ISOLATION AND IDENTIFICATION OF TWO SMOOTH MUSCLE STIMULANTS FROM MENSTRUAL FLUID

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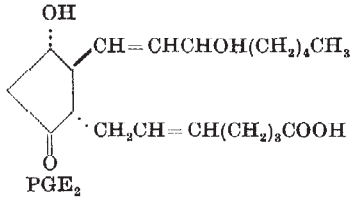
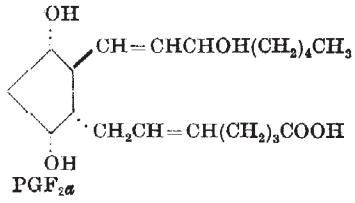
THE presence of a group of lipid smooth-muscle stimulants in menstrual fluid was first reported in *Nature* in 1957 (ref. 1). Since then it has been shown that these substances, which are almost certainly produced by the endometrium in its secretory phase[2], are in part carried to the myometrium, and there stimulate the rhythmical contractions of normal menstruation. An excess of such an effect is probably an important factor in causing the intense uterine cramps of primary dysmenorrhœa[3].

Two physiologically active constituents of high potency have now been isolated from menstrual extracts and, by methods described here, have been identified as prostaglandins PGE_2 and $PGF_{2\alpha}$.



(11*α*,15-dihydroxy-9-keto-prosta-5,13-dienoic acid)



(9*α*,11*α*,15-trihydroxy-prosta-5,13-dienoic acid)

These substances are members of an important class of smooth-muscle stimulants originally detected by von Euler[4], who introduced the name 'prostaglandin'; the recent outstanding investigations of Bergström and his colleagues have resulted in the separation and structural identification of five naturally occurring individuals of this closely related group of compounds[5,9,10,13,14].

The prostaglandins PGE_2 and $PGF_{2\alpha}$ are certainly not the only active constituents present in menstrual fluid, but they do account for the major part of the activity. Although these two components are only present in minute amounts their high activity, detectable at a concentration of 10^{-10} g/ml., permitted the physiological effect to be discerned at an early stage[6].

Preliminary fractionation results gave some indication that the active materials were of the long-chain hydroxy-acid type[7]. This result, together with the nature of the activity shown, gave the first inkling that the substances involved might be members of the prostaglandin series. Accordingly, the separation techniques used so elegantly by Bergström's school in the isolation of the prostaglandins from more abundant sources were applied to the menstrual fluid derived from 3,675 specimens, collected and treated as described previously[7]. Successive extractions with acetone and dichloromethane produced a crude menstrual lipid concentrate (about 75 g).

The treatment of this lipid concentrate is briefly outlined in Fig. 1. Assay of the active constituents was

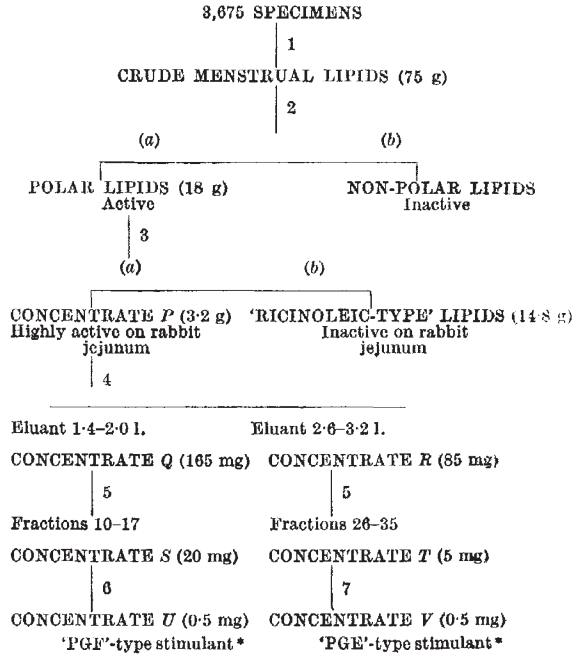


Fig. 1. Fractionation scheme for the isolation of the active constituents from the menstrual specimens

(1) Acetone and dichloromethane extraction (ref. 7). (2) Elution from hydrophobic 'Embacel' with (*a*) aqueous acetone, (*b*) acetone; ether. (3) Reversed-phase partition chromatography on column of hydrophobic 'Embacel' impregnated with paraffin oil (ref. 8). (*a*) 30 per cent aqueous acetone, (*b*) 50–100 per cent aqueous acetone. (4) Reversed-phase partition chromatography using iso-octanol/chloroform//methanol/water, 1 : 1 : 9 : 10 v/v (see Fig. 2). (5) Reversed-phase partition chromatography using iso-amyl acetate/chloroform/methanol/water, 4 : 6 : 35 : 65. (6) Reversed-phase chromatography using the system described in 4 except that a phosphate buffer of *p*H 7·2 replaced the water, followed by re-chromatography using system 4. (7) Extraction from chloroform solution with a phosphate buffer *p*H 7·2 followed by acidification and re-extraction into chloroform

* Biossay of the concentrates obtained at each stage on the guinea pig uterus and the rabbit jejunum indicated that the recovery of prostaglandin-like activity in the final concentrates was at least 90 per cent of that originally present.

carried out *in vitro* on preparations of guinea pig uterus, human myometrium and rabbit jejunum. Extraction of the polar lipids was followed by reversed-phase chromatography[8] to remove the compounds of 'ricinoleic acid type' polarity. This produced a concentrate, *P* (3·2 g), containing all the components which were physiologically active on the rabbit jejunum. Fractionation of *P* using Bergström's first system[9] (Fig. 2) afforded two active fractions *Q* (165 mg) and *R* (85 mg) of contrasting physiological behaviour. Fraction *Q*, a potent stimulant of the human myometrium (Fig. 3), was highly active on the rabbit jejunum, but relatively inactive on the guinea pig uterus. Fraction *R* either completely inhibited or feebly stimulated the contractions of the human myometrium (Fig. 3); it was extremely active on the guinea pig uterus but only moderately stimulating to the rabbit jejunum.

