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A smooth muscle contracting substance in extracts of human umbilical cord

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Aqueous extracts of human umbilical arteries and vein have been shown to contain a smooth muscle contracting substance. The active principle has been distinguished from smooth muscle contracting substances which are found in mammalian tissues, namely esters of choline, histamine, 5-hydroxytryptamine, bradykinin, angiotensin, and darmstoff. Evidence indicates that the smooth muscle stimulating activity of umbilical blood vessel extracts is due to a lipid-soluble unsaturated hydroxy acid. The possible physiological function of the active substance is discussed.

THE umbilical cord is not ligated at the birth of most mammals and in veterinary obstetrics no important haemorrhage follows the division of the cord (Williams, 1931). It appears that in man, nature has also made adequate provision for the cessation of umbilical blood flow since constriction of the umbilical blood vessels follows delivery.

The umbilical blood vessels have been shown to constrict in response to a variety of stimuli. Haselhorst (1929) demonstrated that the walls of cord vessels were sensitive to temperature and to irritation from handling. Schmitt (1926) showed that umbilical vessels contracted when the oxygen content of perfusing fluid was increased. Umbilical vasoconstriction may also be induced by the catecholamines, histamine, posterior pituitary extracts and 5-hydroxytryptamine (5-HT) (Panigel, 1959, 1962). These facts suggested to me the possibility of the involvement of a chemical substance which could act on the umbilical blood vessels after birth. If this speculation is correct it should be possible to recover the active substance from the umbilical blood vessels. In the present investigation, human umbilical cords have been examined for the presence of substances which might cause contraction of the umbilical blood vessels and other smooth muscles.

Experimental

Preparation of aqueous extract. The umbilical arteries and veins were dissected from fresh umbilical cords and were split open along their longitudinal axis. Blood was removed by washing with distilled water, and the vessels were dried between filter paper, weighed and then minced with 4 ml of distilled water (pH 6·5–7) for each gram of tissue. The mixture was left in the refrigerator (4°) for 12 hr before centrifugation for 15 min at 3,000 rpm. The viscous supernatant was decanted into stoppered bottles and the residue re-extracted with a further 4 ml of distilled water per gram of tissue. The two supernatants were combined and extracted with ether as described below.

Purification of aqueous extract. A slight modification of the method described by Ambache (1959) has been successfully used for the purification of the aqueous extracts of umbilical cord vessels. Aqueous extracts

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at pH 6·5–7 were shaken with equal volumes of freshly distilled diethyl ether. The separation of the two layers was assisted by centrifugation and the ether layer was discarded. The aqueous solution was next acidified to pH 3 with N hydrochloric acid and extracted with three volumes of peroxide-free ether. The two phases were separated as before and the ether layer was evaporated to dryness under reduced pressure at room temperature. The dry residue was reconstituted in 0·9% w/v sodium chloride solution just before assay.

Extract of foetal arteries. Aqueous extracts of foetal abdominal and thoracic aortae obtained at post-mortem were prepared in exactly the same way as the extracts of umbilical blood vessels. The aqueous extracts were shaken at pH 3 with 3 volumes of diethyl ether. The dry ether residue after the evaporation of the ether was taken up in 0·9% sodium chloride solution and tested for biological activity.

Extracts of Wharton's Jelly. Wharton's Jelly from fresh cords was similarly extracted.

BIOLOGICAL ACTIVITY TESTS

Rabbit ileum preparation. Segments of rabbit ileum were suspended in a 5 ml organ bath of Tyrode solution. The bath solution at 32° was bubbled with air. Records were made with a frontal writing lever of 8 : 1 magnification with a 3 g load on the muscle.

Guinea-pig ileum. From animals weighing 200–250 g, a piece of terminal ileum was removed and suspended in a 5 ml organ bath of Tyrode solution at 32°. The tension on the tissue was approximately 2 g. The frontal writing lever had a magnification of 10 : 1.

Rat uterus. This was set up as described by Amin, Crawford & Gaddum (1954). The bath fluid was gassed with oxygen at 28°.

Guinea-pig proximal colon. This was set up in a 10 ml organ bath as described by Botting (1965).

Rat colon. The proximal 4–5 cm of colon was suspended in a 5 ml organ bath at 28°. The aerated bath fluid corresponded to that used by Gaddum, Peart & Vogt (1949).

