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ON THE QUESTION OF THE OCCURRENCE AND METABOLISM OF 5-HYDROXYTRYPTAMINE AND RELATED INDOLE COMPOUNDS IN MAMMALIAN SEMEN

BY

T. MANN, R. F. SEAMARK AND D. F. SHARMAN

From the A.R.C. Unit of Reproductive Physiology and Biochemistry, School of Veterinary Medicine, and Molteno Institute, University of Cambridge, and A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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The content of 5-hydroxytryptamine was examined in the semen of man, bull, ram, boar and dog. Spectrophotofluorimetric and chromatographic analysis of extracts prepared by several methods has shown that the semen contains only minute quantities of 5-hydroxytryptamine, if any. One cannot attribute to 5-hydroxytryptamine the uterus-stimulating effect which the human seminal plasma is known to exert. Tryptophan, although present in the seminal plasma, is not converted to 5-hydroxytryptamine on incubation of semen. 5-Hydroxytryptophan is oxidatively deaminated, but not decarboxylated, by spermatozoa. 5-Hydroxytryptamine itself, like tryptamine and tyramine, is not deaminated by either whole semen or washed suspensions of ram spermatozoa.

Among the pharmacodynamic effects of the mammalian seminal plasma is a strong stimulation of smooth-muscle organs such as the uterus and the intestine. This oxytocic activity of semen was investigated in the past by many authors, including Kurzrok & Lieb (1930), Cockrill, Miller & Kurzrok (1935), Goldblatt (1933, 1935a, b), von Euler (1934a, b; 1935, 1936, 1939, 1949), Vandelli (1943), Asplund (1947a, b), Karlson (1949) and Bergström (1949); the general conclusion reached was that the uterine-stimulating property of semen, particularly in man, is probably due to the combined action of several constituents of the seminal plasma, such as choline, prostaglandin and histamine. The early literature has been reviewed in some detail by Mann (1954). Seminal prostaglandin has since been purified and is now believed to be produced, at least in man and ram, by the seminal vesicles and not by the prostate gland (Eliasson, 1959). Two crystalline compounds, both possessing the properties of nitrogen-free unsaturated fatty-acids, have been separated from prostaglandin preparations of vesicular origin; one of these, "prostaglandin E," was shown to exert, in addition to the smooth-muscle stimulating action, a depressant effect on blood pressure of the rabbit, while the other, "prostaglandin F," was found to be devoid of action on blood pressure (Bergström, Eliasson, Euler & Sjövall, 1959; Bergström, Duner, Euler, Pernow & Sjövall, 1959).

Several authors have also considered the possibility that the "oxytocic activity" of semen is due, at least partly, to 5-hydroxytryptamine, which is known to induce

strong uterine contractions, either when applied to the isolated uterus of the rat (Erspamer, 1952) or injected into a living dog (Abrahams & Pickford, 1956). The evidence, however, for the presence of this substance in mammalian semen has so far been confusing and contradictory. Thus, whereas according to Katsh (1959) human seminal plasma contains as much as 135 μg/ml., Hawker, Roberts & Walmsley (1960) could not find 5-hydroxytryptamine either in human or in ram semen.

On the other hand, a recent investigation of the secretory function of male accessory organs in the spiny dogfish, *Squalus acanthias*, has revealed the surprising fact that, in this viviparous elasmobranch fish, one of the accessory organs, the so-called clasper siphon, produces a secretion which is extremely rich in 5-hydroxytryptamine (Mann, 1960). The presence of as much as 7% of 5-hydroxytryptamine in a secretion which forms part of the seminal plasma in the spiny dogfish suggests that in this animal 5-hydroxytryptamine may be involved in the process of reproduction, either by facilitating the ejaculation of semen in the male or perhaps by eliciting contractions of the reproductive tract in the female and thus aiding sperm ascent in the oviducts. Another recent observation relevant to the function of 5-hydroxytryptamine in reproduction concerns certain insects. It was shown that the secretion of the opaque accessory gland in the male of *Rhodnius prolixus*, as well as the secretion of similar glands, the utriculi majores, in the cockroach *Periplaneta americana*, contain a substance which causes contractions of the insects' oviducts. The active principle is destroyed by monoamine oxidase and *o*-diphenol oxidase, and is probably an *o*-dihydroxyindolalkylamine related to 5-hydroxytryptamine (Davey, 1960).

