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THE ISOLATION AND DETERMINATION OF PROSTAGLANDINS IN LUNGS OF SHEEP, GUINEA PIG, MONKEY AND MAN*

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Abstract—Lipid extracts of lungs of sheep, guinea pig, monkey and man were analyzed for smooth muscle stimulating substances. Most of the spasmogenic activity was due to prostaglandins. After preliminary purification the prostaglandins were isolated and identified with silicic acid chromatography, reversed phase partition chromatography, layer chromatography and gas liquid chromatography. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was the main prostaglandin present and was identified in all lungs. Approximate concentrations of $PGF_{2\alpha}$/g wet wt. calculated by means of isotope dilution were; sheep lung 0·5 μg, guinea pig lung 0·5 μg, human lung (autopsy material) 0·02 μg, monkey lung (autopsy material) 0·2 μg. Prostaglandin E_2 was identified in sheep lung.

RECENT work on the occurrence of prostaglandins has shown that these substances are not confined to the vesicular gland and its secretions, but have a more widespread distribution in the body. Thus the presence of prostaglandins has so far been demonstrated in sheep, pig and cattle lung[9, 10], calf thymus,[11] brain,[12] sheep iris[13] and human menstrual fluid.[14] The prostaglandin isolated from sheep and pig lung was identified as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fig. 1). Its biological effects were studied.[15] It was found to stimulate a wide range of isolated smooth muscle organs in concentrations as low as 10^{-9} g/ml. Upon i.v. injection in cats of 15–30 μg/kg increased pulmonary vascular and bronchial resistances were noted. In view of the high smooth muscle stimulating potency and pharmacodynamic effects *in vivo* exhibited by this compound, it seemed of interest to make a quantitative determination of $PGF_{2\alpha}$ in lungs from various species, and to establish whether any other smooth muscle stimulating lipids could be found in this tissue.

The present work describes the isolation and determination of $PGF_{2\alpha}$ in lungs of sheep, guinea pig, monkey and man. In addition prostaglandin E_2 (PGE_2) (Fig. 1) was identified in sheep lung.

MATERIALS

Sheep. Sheep lungs obtained at death were either processed at once or kept frozen at −20° until the work up. Ten batches of about 100 kg were analyzed.

Guinea pig. The lungs from normal adult animals of both sexes were removed

* Prostaglandins and Related Factors 45.

† Preliminary reports of this work have appeared.[1, 2]

‡ The prostaglandins are a class of compounds with effects on smooth muscle and on lipid metabolism (For references see 3,4). Their structures have been determined[5–8] and are given in Fig. 1.

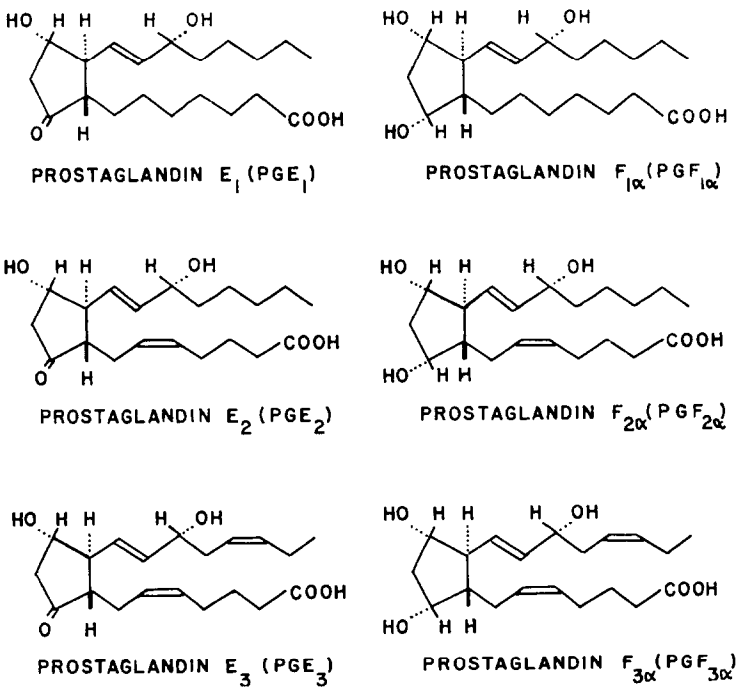

FIG. 1. The structures of PGE_1, PGE_1, PGE_3, PGF_{1a}, PGF_{2a} and PGF_{3a}.

immediately after death and stored at $-20°$. The pooled lungs (2 kg) from several hundred guinea pigs were worked up in one batch.