Umbilical artery preparations. Fresh umbilical cords were used. Approximately 4–5 cm of the spirally cut arteries were suspended in a 5 ml organ bath of Krebs bicarbonate solution gassed with oxygen.

Pithed rat blood pressure. Rats weighing 250–300 g were anaesthetised with ether and pithed by passing a strong wire through the orbit down the cerebrospinal axis. Artificial respiration was maintained by a Palmer small animal pump. Blood pressure was recorded from a carotid artery by means of a condon manometer.

Rabbit blood pressure. Rabbits (1·8–2·5 kg) were anaesthetised with urethane (8 ml of a 20% solution i.v.) and injected with heparin (500 units/kg) as an anticoagulant. Arterial blood pressure was recorded from the carotid artery by a mercury manometer and drugs were injected in the external jugular vein.

Cat blood pressure. Cats weighing between 2·3–4 kg were anaesthetised with ether followed by chloralose (80 mg/kg i.v.). The blood pressure was

recorded from a cannulated femoral artery by a mercury manometer. Drugs were injected into a femoral vein.

Paper chromatography. Ascending chromatograms of the umbilical vessels extracts were run on Whatman No. 1 filter paper. The following solvent systems were used. (1) Organic, upper phase of the following mixture: ethyl methyl ketone-water-diethylamine (60:20:3) (Vogt 1955, 1957c). (2) Butanol-acetic acid-water (4:1:5) (Partridge, 1948). (3) Phenol saturated with 0·01N hydrochloric acid. The chromatograms were developed at room temperature (18–20°) for 22–24 hr in an atmosphere of nitrogen. The localisation of the active substance was detected by biological assay. Strips of paper were cut at variable intervals from the starting line to the solvent front, and eluted by descending chromatography overnight in 10 ml graduated cylinders with distilled water (pH 6·5–7). The eluates were acidified to pH 3 with 0·1N hydrochloric acid and extracted with three volumes of freshly distilled diethyl ether. The ether extracts were evaporated to dryness at room temperature under reduced pressure. The dry extracts were dissolved in Tyrode solution just before assay on guinea-pig proximal colon.

Paper electrophoresis. Paper electrophoresis was on Whatman No. 1 filter paper at pH between 5 and 9 in phosphate buffer as described by Ambache (1957). The position of the active substance on the dry paper was ascertained by assay on the guinea-pig proximal colon.

Enzyme Incubation. (i) *Chymotrypsin.* To 1 ml of ether-purified umbilical blood vessels extract ($\equiv$100 μg dry weight of extract) was added 0·3 mg of crystalline chymotrypsin. The solution at pH 8·0 was placed in a water-bath at 37° for 1 hr. A control tube containing an equiactive concentration of bradykinin and 0·3 mg of chymotrypsin was similarly incubated. Another control tube containing 100 μg of umbilical blood vessels extract at pH 8 was also incubated. At the end of 60 min the three solutions were assayed for biological activity on guinea-pig proximal colon.

(ii) *Trypsin.* To each of two test-tubes containing 100 μg/ml umbilical blood vessels extract and 1 μg/ml angiotensin was added 10 mg of trypsin. The tubes were incubated at 37° for 60 min. At the end of this period the two solutions were tested for activity on the guinea-pig proximal colon.

CHEMICAL TESTS

Chemical tests on umbilical vessels were made in solutions or on chromatograms.

Amino-acid test. Papers were sprayed with 0·1% ninhydrin in water-saturated butanol and left in an oven at 100° for 5–10 min.

Test for -NH- group. The modified method of Reindel & Hoppe (1953) as described by Ambache (1959) was used. Filter paper containing the active extracts was exposed to chlorine dioxide followed by spraying the paper with 1% benzidine solution in 10% acetic acid.

Colour test for higher fatty acids. To 0·1 ml of a saturated solution of Rhodamine B in benzene were added 0·1 ml of 1% aqueous solution of uranyl acetate and 0·2 ml of a benzene solution of ether purified umbilical

blood vessels extract (≡50 μg). A control tube containing the reagents and 0·2 ml benzene without extracts was prepared simultaneously and both tubes were shaken. With this procedure the higher fatty acids form benzene-soluble red additive compounds which fluoresce (Feigl, 1956) under ultraviolet light.