We have attempted to clarify the question concerning the presence of 5-hydroxytryptamine and related compounds in mammalian semen, and to provide more information on the relation of 5-hydroxytryptamine to the "oxytocic activity" of semen.

METHODS

Semen. Semen from rams, bulls and boars was collected by means of the artificial vagina. Dog semen provided by Dr D. Bartlett was collected by the massage technique. Human semen from donors at the Fertility Clinic was made available by Dr H. A. Davidson. The semen samples intended for determination of 5-hydroxytryptamine were kept in solid carbon dioxide until deproteinized. In addition to whole boar semen, analyses of 5-hydroxytryptamine were also carried out on the seminal gel and the epididymal seminal plasma of this species. For measurements of respiration, fresh ram semen was used, either whole or after separation into spermatozoa and seminal plasma.

Spectrophotofluorimetric determination of 5-hydroxytryptamine. This was carried out in the Aminco-Bowman spectrophotofluorimeter on extracts prepared by one of the following three procedures: (1) The sample was deproteinized with zinc sulphate and sodium hydroxide; deproteinization was usually carried out by adding to the sample 1 vol. water, followed by 1 vol. of 10% zinc sulphate ($ZnSO_4.H_2O$) and 0.2 vol. of 10% sodium hydroxide; on occasions when the centrifuged extract was not completely clear, small additional amounts of the deproteinizing agents had to be added; to 2 ml. of the protein-free filtrate was added 1 ml. concentrated hydrochloric acid containing ascorbic acid (0.1 mg/1 ml.); the solution was placed in a quartz cuvette for spectrophotofluorimetric analysis. (2) The sample was extracted by the method of Bogdanski, Pletscher, Brodie & Udenfriend (1956). (3) The sample was treated with 4 vol. acetone and the resultant precipitate and supernatant solution processed further as described by Katsh (1959).

Chromatographic identification of 5-hydroxytryptamine and related indole compounds. Both descending and ascending one- and two-dimensional paper partition chromatography was used. The solvent systems, (i) butanol + acetic acid, (ii) isopropanol + ammonia, (iii) aqueous potassium chloride, and (iv) butanol + pyridine, were used as recommended by Jepson (1960). The developed chromatograms were dried at 25° C in an air oven, all fluorescent areas noted, and an ultra-violet contact photograph taken, using a Hanovia "Chromatolite" lamp and Kodak Duostat 13 reflex paper. The following location reagents, used either singly or in sequence, were applied: acid ninhydrin, ninhydrin pyridine, Ehrlich's reagent, diazotized sulphanilic acid (Jepson, 1960), 5 N hydrochloric acid (Sharman, 1960), and potassium dichromate formaldehyde (Shepherd & West, 1953).

Metabolic studies. Oxygen uptake was determined in Barcroft differential manometers filled with air, at 37° C. Ammonia formation was followed by treating the samples with a saturated solution of sodium borate and subjecting the mixtures to steam-distillation *in vacuo*, at a temperature not exceeding 25° C, in the apparatus of Parnas & Heller (1924). Monoamine oxidase activity was determined by measuring O_2 uptake and ammonia formation as well as by the colorimetric method of Green & Haughton (1961).

Chemicals. The chemicals were of the analytical or microanalytical reagent grade: 5-Hydroxytryptamine creatinine sulphate (Sigma Chem. Co.; May & Baker); dl-tryptophan (Light & Co.); 5-hydroxytryptophan (Roche); tryptamine and tyramine hydrochlorides (British Drug Houses).