In preliminary experiments lungs were obtained 2–4 hr after death from guinea pigs used in tests for tuberculosis. Only animals showing a negative reaction were used. Fifteen kg of this material were processed in two batches.

Rhesus monkey. Lungs were collected from monkeys of both sexes 2–3 hr after death. One batch of 2 kg was processed.

Man. Human lungs (male) were obtained at autopsy 7–24 hr after death. The tissue showed no macroscopic signs of disease, except moderate oedema. One batch of 15 kg was processed.

EXPERIMENTAL AND RESULTS

Extraction and preliminary purification. An outline of the course of the purification and the methods used is given in Fig. 2. For guinea pig and monkey lung steps b–d were omitted. The lungs were ground in semi-frozen condition through a meatgrinder or homogenized in a Waring blendor and 4 volumes of 96% ethanol were added (a, Fig. 2). The suspension was left at room temperature overnight with mechanical stirring. After sedimentation the clear supernatant was siphoned off and the insoluble residue strained through a cheese cloth and filtered. The extract was evaporated under reduced pressure to about one twentieth of the original volume, acidified to pH 3 with 6 N hydrochloric acid and then twice extracted with half its volume of ethyl acetate (b). The aqueous phase was then discarded and the combined ethyl acetate phases were at once extracted several times with small portions of 0·2 M phosphate buffer at

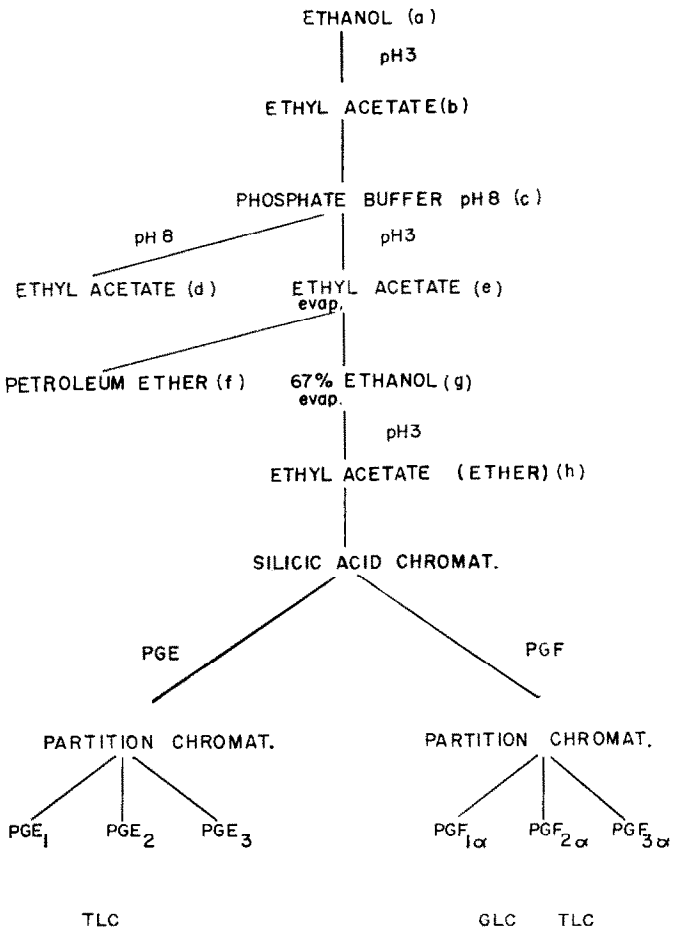

FIG. 2. Scheme showing purification of prostaglandins from lung tissue. For guinea pig and monkey lung steps b, c and d were omitted.