Colour test for hydroxy-fatty acids. 0·1 ml of a freshly prepared 0·5% solution of sym-diphenylcarbazide in tetrachloroethane was added to 50 μg/ml solution of umbilical blood vessels extract. A control tube with same volumes of reagents and without the umbilical extracts was also prepared. The tubes were shaken intermittently and the colour in the lower tetrachloroethane was noted.

Phenyl isocyanate. This test was made as described by Ambache (1959). To 1 ml of umbilical vessels extracts (≡100 μg) was added 0·02 ml of phenyl isocyanate in a test-tube which was then left at room temperature for 1 hr; during this time the tube was shaken intermittently. The solution was centrifuged to remove the precipitated diphenyl urea and the supernatant after decanting was tested for biological activity.

*NN'-carbo-di-*p*-tolyl imide.* To 100 μg of umbilical vessels extracts in diethyl ether was added 1 mg of the reagent. A second sample of the cord extract was used as an untreated control. The two samples were left at room temperature for 30 min and were then evaporated to dryness and taken up in Tyrode solution and tested for activity on the guinea-pig proximal colon.

Potassium permanganate. To 1 ml of aqueous solution of umbilical vessels extract (≡100 μg dry weight) was added 0·025 ml of a 2% solution of potassium permanganate. The pH of the mixture was 7·5. After 5 min at room temperature the solution was acidified to pH 3 and extracted with 3 volumes of diethyl ether. The ether phase after evaporation was reconstituted in 0·9% sodium chloride solution and assayed on guinea-pig proximal colon.

Iodine bromide. 0·2 ml of a saturated solution of iodine bromide was added to 1 ml of umbilical cord extract (100 μg). The solution was left standing for 15 min at room temperature and the excess of iodine bromide was inactivated by adding excess of sodium thiosulphate. The extract was acidified to pH 3 and extracted with 3 volumes of ether. The control was a tube with the same amount of iodine bromide inactivated with sodium thiosulphate before the addition of the active extract. It was partitioned under identical conditions.

DRUGS USED

Acetylcholine chloride, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, bradykinin, angiotensin, diphenhydramine, atropine sulphate, methysergide bimaleate, trypsin and chymotrypsin.

Results

Extraction. After extraction of the minced umbilical blood vessels twice with 4 ml of distilled water per gram of the tissue, less than 20% of

the biological activity was left. For this reason the residue was not further re-extracted.

Purification by partition with ether. Fig. 1 shows the recovery of the active substance from the aqueous extracts of the umbilical blood vessels

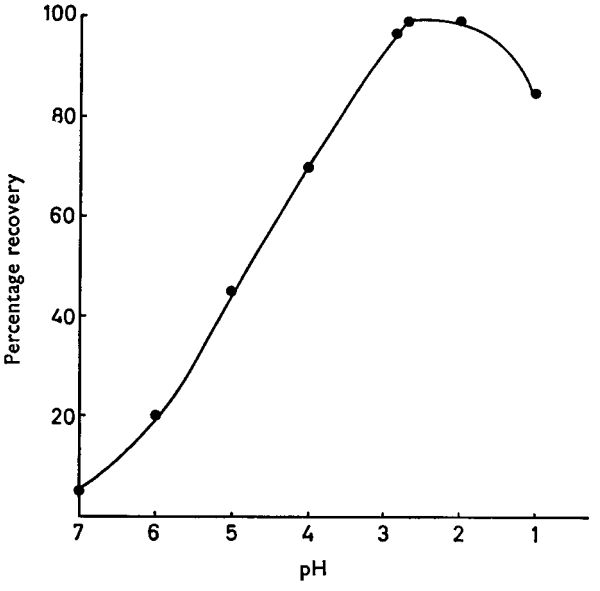

FIG. 1. Recovery of the active substance after partitioning the aqueous extracts of umbilical blood vessels with three volumes of diethyl ether at various pH values, expressed as percentage of the original activity found per ml of ether phase.

after partition with three volumes of diethyl ether at various pH values. The yield of the active material was almost 100% between pH 2 and 3 (Fig. 1). The low recovery at pH 1 was found to be due to the active substance being partly destroyed. The yields of the active substance after purification are given in Table 1.

