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Smooth Muscle Stimulating Factors in Ram Semen

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Extraction and chromatographic separation of lipid soluble, acidic material in ram seminal plasma has shown the presence of at least two different factors with smooth muscle stimulating and blood pressure lowering effects. One of the factors shows certain properties similar to those of the PGE previously isolated from sheep prostate glands.

The pharmacodynamic effects of extracts of seminal fluid and male accessory glands of humans and some animal species were observed independently by Goldblatt [1] and U.S. von Euler [2,3]. The active principle was called prostaglandin.

We have recently described the isolation of two factors, PGE and PGF, from the prostate glands of sheep in crystalline form [4,5]. The compound PGE predominates in the extracts of frozen glands, while roughly equal amounts of the two compounds were found in vacuum-dried glands.

In order to ascertain the nature of the active compounds present in the seminal plasma, we have made a preliminary study of the chemical properties of the physiologically active factors normally present in ram semen plasma.

EXPERIMENTAL AND RESULTS

Ram seminal plasma was obtained through the courtesy of Dr. T. Mann, Molteno Institute, Cambridge, to whom we are greatly indebted for his help. The ram semen had been collected at the A.R.C. Unit of reproductive Physiology and Biochemistry, Cambridge. The seminal plasma was obtained by centrifugation of 23 ejaculates (30 ml). The supernatant seminal plasma (24 ml) was precipitated with two volumes of 96 % ethanol and was sent by air mail to our laboratory.

Extraction and chromatography. The ethanolic solution was filtered and the precipitate washed several times with 96 % ethanol. About 150 ml of clear solution was obtained and evaporated *in vacuo* to a volume of a few ml After dilution to about 10 ml with water, the solution was acidified with hydrochloric acid to pH 3 and extracted three times with equal volumes of ether.

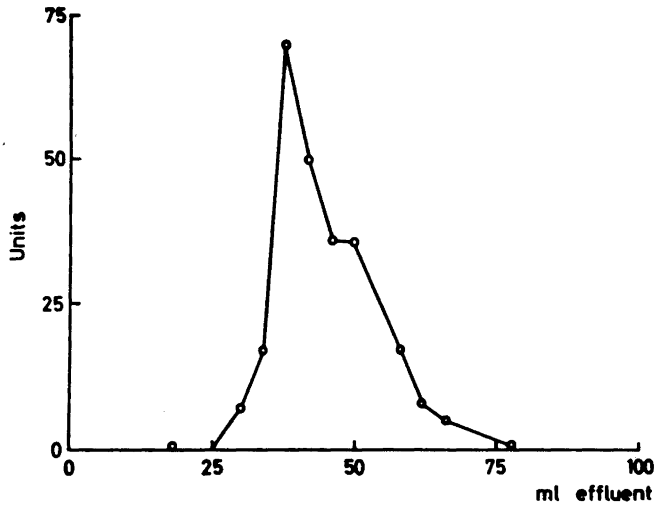

Fig. 1. First chromatography of the physiologically active material in ram seminal plasma.

The combined ether extracts were extracted twice with half the volume of 0.2 M sodium phosphate pH 8, the pH having been corrected to 8 with sodium carbonate during the first extraction.

The buffer solution was acidified to pH 3 with hydrochloric acid and extracted twice with equal volumes of ether. After washing with small portions of water, each of which was passed through a second ether phase, evaporation of the combined ether extracts left about one mg of residue.

This material was subjected to reversed-phase chromatography. 150 ml methanol, 150 ml water, 15 ml chloroform and 15 ml *iso*octanol were shaken in a separatory funnel and left to separate at 23°C. The less polar phase was used as the stationary phase; 3 ml were supported on 4.5 g of Hostalen (Hoechst) that had been purified by continuous ethanol extraction in a Soxhlet apparatus for two days. The column was prepared as described earlier [4]. The residue was dissolved in a few ml of the moving phase and transferred onto the top of the column. 100 ml of the moving phase were allowed to pass through the column and 2 ml fractions were collected. The activity of every third fraction was determined on duodenal intestinal strips; results are shown in Fig. 1. The activity appeared at the same place in the chromatogram as PGE and PGF which move together in this system. The testing was done against a standard of pure PGE, *1 μg being defined as one PGE rabbit intestinal unit.* The total activity corresponded to approximately 500 μg of PGE. The effect of the fractions at 34 and 58 ml effluent was tested on rabbit blood pressure and it was found that 2—4 intestinal units produced a distinct lowering of blood pressure.

The fractions between 25 and 75 ml effluent were combined and subjected to reversed-phase chromatography, using methanol/water/*iso*amylacetate/chlo-

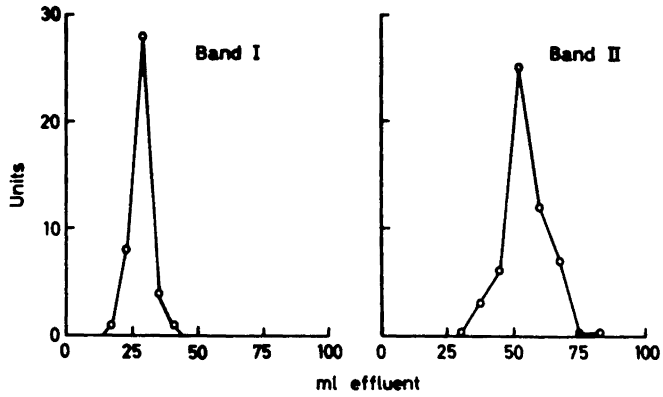

Fig. 2. Chromatography of the two active bands separated by chromatography of the active band shown in Fig. 1. See text.

roform (70/130/8/12/ (v/v)). Four ml of the less polar phase were supported on 4.5 g of silane treated hydrophobic Supercel. On testing the fractions on the intestinal strip, it was found that the activity had been separated into two bands. The peak of the first band appeared at 27 ml effluent and that of the second band at 56 ml effluent. Both bands contained material with a depressing effect on rabbit blood pressure.