pH 8 (c) until less than 10 per cent of the biological activity was left in the ethyl acetate. During the first extraction, the pH of the buffer had to be adjusted back to pH 8 with 2 N sodium hydroxide. The combined buffer phases of pH 8 were then washed with a small quantity of ethyl acetate (d). This contained no smooth muscle stimulating activity and was discarded. After acidification to pH 3 with 6 N hydrochloric acid the buffer was extracted several times with one fifth of its volume of ethyl acetate (e) until all smooth muscle stimulating activity had passed into the organic phase. The combined ethyl acetate phases were then washed neutral with water and evaporated to dryness under reduced pressure. This material was then subjected to a three or four stage counter current distribution between equal volumes (500 ml/10 g extract) of petroleum ether and ethanol–water (2:1) (f,g). In runs with tritium labelled prostaglandin (see below) all of the radioactivity and most of the smooth muscle stimulating activity were found in the aqueous alcohol (g). Some biological activity (less than 10 per cent) was present in the petroleum ether (f). Since no labelled prostaglandin could be found in this phase the activity must have

been due to nonpolar acidic lipids. No attempt was made to identify these compounds. The combined alcoholic phases were evaporated at reduced pressure with a nitrogen leak at 40°, the pH adjusted to 3 and extracted three times with one volume of ethyl acetate or ether (h). The combined extracts were washed neutral with water and were evaporated to dryness leaving 1–5 g of a thick brownish green paste. This constituted the starting material for subsequent chromatography. In the tracer experiments the recovery of tritium labelled prostaglandin at this stage (h) was 20–50 per cent in sheep lungs and human lungs, and 40–80 per cent in lungs from guinea pigs and monkeys. The recovery of smooth muscle stimulating activity was of the same order of magnitude. The presence of impurities with unspecific effects on the test organ and the large volumes of solvent rendered the biological assay of the first extracts difficult.

Silicic acid chromatography. The adsorbent (Malinckrodt, 100 mesh) was activated at 115°. The amount of silicic acid employed in a single column varied between 1 and 500 g. The loading factor was about 1:50 for preliminary fractionations, later about 1:100. The volume of the fractions was kept at 2–5 ml/g of silicic acid. To improve the flow rates a moderate pressure (N_2) was applied on top of the columns. A discontinuous gradient elution with ethyl acetate in benzene was used, the ethyl acetate concentration being increased from 30% to 100%. This system separates the PGE-compounds from the PGF-compounds.[16] With small columns (1–30 g) the dihydroxy-monoketoprostaglandins (PGE) were eluted with 60% ethyl acetate and the trihydroxy prostaglandins (PGF) with 80% ethyl acetate in benzene. With larger columns this elution pattern had to be made somewhat more polar to effect the same separation. The fractionation of a lipid extract from guinea pig lung on a silicic acid column is shown in Fig. 3. A band of smooth muscle stimulating activity was eluted with ethyl acetate-benzene 70:30 whereas the major part, coinciding with the tracer $PGF_{2\alpha}$, was eluted with pure ethyl acetate. This elution pattern corresponds to that found in model experiments for PGE- and PGF-compounds, which appear in ethyl acetate-benzene 70:30 and ethyl acetate respectively on this size of column. It is also evident that some smooth muscle stimulating activity due to more polar compounds was eluted with the methanol. This material was not further characterized.

Reversed phase chromatography. After group separation of the PGE-and PGF-compounds had been achieved, the prostaglandins in each group were separated by reversed phase partition chromatography as described earlier.[17] Methanol–water [38–47·5% (v/v)] was equilibrated with one tenth of its volume of isooctanol-chloroform [50% (v/v)] The stationary phase. (4 ml/4·5 g support) was supported on hydrophobic Super-Cel. The columns were loaded with up to 50 mg of lipid material/4·5 g support. A methanol concentration of 38–43% (v/v) in the moving phase separated the individual PGF-compounds. For the separation of PGE compounds the concentration of methanol in the moving phase was increased to 45–47·5 per cent.

A chromatogram of the PGF-fraction from a silicic acid column in a run of guinea pig lung is shown in Fig. 4. The main peak of the smooth muscle stimulating activity coincided with that of the labelled $PGF_{2\alpha}$. However, small amounts of spasmogenic activity were observed both before and after the main peak.