RESULTS

Spectrophotofluorimetric observations

Experiment no. 1

This was carried out on a series of extracts prepared from human, ram, bull and boar semen by deproteinization with zinc hydroxide, and the supernatant then rendered 3 N with respect to hydrochloric acid. When these extracts were examined in the spectrophotofluorimeter using a Chance OY 4 filter in the fluorescent light path, a distinct fluorescence was observed with an activation maximum at 295 mμ and a fluorescence maximum at 525 mμ. This fluorescence maximum, however, was shown to be an artefact, due to the transmission characteristics of the filter, since, when the determinations were repeated with the filter omitted, the wavelengths for maximal activation and fluorescence of the seminal extracts were 295 mμ and 430 mμ, respectively. On the instrument used, 5-hydroxytryptamine, in common with other 5-hydroxyindolyl derivatives, showed a fluorescence in 3 N HCl, characterized by an activation maximum at 285 mμ and a fluorescence maximum at 540 mμ. Thus the seminal constituent which fluoresced with a maximum at 430 mμ could not be identical with 5-hydroxytryptamine, even though its activation peak of 295 mμ is close to that of 5-hydroxytryptamine.

In the light of this observation, subsequent fluorimetric analyses of the seminal extracts were made using an activation wavelength of 285 mμ and a fluorescence wavelength of 540 mμ, and calibrating each time the observed fluorescence against one produced by a standard solution of pure 5-hydroxytryptamine. When this was done the following results were obtained, the 5-hydroxytryptamine content being expressed in ng/2 ml. extract: three different samples of human semen, 0 to 5; ram semen, 0; ram seminal plasma, 0; two different samples of bull semen, 0 and 60; boar semen (separated from the gel), 250; boar seminal gel, 0; boar epididymal seminal plasma, 0.

As can be seen, the fluorescence values were of an exceedingly low order in all species. Moreover, these values represent not only the maximum fluorescence attributable to 5-hydroxytryptamine, but include, in all probability, the fluorescence due to some other substances which are present in semen.

Experiment no. 2

For the detection of trace amounts of 5-hydroxytryptamine in semen, an experiment was carried out on two 1.5 ml. samples of human semen, to one of which 5 μg 5-hydroxytryptamine was added before deproteinization. Both samples were extracted with butanol according to Bogdanski *et al.* (1956), and the purified and acidified extracts used to determine the activation scans with fluorescence at 540 mμ, as well as the fluorescence scans with activation at 280 mμ. The results of these determinations are recorded in Fig. 1, which shows the characteristic fluorescence