TABLE 1. YIELDS OF ACTIVE SUBSTANCE PURIFIED BY PARTITION FROM 100 G OF TISSUE

pH of the partition	Weight of active residue	Purification—from original weight of tissue
2·5	10 mg	10,000 ×
2·3	12 mg	8,333 ×
3·0	8 mg	12,500 ×
3·0	9 mg	11,111 ×

Activity test. The ether-purified umbilical vessels extracts contracted smooth muscles, namely guinea-pig ileum and colon, rat colon and uterus, rabbit ileum and isolated umbilical artery preparations (Fig. 2). Guinea-pig proximal colon (Botting, 1965) was found to be the most sensitive

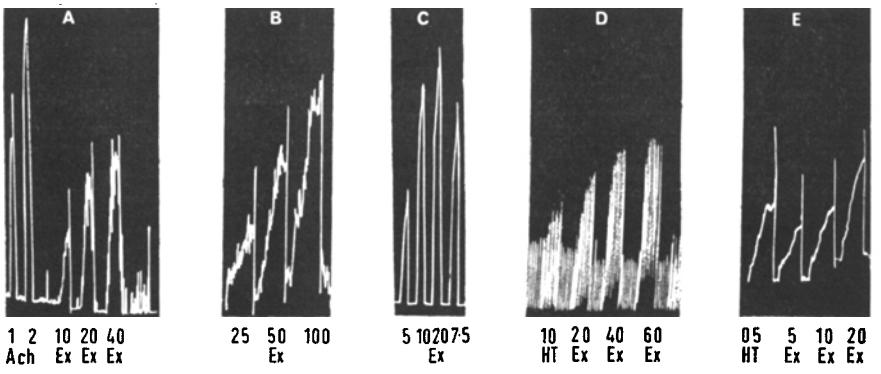

FIG. 2. Effect of umbilical cord vessels extracts on various smooth muscle preparations suspended in 5 ml isolated organ baths. A. Guinea-pig terminal ileum. B. Rat colon. C. Rat uterus. D. Rabbit ileum. E. Umbilical artery preparation. Ex. Doses of ether purified extracts in μg. HT. 5-Hydroxytryptamine. Ach. Acetylcholine. All doses in μg.

preparation and has been used for the routine biological assay of the active substance. Extracts of foetal aortae and of Wharton's Jelly in a concentration of 200 μg/ml had no smooth muscle stimulating activity.

Other pharmacological actions. The active substance in the umbilical blood vessels produced vasodepression in the cat, rat and the rabbit (Fig. 3). This property has been used to distinguish the active principle from other known substances with similar properties.

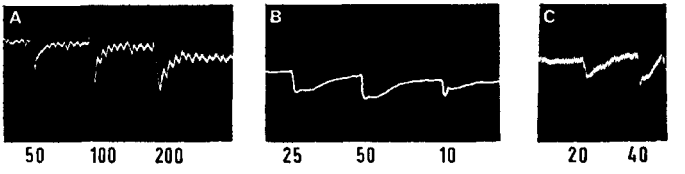

FIG. 3. Effect of intravenous injections of umbilical cord vessels extract on the blood pressure responses of (A) anaesthetised cat (2·2 kg), dose interval 5 min; (B) anaesthetised rabbit (1·9 kg), dose interval 20 min; (C) pithed rat (300 g), dose interval 10 min. All doses in μg/kg.

Dialysis. The active substance in the umbilical blood vessels slowly dialysed through a cellophane membrane. In dialysis experiments of extracts against distilled water, equilibrium was reached slowly. After 6 hr the activity in the water was almost nil and there was no loss of activity in the extract. After 14 hr the activity was equally distributed on both sides.

Stability. The stability of the active substance in the umbilical blood vessels depended on pH and temperature. After 7 days at room temperature at pH 7 there was no loss of activity, whereas at pH 1 or 2 more than 50% of the activity was destroyed after standing at room temperature for 8 hr. The effect of boiling the aqueous extracts in sealed containers at various pH is shown in Fig. 4. The active substance was not destroyed

by boiling for 30 min at pH 3–9. At pH 7, one sample was left boiling for 2 hr without loss of activity.

Solubility. The active substance is soluble in water, ethanol and acetone. It is also soluble in ether and chloroform at acid pH but almost insoluble in light petroleum.