Thin layer chromatography. The isolated samples were chromatographed with reference prostaglandins on $AgNO_3$-impregnated silica gel as previously described.[18] The adsorbent was activated at 115° for 30 min. The spots were rendered visible by

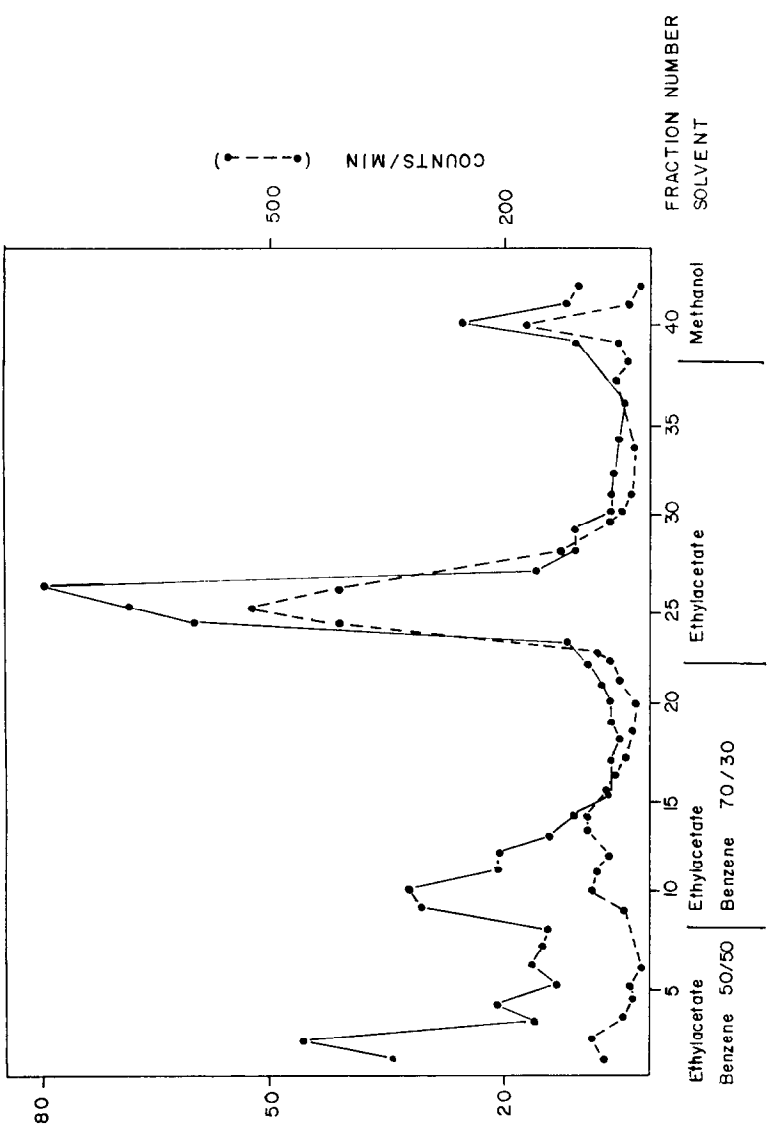

FIG. 3. Silicic acid chromatography of smooth muscle stimulating material from an extract (1·93 g) of guinea pig lung. The radioactivity is due to tritium labelled $PGF_{2\alpha}$ added at the beginning of the extraction. Column 100 g, fraction volume 250 ml.