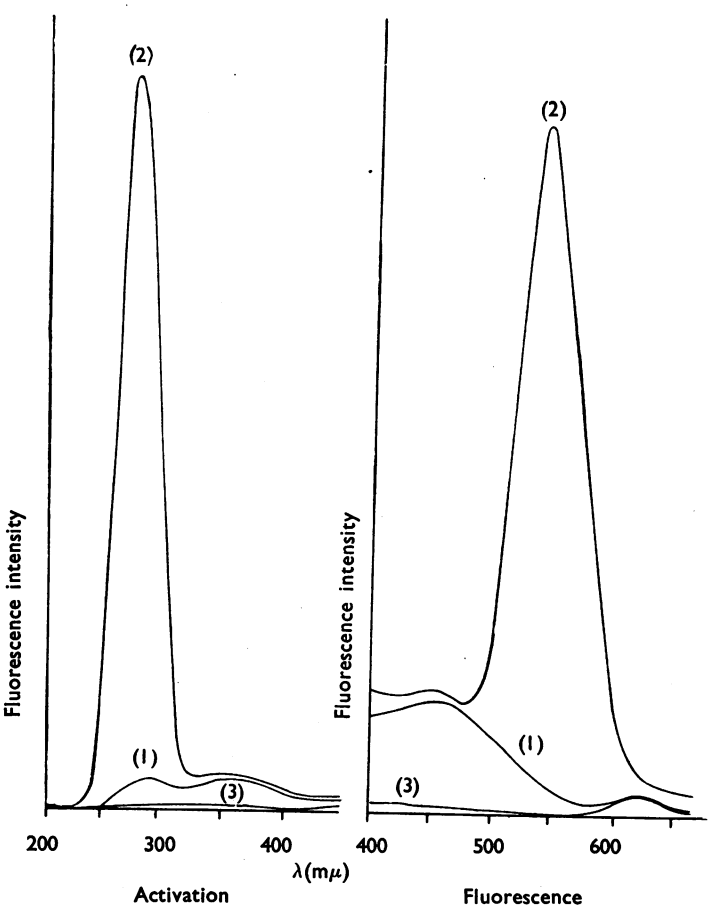

Fig. 1. Activation and fluorescence spectra of extracts from human semen: (1) extract from 1·5 ml. human semen; (2) extract from 1·5 ml. human semen to which 5 μg 5-hydroxytryptamine was added; and (3) reagent blank.

due to added 5-hydroxytryptamine and the lack of fluorescence in the sample without 5-hydroxytryptamine. These results clearly indicate that the content of 5-hydroxytryptamine in human semen is either negligible or nil.

Experiment no. 3

The fluorimetric analyses of human, bull, boar and ram semen were repeated, using larger quantities of semen and the extraction procedure of Bogdanski *et al.* (1956) which allows the preparation of more concentrated extracts. In addition, dog semen was examined by the same method. In each instance two measurements were taken: (i) of the wavelengths of maximal activation and fluorescence of the seminal extracts; and (ii) of the "5-hydroxytryptamine equivalent" of any fluorescence present, determined at the activation wavelength of 285 mμ, and the fluorescence wavelength of 540 mμ. The results were as follows.

Human semen. 9 ml. representing pooled ejaculates from four donors; two normal, one completely azoospermic, and one with sperm density less than 10,000 cells/μl.; final volume of the hydrochloric acid extract, 5 ml. When examined in the spectrophotofluorimeter, the extract exhibited a characteristically strong fluorescence with an activation maximum of 295 mμ and a fluorescence maximum at 430 mμ; 2 ml. of the hydrochloric acid extract, when examined at the 285 and 540 mμ wavelength, corresponded to a content of 540 ng "5-hydroxytryptamine equivalent," that is, 150 ng/ml. semen.

Bull semen. 13 ml. obtained from two bulls. The acid extract exhibited the typical 295/430 mμ fluorescence. The "5-hydroxytryptamine equivalent" content was 1,000 ng (1 μg)/ml. semen.

Ram semen. 15 ml. representing pooled ejaculates from ten rams. The acid extract exhibited the typical 295/430 mμ fluorescence. The "5-hydroxytryptamine equivalent" was 500 ng/ml. semen.

Boar semen. Out of a total ejaculate of 270 ml., a 15 ml. sample was used for extraction. The extract showed the typical 295/430 mμ fluorescence, and contained a "5-hydroxytryptamine equivalent" of 50 ng/ml. semen.

Dog semen. 15 ml. representing two ejaculates. The extract prepared from dog semen clearly showed the blue fluorescence previously observed in human, bull, ram and boar semen, namely, at the 295 and 430 mμ wavelengths. It contained a "5-hydroxytryptamine equivalent" of 150 ng/ml. semen.

The general conclusion to be drawn from experiment no. 3 is that there is very little 5-hydroxytryptamine, if any, in the semen of man, boar, ram, bull and dog. This was confirmed by the following experiment.