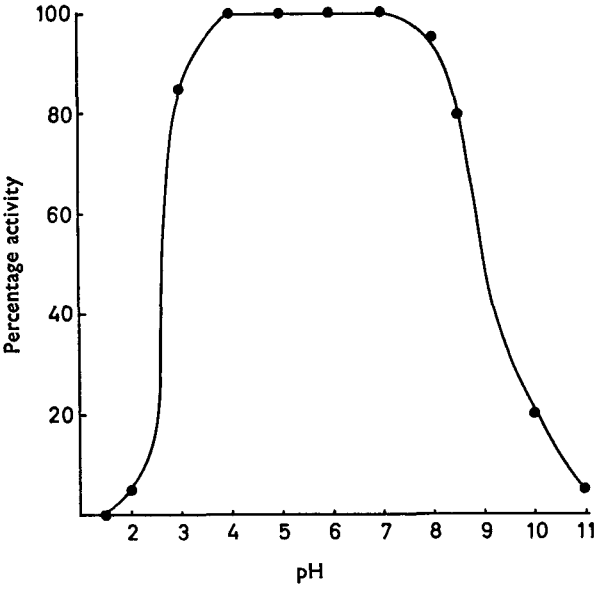

FIG. 4. Percentage smooth muscle stimulating activity left after standing umbilical cord vessels extracts in a boiling water-bath for 30 min at different pH values.

Electrophoresis. The active substance tended to move towards the anode; this tendency was increased at alkaline pH. Thus at pH 6·3 under a potential gradient of 12 V/cm the active substance had moved 0·5 cm towards the anode after 4 hr. At pH 8·5 under a similar potential gradient the activity was found 4·0 cm from the origin towards the anode after 4 hr. Endosmosis was in the direction of the cathode as shown by glucose and the above values were arrived at after correction in the migration values had been made for this effect.

Paper chromatography. Chromatograms of ether-purified extracts of umbilical blood vessels were developed in three solvent systems. The active substance was detected by biological activity as described under Methods. The results of these experiments are summarised in Table 2.

TABLE 2. Rf VALUES OF ACTIVE SUBSTANCE IN CORD VESSELS

Solvent system	Rf values	Recoveries
		%
Phenol-hydrochloric acid (saturated)	0·8 –0·92	80
Butanol-acetic acid-water (4:1:5)	0·85–0·95	70
Ethyl methyl ketone-diethylamine-water (60:3:20)	0·78–0·96	76

DISTINCTION OF THE ACTIVE MATERIAL FROM PHARMACOLOGICALLY ACTIVE SUBSTANCES OF NATURAL OCCURRENCE

The contractions of various smooth muscle preparations produced by umbilical blood vessels extract could be distinguished from those produced by acetylcholine, 5-HT and histamine.

Acetylcholine. The contractions of the guinea-pig proximal colon produced by the umbilical cord extracts were resistant to atropine. Concentrations of atropine (10^{-7}–10^{-6} g/ml), while abolishing the responses of the guinea-pig colon to acetylcholine, did not modify responses to the umbilical cord extracts (Fig. 5).

Histamine. In a similar manner the active substance could be distinguished from histamine. Diphenhydramine (10^{-7} g/ml) while abolishing the contractions of the guinea-pig proximal colon produced by histamine did not affect responses to the umbilical vessels extract (Fig. 5).

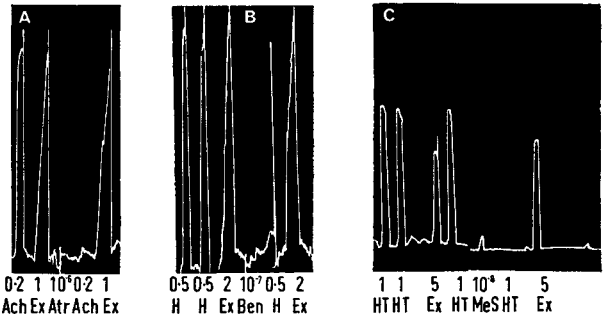

FIG. 5. Comparison of the effects of acetylcholine (Ach) and umbilical vessels extract (Ex) before and after (A) atropine (Atr) on guinea-pig proximal colon; contact time 90 sec, dose interval 10 min. (B) Histamine (H) and Ex on a similar preparation before and after diphenhydramine (Ben) and (C) 5-hydroxytryptamine (HT) and Ex before and after methysergide (MeS) on non-pregnant rat uterus; dose interval 5 min, contact time 2 min.

In all experiments the antagonists were injected 1 min before the doses of the stimulant drugs. All doses in μg.