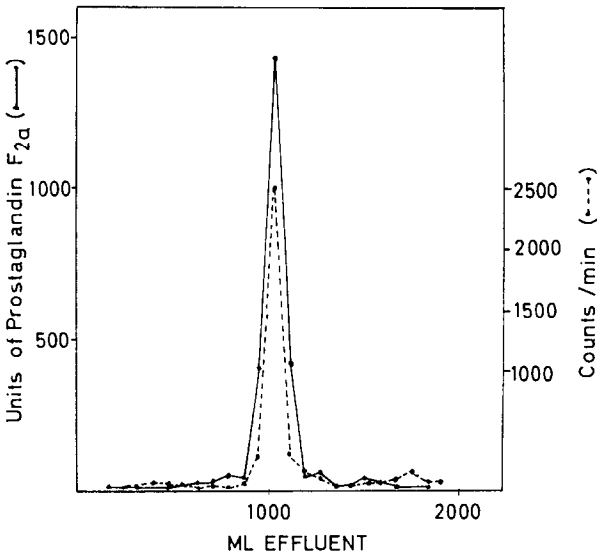

FIG. 4. Reversed phase partition chromatography of the PGF-fraction of an extract of sheep lung. Column: 45 g of hydrophobic Hyflo Super-Cel. Moving phase: 43% aqueous methanol. Stationary phase: 40 ml. of chloroform-isooctanol (1:1). The radioactivity is due to tritium labelled $PGF_{2\alpha}$. The smooth muscle stimulating activity was assayed on the isolated rabbit duodenum. One unit is equivalent to one μg of $PGF_{2\alpha}$.

spraying with 10% alcoholic phosphomolybdic acid and heating to 90° for 15 min. The solvent systems used are given in Table 1.

In preparative runs up to 10 mg of lipid material was applied along the start line with reference prostaglandins on either side. After development the plates were sprayed with water. The opaque zones that appeared were scraped off and transferred to test tubes. Active material could then be recovered by elution with suitable solvents.[18]

Gas liquid chromatography. A Pye Argon Chromatograph modified for use with a 2 m × 5 mm U-tube column was used. The instrument was equipped with separately heated flash heater and argon ionization detector. The conditions were the same as those described by Bygdeman and Samuelsson[19] (0·5% Hi Eff-8B, 184°). The

TABLE 1. SOLVENT SYSTEMS FOR THIN LAYER CHROMATOGRAPHY

System	Composition (ml.)	Adsorbent
A I	Benzene-dioxane-acetic acid (40:40:2)	Silica gel G*
A II	Ethyl acetate-methanol-acetic acid-2,2,4-trimethylpentane-water (110-25-30:10:100)†	Silica gel G*-$AgNO_3$ (40:1)
A VII	Ethyl acetate-methanol-acetic acid-2,2,4-trimethylpentane-water (110:20:30:10:100)†	Silica gel G*-$AgNO_3$ (40:1)
M IV	Ethyl acetate-methanol-water (160:10:100)†	Silica gel G*-$AgNO_3$ (40:1)

* Merck, A. G., West Germany.
† The solvent mixtures were equilibrated for 1 hr and the upper phase used.

samples were esterified with diazomethane and converted to the trimethylsilyl ether derivatives.[19]

Determination of tritium labelled prostaglandins. An isotope dilution method was used to determine the amount of prostaglandins originally present in the lungs. Tritium labelled PGE_1 (50 mc/μmole)[20] and $PGF_{2\alpha}$ (20 mc/μmole) were kindly made available by Dr. B. Samuelsson. A known small amount of the labelled compound was added during the first extraction (a in Fig. 2) and the radioactivity was then determined throughout the isolation procedure by liquid scintillation counting (Packard Tri-Carb Model 314). Corrections for quenching were made by the addition of an internal tritium standard to each sample. By determining the corrected radioactivity and the smooth muscle stimulating activity recovered in the final product, the amount of the corresponding prostaglandin originally present in the lungs was calculated.

Ultraviolet spectrometry. PGE-compounds develop an absorption band with λ_{max} at 278 mμ after treatment with weak alkali at room temperature.[5] To scan chromatographies for PGE activity, aliquots of fractions were treated with alcoholic 0·5 N NaOH and the absorbance at 278 mμ determined in a spectrophotometer (Zeiss PMQ II).

Biological assay. The smooth muscle stimulating activity was tested on isolated rabbit duodenum and on rabbit blood pressure against standards of pure prostaglandins. One unit of biological activity is equivalent to one μg of the prostaglandin standard used. For the accurate determination of $PGF_{2\alpha}$ a four point assay[21] was carried out, based on the linear log dose response relationship demonstrated earlier for $PGF_{2\alpha}$ on the rabbit duodenum.[15]

Identification of prostaglandin E_2 in sheep lung. Only comparatively small amounts of smooth muscle stimulating activity were seen where PGE-compounds were expected to appear. To facilitate the isolation of these substances, tritium labelled PGE_1 was added to one run of 94 kg of sheep lung.