Experiment no. 4

Both the activation and fluorescence scans were determined using side by side the hydrochloric acid extracts obtained from human and canine semen, and a similarly prepared extract from the nucleus caudatus of the dog brain, of which the 5-hydroxytryptamine content had previously been determined and found to be

0.3 μg/g tissue. The results of this experiment are recorded in Fig. 2. In the left part of this figure the activation scan of the extract from dog semen is compared with that obtained from the nucleus caudatus. There is only a small difference in the maximum activation wavelength (295 mμ and 285 mμ, respectively). In the right part of Fig. 2, the fluorescence scan of human semen is contrasted with that of the dog nucleus caudatus; here it is obvious that the peak characteristic of 5-hydroxytryptamine (540 mμ) is entirely absent in the semen.

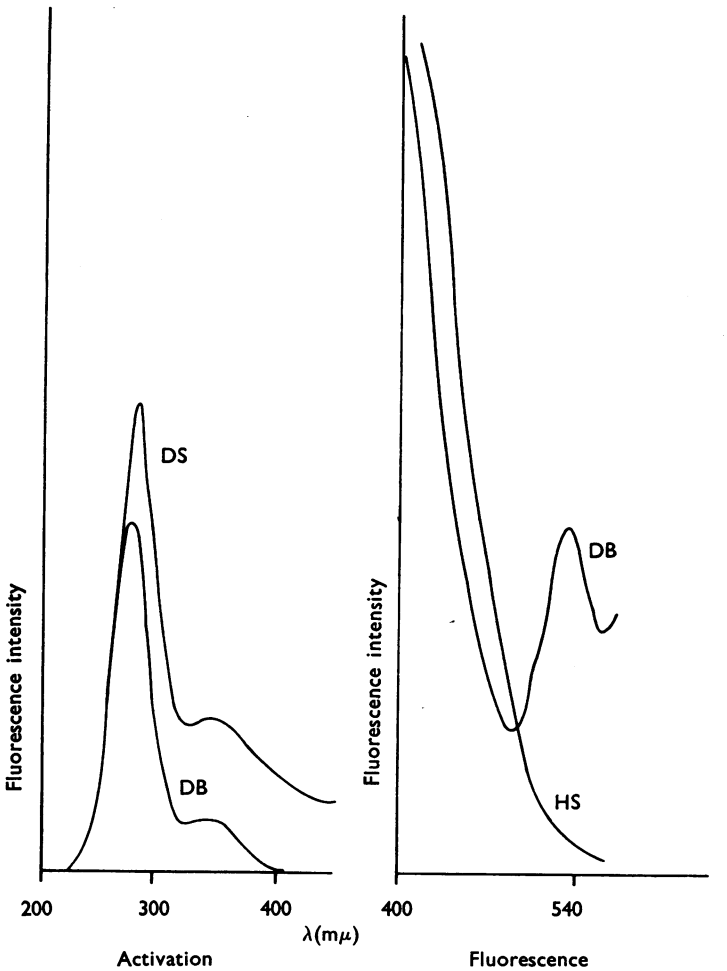

Fig. 2. Activation and fluorescence spectra of extracts from dog semen (DS), human semen (HS) and the nucleus caudatus of the dog brain (DB). The amplification used in recording the fluorescence scan of the extract from human semen (HS) was reduced to enable the record to cover the same wavelength range as that obtained with the nucleus caudatus extract (DB). If HS had contained 1 μg 5-hydroxytryptamine, a peak of the same size as that shown b y DB would have been observed.

Chromatographic observations on fresh semen

Several attempts were made to detect 5-hydroxytryptamine by chromatography in concentrated extracts from either whole semen or freshly prepared washed sperm suspensions and seminal plasma of man, bull, ram, boar and dog. The chromatograms were examined by the various methods already cited, but no evidence was obtained for the occurrence of 5-hydroxytryptamine.