Apart from this the active substance in the cord vessels could be distinguished from histamine for the following reasons. (1) Histamine is a base: the active substance in the cord vessel behaves as a weak acid. (2) Whereas the umbilical cord extracts caused the rat colon and uterus preparations to contract, histamine even in very large doses (100 μg) failed to contract these preparations.

5-Hydroxytryptamine. Antagonists of 5-HT do not effectively abolish the contractions of the guinea-pig ileum and colon produced by this amine. For this reason the isolated rat uterus preparation was used to demonstrate that the smooth muscle stimulating activity of umbilical cord vessels extract was not due to 5-HT.

Methysergide (10^{-8} g/ml) abolished the contractions of the rat uterus produced by 5-HT whereas contractions of this preparation produced by umbilical vessels extract were unaffected by even larger doses (10^{-8}–10^{-5} g/ml) of methysergide (Fig. 5).

Effect of incubation with chymotrypsin and trypsin. There was no loss of activity on incubating the extracts of umbilical blood vessels at pH 8·0 with trypsin, or with crystalline chymotrypsin for 60 min at 37°. Controls consisting of chymotrypsin and bradykinin or angiotensin and trypsin when similarly incubated were inactive after 60 min (Fig. 6).

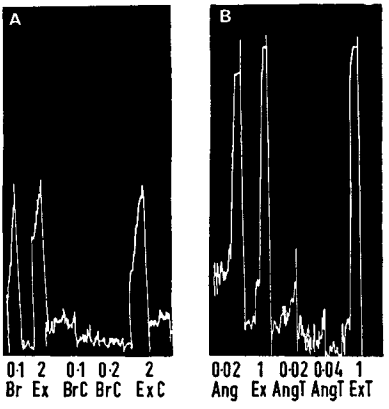

FIG. 6. Guinea-pig proximal colon. Effect of incubation with chymotrypsin and trypsin on the smooth muscle stimulating activity of umbilical cord vessels extract and of bradykinin and angiotensin. A. Br and Ex control responses to bradykinin and cord extracts. BrC and ExC responses after incubation of bradykinin and cord extract with chymotrypsin for 60 min. B. Ang and Ex control responses to angiotensin and cord extract. AngT and ExT responses after 60 min incubation with trypsin. Doses in μg.

This together with the negative ninhydrin and -NH- group test appears to show that the active substance is unlikely to be a polypeptide.

CHEMICAL NATURE OF THE ACTIVE SUBSTANCE IN THE UMBILICAL CORD VESSELS

The solubility of the active substance in the umbilical blood vessels in various organic solvents, its behaviour on partition with ether and its migration towards the anode at alkaline pH, all suggest that it is a lipid soluble acid. The following evidence based on chemical tests and colour reactions is produced to suggest that the active substance is an unsaturated hydroxy acid and that the integrity of hydroxyl and carboxyl groups and at least one double bond are essential for the smooth muscle contracting action.

Inactivation of the active substance by NN'-*carbo-di*-p-*tolyl imide.* The carboxyl binding reagent *NN'*-carbo-di-*p*-tolyl imide converts carboxylic acids into mono-acylated di-ureides (Zetsche, Lüsher & Meyer, 1938). This test for the presence of the carboxyl group was made on three different samples of ether-purified umbilical cord extracts. When the samples treated with reagents were assayed on the guinea-pig proximal colon it was found that the reagent had inactivated the treated samples to the extent of 80–90%.

Colour test for carboxylic acid. This test was made on several batches of ether-purified umbilical vessel extracts. With 100 μg of the active substance dissolved in benzene, in presence of the reagents uranyl acetate and Rhodamine B a pink colour appeared in the benzene layer.

Inactivation of the active material by phenyl isocyanate. (i) *Presence of OH group.* Phenyl isocyanate reacts with free hydroxyl groups to form phenyl urethane. Umbilical cord extracts treated with this reagent were inactivated after standing at room temperature for 1 hr.

(ii) *Evidence of unsaturation.* Inactivation by potassium permanganate. The active substance in the umbilical cord vessels was inactivated by treating ether purified extracts with neutral potassium permanganate. A control sample without permanganate retained its activity.

Inactivation by iodine bromide. Samples of extracts treated with iodine bromide showed a loss of almost 100% activity. Controls showed no loss in activity.

The results of these experiments suggest that the active substance is a lipid soluble unsaturated hydroxy acid. The active substance in the umbilical blood vessels, however, can be distinguished from various lipid soluble acids of natural occurrence.