After preliminary purification (h in Fig. 2) the PGE-compounds were separated from the PGF-compounds on a 500 g silicic acid column. A peak of biological activity coincided with that of the added radioactive PGE_1. This material (140 mg) was run on an 18 g Super-Cel column with 45% (v/v) aqueous methanol as the moving phase. A peak of spasmogenic activity and absorption at 278 mμ appeared before the radioactivity due to the tracer PGE_1. This suggested the presence of PGE_2. Further chromatography of this material (23 mg) on silicic acid confirmed that the compound was of the PGE-type and also removed residues of PGF-compounds. A final reversed phase chromatogram gave a single peak of biological activity and ultraviolet absorption at the expected position of PGE_2. This fraction contained smooth muscle stimulating activity corresponding to 170 μg of PGE_2 when assayed on the rabbit duodenum. Furthermore, this material was found to depress rabbit blood pressure in the same dose as PGE_2 (0·5 rabbit intestinal units). The isolated sample and pure PGE_2 both gave ultraviolet absorption with λ_{max}^{EtOH} at 278 mμ after treatment with alkali. The intensity of their absorption at 278 mμ was practically the same when based on their smooth muscle stimulating activities. Thin layer chromatography clearly established that the isolated material contained PGE_2. In solvent systems AI, AII and AVIII the main component was indistinguishable from PGE_2. After esterification with diazomethane, the methyl ester, when run in solvent system M IV gave a spot at the position

of the methyl ester of PGE_2. In a preparative thin layer chromatography of the methyl ester, 80 per cent of the recovered smooth muscle stimulating activity could be eluted from the zone corresponding to the methyl ester of PGE_2. Taken together the results established that the isolated material was identical to PGE_2. Judging from the values of the recovery of the labelled PGE_1 and the smooth muscle stimulating activity of the isolated PGE_2 fraction, the concentration of PGE_2 in the lung would be about 0·04μg/g (wet wt.). However, since PGE_2 would be more susceptible to destruction during the isolation than PGE_1, the figure might be somewhat higher.

Isolation and determination of $PGF_{2\alpha}$. $PGF_{2\alpha}$ was found to be the major lipid with smooth muscle stimulating activity as judged by the appearance of spasmogenic activity in the silicic acid and partition chromatographies. The identification of $PGF_{2\alpha}$ was based on several chromatographic procedures. In silicic acid and reversed phase chromatography the main peak of spasmogenic activity always coincided with the radioactivity from the labelled $PGF_{2\alpha}$ (Fig. 2 and 4) When the isolated samples were subjected to preparative thin layer chromatography on $AgNO_3$-impregnated silica gel (solvent system AII), 80–100 per cent of the recovered smooth muscle stimulating activity and radioactivity was found in the area corresponding to $PGF_{2\alpha}$ Final identification was achieved by means of gas liquid chromatography after esterification with diazomethane and conversion into the trimethylsilynethers.

The recovery of radioactivity and the smooth muscle stimulating activity of the isolated $PGF_{2\alpha}$ fraction were used to calculate the amount of $PGF_{2\alpha}$ originally present in the lungs from sheep, guinea pig, monkey and man. The results are given in Table 2.

TABLE 2. APPROXIMATE CONCENTRATIONS OF $PGF_{2\alpha}$ IN LUNGS FROM DIFFERENT SPECIES

Origin	Concentration of $PGF_{2\alpha}$ (μg/g)	Remarks
Sheep lung	0·5	Fresh
Guinea pig lung	0·5	Fresh
Monkey* lung	0·2	Obtained 2–3 hr after death
Human lung	0·02	Obtained 7–24 hr after death

* Macaco Rhesus

Evidence for the presence of $PGF_{1\alpha}$ and $PGF_{3\alpha}$. In some runs of sheep lung, small amounts of smooth muscle stimulating activity could be detected both before and after the peak of $PGF_{2\alpha}$ in reversed phase partition chromatographies. When these fractions were analyzed by thin layer chromatography on $AgNO_3$-impregnated silica gel (solvent system AIII), spots corresponding to $PGF_{3\alpha}$ and $PGF_{1\alpha}$ respectively, appeared. After preparative thin layer chromatography in the same solvent system it was found that significant amounts of smooth muscle stimulating activity could be eluted from the areas corresponding to $PGF_{3\alpha}$ and $PGF_{1\alpha}$. These results suggest that these prostaglandins are present in small amounts in normal sheep lung.