In order to repeat the observation of Katsh on the presence of 5-hydroxytryptamine in human semen, two separate experiments were carried out, with 3 ml. and 12 ml. human seminal plasma, following closely the acetone extraction procedure. These experiments, too, failed to yield positive evidence for the presence of 5-hydroxytryptamine in human seminal plasma. It may be added that chromatograms developed with the various systems used revealed several substances reacting with one or another of the spraying reagents. However, no indole compounds other than tryptophan, no substituted tryptamine derivatives such as 5-hydroxytryptamine, nor any phenolic indole compounds were detected. Similarly, we obtained negative results with human semen using different deproteinization and extraction procedures (Udenfriend, Weissbach & Brodie, 1958).

We did not succeed in demonstrating 5-hydroxytryptamine chromatographically in bull, ram, boar and dog semen by any of the methods applied in this work.

Metabolism of 5-hydroxytryptamine and other indole compounds in semen

A series of chromatographic analyses was performed on ram semen, washed sperm and seminal plasma, which had been incubated in the presence of added 5-hydroxytryptamine or certain other related compounds, to find out whether 5-hydroxytryptamine itself is metabolized in semen, and also to explore the possibility that it might be formed in semen as the result of the metabolic breakdown of substances such as tryptophan and 5-hydroxytryptophan. Most of these experiments were combined with measurements of the oxygen uptake and ammonia evolution during the incubations.

5-Hydroxytryptamine. The experimental samples contained 5 mg 5-hydroxytryptamine creatinine sulphate dissolved in 1 ml. Ringer-phosphate, and 2 ml. of either a washed sperm suspension (10^9 cells) in Ringer or 2 ml. of correspondingly diluted whole semen ; aerobic incubations were done in Barcroft manometers for periods ranging from 1 to 4 hr at 37° C. Under these conditions the addition of 5-hydroxytryptamine had no detectable effect on the rate of oxygen uptake, and there was no rise in free ammonia. After deproteinization with barium hydroxide and zinc sulphate {1.5 ml. incubation mixture + 0.5 ml. 0.3 N barium hydroxide + 0.5 ml. 5% zinc sulphate ($ZnSO_4.7H_2O$)}, 50 to 100 μl. aliquots of the protein-free filtrates were analysed by paper chromatography in various solvents. However, as a result of the aerobic incubation there was no evidence that either washed spermatozoa or whole semen produced any change in the 5-hydroxytryptamine content. In particular, there was no formation of 5-hydroxyindole acetic acid as an oxidative breakdown product of 5-hydroxytryptamine.

Tryptophan. The oxidative deamination of tryptophan by bull spermatozoa has been the subject of a study by Tosic & Walton (1950) and Tosic (1951), who have

pointed out that the formation of hydrogen peroxide during the oxidative deamination of aromatic amino-acids is responsible for the gradual decline in the oxygen uptake of incubated sperm mixtures. Using the same conditions as in the experiments with 5-hydroxytryptamine, we found that whole ram semen and washed ram spermatozoa can deaminate dl-tryptophan aerobically; moreover, whilst the same amount of ammonia was produced in the presence as in the absence of pure catalase, the rate of oxygen consumption was much higher with catalase present, owing to the decomposition of the toxic hydrogen peroxide. Chromatographic analyses of the incubation mixtures of dl-tryptophan and either whole semen or washed sperm suspensions showed that ram spermatozoa metabolize tryptophan aerobically, but not anaerobically, to several products. The major product was identified chromatographically as indoleacetic acid. The identification rested on the use of both descending and ascending chromatography in butanol + acetic acid (R_F 0.88 and 0.92, respectively), as well as in two other solvents, namely, isopropanol + ammonia (R_F 0.28) and potassium chloride (R_F 0.60). No tryptamine, 5-hydroxytryptophan or 5-hydroxytryptamine was detected.

5-Hydroxytryptophan. Mixtures of sperm and 5-hydroxytryptophan were incubated under the same conditions as in experiments with 5-hydroxytryptamine and tryptophan, in the presence and absence of catalase. A small amount of ammonia was formed, but there was no evidence of decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine.

