distilled water. The combined filtrate *plus* washings was acid, and on making slightly alkaline to litmus by the cautious addition of 40 % sodium hydroxide, a further small pigmented precipitate was generally obtained and was filtered off after adding decolorising charcoal.

The final solution of spermine hydrochloride was treated with a concentrated solution of sodium picrate (picric acid 23 g., *N*/1 NaOH 100 cc., water 500 cc.) until no further precipitate could be obtained. After standing at room temperature for a few hours (generally overnight) the crude spermine picrate was filtered off, dried and weighed. (The mother-liquor, after concentration, as a rule yielded no significant quantities of spermine picrate.)

The dry crude picrate was suspended in sufficient acid alcohol (concentrated HCl 7 cc., water 43 cc., alcohol to 200 cc.) to dissolve it after heating on the boiling water-bath for about 10 minutes. For each 1 g. of picrate 15 to 25 cc. of the acid alcohol were generally required. The hot solution was poured into about 10 volumes of acetone, the glass vessel being washed out with a further few cc. of hot acid alcohol. The mixture was kept until the precipitate clumped and was then filtered with suction. The precipitate was washed with acetone until free from picric acid.

This precipitate of crude spermine hydrochloride rapidly deliquesced; the funnel was therefore promptly connected up with another filter-flask, a small volume of water was added and the solution sucked through. To this solution the calculated amount (weight of picrate $\times$ 0.236) of diammonium hydrogen phosphate, dissolved in a small volume of warm water, was added. An equal volume of absolute alcohol was added to the mixture which was kept at room temperature overnight and then filtered. The precipitate was dissolved in the minimum of boiling water and the solution treated with charcoal and filtered hot.

To the final filtrate which was colourless, a few drops of phenol red were added, and (if necessary) 10 % ammonia until the reaction was p_H 7.0 to 7.2 (warm brown tint). Crystallisation of spermine phosphate often started rapidly but was completed by adding $\frac{1}{3}$ vol. of absolute alcohol. After standing overnight at room temperature, the precipitate of spermine phosphate

was filtered off, dried and weighed. The mother-liquors after addition of alcohol to 50 % and to 25 % respectively, in the last two stages, were always worked up, but seldom yielded significant amounts of spermine phosphate.

Notes on method.

When the first filtrate, after removal of protein, contains appreciable quantities of fatty material in suspension, it is necessary to introduce one or more extractions with ether.

Treatment with lead may be omitted, as in the original description [Dudley *et al.* 1924], but if it is, considerable quantities of ammonia are formed owing to the action of sodium hydroxide on the protein, and frothing is likely to be much more troublesome. Filtration of the lead precipitates is in some cases very tedious or difficult; centrifuging is a great improvement.

If the steam-distillate is treated with phosphoric acid as described by Dudley *et al.* [1924], and is then evaporated to a small volume, it becomes very acid owing to loss of ammonia. If the reaction is not readjusted, spermine phosphate will generally not crystallise out at all. If concentration is effected before adjustment of reaction with phosphoric acid, spermine phosphate crystallises out, but in the writer's experience the yield is lower than in the method above described, possibly owing to the effect of the other phosphates on the solubility of spermine phosphate. The preliminary distillation after addition of sodium hydroxide to 10 % largely eliminates this difficulty.

The conversion of the crude hydrochloride (obtained by acidification and evaporation to dryness of the steam-distillate), into picrate, into hydrochloride and then into phosphate, may at first sight appear a tedious and unnecessarily complicated procedure, but gives a comparatively clean and pure preparation with more certainty, in the writer's opinion, than the original method of Dudley *et al.* [1924]. Incidentally it may be noted that the weight of picrate has generally been some four to ten times the weight corresponding to the yield of phosphate finally obtained (except in the case of the prostate). This presumably is due in part at any rate to the presence of picrates of bases other than spermine [*cf.* Dudley, Rosenheim and Starling, 1927].

A better yield was obtained by first precipitating crude spermine phosphate in the presence of 50 % alcohol and then recrystallising from 25 % alcohol, than by precipitating it initially with $\frac{1}{2}$ vol. of alcohol.

Finally, decoloration with charcoal of extracts, which are generally deep brown, allows phenol red to be used and therefore a close adjustment of reaction to be made.

Conversion of spermine into phosphate via picrate and hydrochloride.

In order to gain some idea of the magnitude of losses which might be expected in the process of conversion of crude spermine hydrochloride into picrate, into hydrochloride and finally into phosphate, the following experiment was performed on pure spermine phosphate¹.

0.4315 g. of spermine phosphate was suspended in 10 cc. of water and sufficient sodium hydroxide solution was added to cause solution (12 drops of 40 % NaOH). The solution was then treated with sodium picrate, and the subsequent stages were carried through exactly as described for a tissue extract.