DISCUSSION

Most of the smooth muscle stimulating activity in a lipid extract of lungs from sheep and man was recovered in the acidic fraction. In all species examined prostaglandins accounted for the major part of the smooth muscle stimulating activity. Some spasmogenic activity was also found in both more and less polar fractions. This might be due to acidic phosphatides and autooxidized unsaturated lipids, since such substances are known to contract smooth muscle.[22, 23]

$PGF_{2\alpha}$ was found to be the dominating prostaglandin in lungs from sheep, guinea pig, monkey and man. The concentration of $PGF_{2\alpha}$ was determined to be about 0·5 μg/g tissue in the lungs analyzed immediately after the death of the animal (sheep and guinea pig). In lungs obtained at autopsy (monkey and man) these values were considerably lower. It is likely that some degradation had occurred in these tissues before they were analyzed.

The concentration of $PGF_{2\alpha}$ in the lung is of about the same order of magnitude as in bovine brain (0·3 μg/g)[12] and slightly lower than in human seminal fluid (2·2 μg/ml.).[19] For comparison it may be mentioned that of substances with equal pharmacodynamic potency, noradrenaline occurs in sheep lung at a concentration of 0·1 μg/g[24] and acetylcholine in guinea pig lung at a concentration of about 0·4 μg/g (wet wt).[25]

$PGF_{3\alpha}$ has previously been identified in cattle lung.[10] The results described in the present paper indicate that both $PGF_{1\alpha}$ and $PGF_{3\alpha}$ are present in sheep lung. However, in comparison with $PGF_{2\alpha}$, the amounts of these PGF-compounds were insignificant.

It was also possible to isolate PGE_2 from sheep lung. This compound was identified by its behaviour in various chromatographic systems, by the formation of a chromophore with λ_{max} at 278 mμ and by differential pharmacological assay. The amount of PGE_2 was small in comparison to that of $PGF_{2\alpha}$. No PGE_1 or PGE_3 could be detected by the methods used.

In lung the PGF-compounds are quantitatively more important than the PGE-compounds. In other tissues, e.g. human seminal plasma and calf thymus, the PGE-compounds dominate.[11, 16] These quantitative differences can be explained by recent findings on the biosynthesis and metabolism of prostaglandins.

In homogenates of sheep vesicular glands, certain essential fatty acids act as precursors in the biosynthesis of PGE-compounds. Thus PGE_1 is formed from homo-γ-linolenic acid[28, 29] (8, 11, 14-eicosa-trienoic acid), PGE_2 from arachidonic acid[26, 27] (5, 8, 11, 14-eicosa-tetraenoic acid) and PGE_3 from 5, 8, 11, 14, 17-eicosa pentaenoic acid.[29] In homogenates of guinea pig lung arachidonic acid is converted to $PGF_{2\alpha}$, PGE_2 and metabolites of PGE_2.[30]

However, it has been shown that each of PGE_1, PGE_2 and PGE_3 are converted into two metabolites by enzymes present in homogenates of guinea pig lung.[31–33] The transformations involve reduction of the Δ^{13}-double bond and oxidation of the secondary alcohol at C-15. These reactions have been demonstrated *in vivo*[20] and seem to be of importance in the metabolism of PGE-compounds. $PGF_{2\alpha}$ is not transformed to the same extent by a homogenate of guinea pig lung.[30]

The enzymes concerned with the biosynthesis and metabolism of prostaglandins in the lung should thus favour the accumulation of PGF- rather than PGE-compounds in this tissue. This conclusion is in agreement with the results reported in the present paper.

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