Tryptamine and tyramine. No ammonia formation occurred when a solution containing 5 mg tryptamine was incubated aerobically for 3 hr at 37° C, with either a suspension of washed spermatozoa (10^9 cells/2 ml. Ringer) or a sample of correspondingly diluted whole ram semen. When the experiment was repeated using tyramine instead of tryptamine, only a trace of ammonia was produced. These experiments provided a good indication that the monoamine oxidase activity of ram semen must be low. Supporting evidence for this view was obtained in the following manner. Tyramine oxidation was examined by the more sensitive procedure of Green & Haughton (1961), which depends on trapping with semicarbazide the aldehyde formed during the enzymic oxidation of tyramine, and converting the semicarbazone into the corresponding 2:4 dinitrophenylhydrazone. The light absorption of the orange-coloured alkaline solution of the dinitrophenylhydrazone was determined in the Unicam spectrophotometer at 450 mμ. Aerobic incubation for 2 hr at 37° C of a mixture (4 ml.) containing 1 ml. whole semen, 1 ml. 0.5 M semicarbazide solution (*p*H 7.4), 0.4 ml. 0.1 M solution of tyramine hydrochloride (*p*H 7.4), and 1.6 ml. Ringer phosphate produced less than 1/25 of the amount of semicarbazone which had been formed, under identical experimental conditions, by a preparation of mitochondria from 1 g of rat liver.

DISCUSSION

It was thought at one time that spermatozoa deposited at ejaculation in the vagina or cervix rely on their own motility in order to traverse the uterus and reach the oviducts. More recently, however, when measurements were made of the time interval required for some spermatozoa, at any rate, to arrive at the site of fertili-

zation, this proved to be remarkably short, in some species no more than a few minutes. This and subsequent work has shown that ejaculated spermatozoa are propelled to their final destination in the oviducts not so much by their own movements as by the concomitant uterine contractions.

The mechanism responsible for the increase in uterine motility following copulation and sexual stimulation is still under investigation. Some investigators attribute it mainly to the release of oxytocin by the pituitary gland ; others believe that semen itself may provide, at least partly, the required stimulus in the form of pharmacologically active constituents. Similarly, the chemical identity of the various " oxytocic " substances in semen is still under dispute, as shown by the perusal of recent literature (Mann, 1954 ; Eliasson, 1959 ; Hawker *et al.*, 1960).

The possibility that 5-hydroxytryptamine is one of these substances has been examined. The present study does not support the claim that mammalian semen has a high content of 5-hydroxytryptamine ; our spectrophotofluorimetric as well as chromatographic analyses have shown that the amine is either altogether absent from the semen of man, bull, boar, ram and dog, or present only in minute quantities. 5-Hydroxytryptamine may, perhaps, appear in human semen in measurable quantities in special circumstances. One need only recall the well-known effect which 5-hydroxytryptamine contained in certain foods exerts on the excretion of indole derivatives in the human urine. Certain other substances administered orally can pass unchanged into the seminal plasma ; for instance, when ergothioneine, a normal constituent of the seminal plasma, is fed to a boar the administered compound is found unchanged in the ejaculated semen (Heath, Rimington, Glover, Mann & Leone, 1953). The fact remains that 5-hydroxytryptamine is not a normal constituent of seminal plasma, either in man or in ram, bull, boar and dog. It is therefore unlikely that the uterine-stimulating property of semen depends upon the presence therein of 5-hydroxytryptamine.

An indole compound which is a normal constituent of mammalian seminal plasma is tryptophan. This amino-acid, however, and the closely related 5-hydroxytryptophan, do not appear to undergo any appreciable decarboxylation in semen. 5-Hydroxytryptamine itself, and related amines such as tryptamine and tyramine, when added to semen, are also relatively stable, as shown by incubation experiments carried out with whole ejaculated semen or washed sperm suspensions.

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