The 0.4315 g. of phosphate gave 0.92 g. of picrate (theory, 0.953 g.), 0.285 g. of hydrochloride, 0.394 g. of "crude" phosphate, and 0.370 g. of once recrystallised phosphate. That is to say approximately 86 % of the original phosphate was finally recovered. Since this experiment further experience

¹ Kindly presented by Dr Dudley.

has been obtained in handling spermine salts, and it is now considered that this recovery should be regarded as a minimum with ordinary care.

Yields.

The yield of spermine, as phosphate, in mg. per 100 g. of tissue is shown in Table I. The result of each experiment is given, except in the case of liver on which eleven sets of analyses were made. The results of Dudley and Rosenheim [1925, 2] for certain animals are quoted for comparison.

Wrede [1926] separated spermine as the chloroaurate from several organs of man, cow and bull. It is probable that his chloroaurate contained small quantities of bases other than spermine; on the other hand the maximum weight of organ employed by him was 2 kg., which would tend to give low yields. His results (recalculated as spermine phosphate) for human testis (0.75), muscle (2.25), brain (3.75), spleen (5.75), liver (12.5), pancreas (13.5) and semen (95 mg. per 100 g.) are of the same order as the writer's (see Table I).

Table I.
Spermine as phosphate, mg. per 100 g.

Tissue	In man	In animals (Dudley and Rosenheim)
Semen	90, 100, 117, 260*	0 (bull)
Testis	1.2, 9.3	6 (bull)
Prostate	130	—
Ovary	—	14 (cow)
Pancreas	16.1	25 to 30 (ox)
Liver	2.3 to 11.4	16 (ox)
Kidney	5.0, 5.9	15 (ox)
Spleen	3.0, 8.0	11 (ox)
Lung	3.3, 3.3, 4.7	7 (ox)
Heart	1.5, 2.3	—
Muscle	—	12 (calf), 4 (sheep) 2 (ox), 2 (horse)
Brain	1.8, 2.0, 3.1	7 (ox)
Thymus	—	5 (calf)
Adrenal	—	5 (ox)
Thyroid	—	3 (ox)
Bone marrow	0	—
Blood	0, 0	0 (ox), 0 (sheep)†
Serum	0	0 (ox)
Sputum	0, 0	—
Faeces (dried)	Trace, trace	—
Milk	—	0 (cow)
Egg	—	0 (hen)

* Result of Rosenheim. † Personal finding, 7791 cc. sheep's blood.

Notes on Table. With the following exceptions 5 kg. or slightly more of tissue were used for each analysis: testis, 975 and 1551 g.; prostate, 1594 g.; pancreas, 3361 g.; whole blood, 400 and 600 cc.; serum, 250 cc.; sputum, 380 and 200 cc.; bone marrow, 15 cc.; dried faeces, 450 and 190 g. The testes and prostates took over a year to collect. Further details as regards the yields from semen are as follows: (a) 18.3 cc. collected under alcohol; steam-distillation method applied to precipitate; yield 90 mg. per 100 cc. (b) 4.9 cc. of fresh seminal plasma (spermatozoa removed by centrifugation); direct separation of Böttcher's crystals; yield 100 mg. per 100 cc. (c) 60 cc. semen, pooled, stored in ice-room for several weeks without preservative; steam-distillation from alkali applied direct to untreated semen; yield 117 mg. per 100 cc. In addition spermine phosphate (Böttcher's) crystals have several times been separated from whole semen, both fresh and stale, by careful adjustment of reaction, but not quantitatively.

DISCUSSION OF RESULTS.

The yields obtained for the majority of tissues need no special comment. Spermine is present in almost every tissue of man just as in animals [Dudley and Rosenheim, 1925, 2], but in most organs of man it is in slightly lower concentration.

The two points of chief interest arising out of the present investigation are first, the large yield of spermine from the prostate, and second the presence of the base in the faeces.

The prostate consists approximately of two parts of muscle and one part of glandular tissue. If it may be assumed that most of the spermine is derived from the glandular portion of the gland, then the yield of 130 mg. per 100 g. of whole gland represents a yield of 300 to 400 mg. per 100 g. of glandular tissue; in other words it makes it likely that the main part, if not the whole, of the spermine of human semen is derived from the prostatic secretion.