The peak fractions of both the bands were used for paper chromatography with ethylene chloride/heptane 5:5 as moving phase and acetic acid/water 7:3 as stationary phase at a constant temperature of 23°C. After the developed chromatograms were sprayed and heated at 80° for a few minutes, one blue spot was revealed which had moved as PGE in the case of the second band and slightly slower in the case of the first one.

The fractions of each peak from the reversed-phase chromatography were then combined and rechromatographed with the same solvent system as that used for their separation (Fig. 2).

Spectroscopic properties. The peak fractions of the chromatograms shown in Fig. 2 were used for a study of the spectroscopic behaviour of the compounds in alkaline and acid solution. After evaporation of the solvents, the residues were dissolved in spectroscopically pure absolute ethanol and the U.V. spectra were recorded with an automatic spectrophotometer (Perkin-Elmer, Spectracord 4 000). The ethanol was then evaporated and the fractions dissolved in 0.01 N sodium hydroxide in 50 % ethanol and heated from 2 h at 100°C in sealed tubes. The U.V. spectra were again recorded against an appropriate solvent blank. After the addition of 50 μl of 0.1 N sodium hydroxide and having been heated for one hour at 100°C in sealed tubes, a third recording of the spectra was made. After addition of 0.5 ml of 4 N hydrochloric acid to each sample and having been heated at 100°C for one hour in sealed tubes, a final recording of the spectra was made.

The results with the peak fraction of band II (Fig. 2) are shown in Fig. 3. The original ethanolic solution of the substance showed a low, noncharacteristic

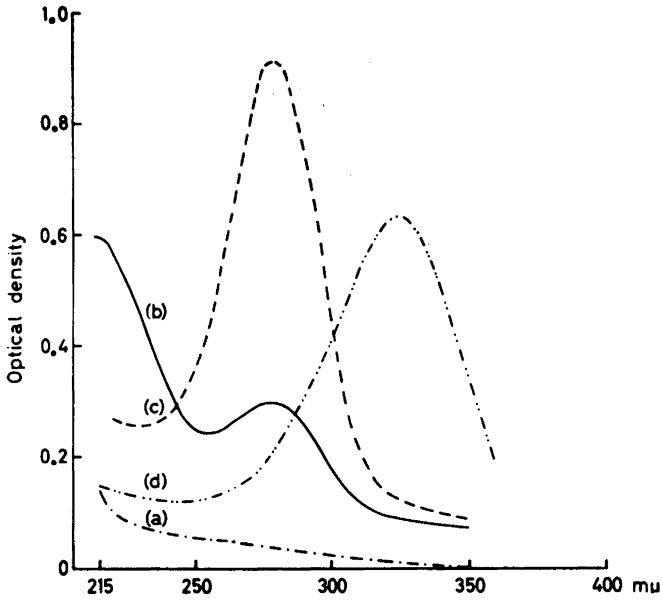

Fig. 3. Absorption spectra of the peak fraction of band II, Fig. 2: (a) in ethanol, (b) after 2 h at 100°C in 0.01 N NaOH, (c) after another hour at 100°C after addition of 50 μl of 0.1 N NaOH, (d) after another hour at 100°C after the addition of 0.5 ml of 4 N HCl. For further explanations see text.

increase of the extinction towards shorter wavelengths (Fig. 3a). After it was heated in 0.01 N sodium hydroxide, a large increase in the absorption at 215 mμ was observed and a lower maximum was present at 280 mμ (Fig. 3b). The latter band increased several times on reheating with more alkali, whereas the absorption at 215 mμ decreased (Fig. 3c). After heating in hydrochloric acid, the maximum at 280 mμ had disappeared and a new maximum at 325 mμ was found (Fig. 3d). All the changes observed are typical for PGE.

In the case of the peak fraction of band I (Fig. 2), no such typical changes in the spectra were found. There was only a noncharacteristic, continuous increase in extinction values towards lower wave-lengths, and heating in alkali and acid increased these values only slightly.

DISCUSSION

The extraction and the chromatographic separation of the lipid soluble, acidic material present in ram seminal plasma has shown that at least two different factors are present in comparable amounts, judging from the activity on intestinal strips from rabbits.

One of these behaves in the column chromatographic separations as well as on paper chromatograms, as the PGE isolated from sheep prostate glands. The purified fractions also show the same typical U.V. spectra on heating with

alkali and acid. The extinctions observed are in rough agreement with those expected from the biological activity.

The observed activity corresponds to a content of about 10 μg of PGE per ml of seminal plasma, *i.e.* the avarage content of prostaglandin E per ejaculate would be slightly higher.

The second active compound is likewise active, both on the intestinal strip on rabbit blood pressure, *i.e.* it is not identical with PGF which shows no activity on the blood pressure. It is furthermore differentiated from PGF by the absence of a maximum at about 225 mμ after being heated with acid. Possibly minor amounts of PGE may be present, but if so, the third physiologically active compound observed in this paper predominates.

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