SLOW REACTING SUBSTANCE A

The smooth muscle stimulant (SRS-A) released from the lungs of perfused sensitised guinea-pigs has been examined by Brocklehurst (1953, 1955). Like the active substance in the umbilical vessel extract it is soluble in water and behaves similarly on electrophoresis. However SRS-A can be distinguished from the umbilical cord extracts because unlike the cord extract it causes no fall in arterial blood pressure and has no stimulant effect on the isolated rat uterus preparation (Vogt, 1957a).

The unsaturated fatty acid SRS which is split off from lecithin by cobra venom and which stimulates smooth muscles (Vogt, 1957b) can also be excluded because its action on guinea-pig ileum decreases with successive applications and finally disappears; no such tachyphylaxis is seen with the umbilical vessels extracts.

Irin. Extracts of rabbit iris contains a smooth muscle stimulating substance to which the name irin has been given (Ambache, 1957). Ambache (1959) has shown that irin is a long chain unsaturated hydroxy fatty acid. The active substance in the umbilical cord extracts can be distinguished from irin because unlike the cord extracts irin does not stimulate the rat uterus preparation. Also irin is thermolabile and at pH 9 it is inactivated even at room temperature. The active substance in the umbilical cord is not inactivated by boiling for 30 min at pH 3–9.

Darmstoff. The lipid soluble darmstoff (Vogt, 1957a, b) consists of acetal or semi-acetal phosphatidic acid. It can be distinguished from umbilical blood vessels extracts because unlike the cord extracts it stains with fuchsin-sulphurous acid. It can also be distinguished from darmstoff by paper chromatography in ethyl methyl ketone-diethylamine-water (60:3:20). In this solvent system darmstoff has an Rf of 0·4–0·45 on Whatman No. 1 paper (Vogt, 1957b) whereas the active substance in the

umbilical vessels run in this solvent system has an Rf of 0·8–0·9. Unlike the umbilical cord extracts, Darmstoff is not vasodepressor in rabbits and cats (Vogt, 1958).

Prostaglandin. The lipid-soluble acidic material with smooth muscle stimulating and vasodepressor activity present in the seminal plasma and in accessory genital glands (prostaglandin) has been shown to include several closely related unsaturated fatty acids (Euler, 1936; Goldblatt, 1935; Green & Samuelsson, 1964). Like the active principle in the cord vessels the prostaglandins are lipid soluble acids and migrate towards the anode on paper electrophoresis. The prostaglandin and the active substance under study produce vasodepression in the cat, rat and rabbit (Fig. 3). However they differ because: (1) the active material in the human seminal plasma has an Rf value of 0·45 on Whatman No. 1 paper when run in ethyl methyl ketone-diethylamine-water (60:3:20). The Rf value of the active substance in the cord vessels under identical conditions is 0·8–0·9 (Table 2). (2) According to Euler (1936) the active material in the seminal plasma is stable to boiling at pH 1–7 for 20 min. Umbilical cord extracts have been shown to be inactivated by boiling at pH 1 and 2.

Discussion

The solubility of the active substance present in the umbilical vessels in various organic solvents, its extraction by partition with ether at acid pH and its migration towards the anode at alkaline pH, all suggest that it is a lipid soluble acid. The inactivation of the active material by chemical reagents which bind with specific functional groups has revealed the presence of hydroxy and carboxyl groups and at least one pair of double bonds in the molecule. The activity of the extract is dependent upon the integrity of these functional groups and double bonds.

The active substance in the cord vessels can be distinguished from various lipid soluble acids of natural occurrence which stimulate smooth muscles including irin, darmstoff, SRS-A and prostaglandins.

Widely diverse functions have been suggested for the various lipid soluble acids extracted from tissues. Prostaglandins could facilitate impregnation by motor effects on the male and female genital tract or accessory glands. Darmstoff is assumed to be involved in the physiological movements of the gut (Vogt, 1958). Irin possibly plays a role in the function of the iris (Ambache, 1957). Thus the functions attributed to the lipid soluble acids are those of local hormones. The physiological significance of the active material in the umbilical cord vessels is a matter for speculation but it provides a possible explanation of the contractions of the cord vessels at birth by acting as a local hormone. It is hoped to show whether this is so. Because of the absence of innervation in the vessels of the umbilical cord the question of the active substance acting as a neurohormone does not arise.

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