Bull's semen contains no spermine [Dudley and Rosenheim, 1925, 2]; the bull does not possess a true functional prostate, but only a rudimentary collection of compound acinar glands which are regarded as the equivalent of the human prostate. This may account for the absence of spermine from the semen of the bull. Wrede [1926] recovered 70 mg. of spermine chloroaurate from 600 g. of bull's "prostate," which is equivalent to a yield of 3.5 mg. of spermine phosphate per 100 g. of tissue. Whether the "Rinder prostata" used by Wrede was rudimentary prostate or seminal vesicles or both, is immaterial; his yield was so low (of the same order as found in other organs of the body) that it is compatible with the absence of spermine from bull's semen.

The writer's observation (see notes on Table I) that fresh human seminal plasma yields at least as much spermine as whole semen, makes it unlikely that the spermatozoa are a significant source of the base.

Fürbringer [1886] stated that human prostatic secretion contained an organic base, the phosphate of which constituted "Böttcher's crystals" or "prostatic crystals." It is only recently [Rosenheim, 1924] that "Böttcher's crystals" from human semen were shown conclusively to consist of spermine phosphate, but it is curious that no worker seems to have explored further Fürbringer's statement that in man "Schreiner's base" (as spermine was then called) was derived from the prostate. Fürbringer [quoted by Von Frisch, 1899] found that the prostatic fluid squeezed out of prostates obtained *post mortem* contained Böttcher's crystals in 56 cases (90 % of cases examined). Fürbringer failed to recover Böttcher's crystals directly from fresh prostatic secretion obtained by massage of the gland during life, but he found that the crystals could be made to appear by adding ammonium phosphate. He concluded therefore that the prostatic secretion supplied the base, and that the phosphoric acid was derived from other components of the semen. He explained his finding of Böttcher's crystals in *post mortem* prostatic fluid by assuming that autolysis of epithelium supplied the phosphoric acid.

Although only some 1 to 5 drops of prostatic fluid are usually obtained by massage during life, it is easy to confirm Fürbringer's experiment of producing Böttcher's crystals by adding ammonium phosphate, though the crystals are of the characteristic shape only if the final reaction of the mixture is close to p_H 7.0. The writer has several times confirmed Fürbringer's work both by adding ammonium phosphate and by adding phenol red (as indicator) and dilute phosphoric acid to p_H approximately 7.0 to expressed prostatic fluid. In both cases the addition of a little alcohol (about 0.1 vol.) facilitated crystallisation. In one instance the addition of acetic acid (and phenol red) produced a few crystals of the typical lenticular shape, so that Fürbringer's failure to obtain Böttcher's crystals from prostatic fluid expressed during life may in part at any rate have been due to failure to secure the correct reaction.

It has been claimed that the prostatic secretion has an activating action on the spermatozoa [see Fürbringer, 1886, and Von Frisch, 1899]. There is no doubt of the effect of adding prostatic secretion to spermatozoa which have previously not come into contact with such secretion; the spermatozoa remain active for a much longer period in the presence of the prostatic fluid. Conversely, human spermatozoa, which have been separated from semen by centrifuging and washing with 0.85 % NaCl, do not remain motile for nearly so long as the spermatozoa left in suspension in the (diluted) seminal plasma (an observation several times confirmed by the writer). But neither of these observations of necessity proves that the normal function of the prostatic secretion is to activate the spermatozoa, though they support the idea.

It was however considered to be worth while to try the action of different concentrations of spermine phosphate in physiological saline on the motility of human spermatozoa separated from semen and washed three times with 0.9 % NaCl. Equal volumes of the suspension of spermatozoa and of spermine phosphate in physiological saline were mixed; control observations were made without the spermine phosphate. It was found that spermine phosphate in a final concentration of 19, 13, 1.3 and 0.13 mg. per 100 cc. had no activating action on the spermatozoa as compared with the controls.

The discovery of spermine in human faeces may be of significance. Just as putrescine in faeces is derived from the putrefaction of the arginine (ornithine) component of proteins, so spermine might be derived from the putrefaction of an appropriate component of proteins, although no parent substance, capable of yielding it, has yet been observed in protein hydrolysates. In a preliminary observation the writer has obtained traces of spermine after incubating a mixture of a platinum loopful of normal faeces with 2000 cc. of 2 % Witte's peptone for 63 hours, and 7 days respectively, under both aerobic and anaerobic conditions. Control tests, in which inoculation with organisms was omitted, were negative. This subject is under investigation.

SUMMARY.

1. A description of the steam-distillation method of separating spermine from tissues is given, modifications having been introduced to obtain the base as phosphate more readily in a relatively pure state.
2. A study of the distribution of the base in human tissues shows that it is virtually the same as that found in animals by Dudley and Rosenheim.
3. The spermine content of the human prostate is remarkably high, and probably most of the spermine of human semen is derived from this organ.
4. The absence of spermine from the semen of the bull is probably to be explained by the absence of a true functional prostate in that animal.
5. Spermine occurs in human faeces.

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