Separate fractionation of *Q* and *R* then followed using Bergström's second system[10], which is effective in discrimi-

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nating between the PGE and PGF types. The main active constituent of *Q* was concentrated by this means into fractions 10–17 (concentrate *S*; 20 mg) of a 60-component fractionation (Fig. 4) and chromatographically resembled a PGF. Successive further fractionation with change in *p*H finally produced an acidic concentrate *U* (~0·5 mg) showing intense PGF-type activity corresponding to an equivalent content of 2 mg of $PGF_{1\alpha}$. A similar process applied to concentrate *R* led to concentrate *V* (~0·5 mg) with pronounced PGE-type activity, corresponding to an equivalent content of 0·2 mg of PGE_1.

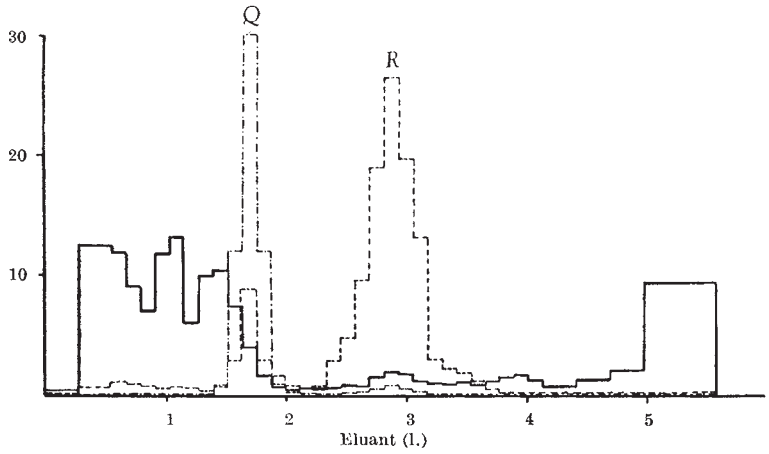

Fig. 2. Reversed-phase partition chromatography of concentrate *P*. Stationary phase: 70 ml. iso-octanol/chloroform (1 : 1) on 100 g of hydrophobic 'Embacel'. Moving phase: 47·5 per cent aqueous methanol. —— Weight distribution; abscissa units 1 mg/ml.; ·—·—· bioassay on rabbit jejunum; abscissa units 6 μg $PGF_{1\alpha}$ equiv./ml.; – – – – bioassay on guinea pig uterus; abscissa units 15 ng PGE_1 equiv./ml.

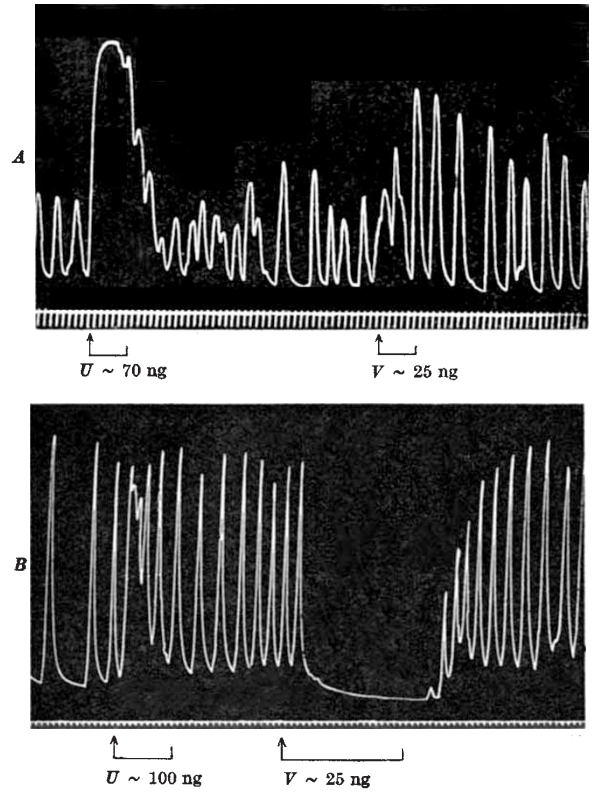

Fig. 3. Effects of menstrual stimulants on human myometrial preparation *in vitro* (bath-volume 4 ml.; time marker, 1 min). *A*, preparation showing good positive responses; *B*, preparation showing well-marked inhibitory responses to concentrate *V*

The next step involved the precise identification of the actual active principles in concentrates *U* and *V*. So far the tentative correlation with the two prostaglandin types had rested on the close parallel between the chromatographic behaviour and the type of biological activity. A routine chemical identification by preparation of derivatives was obviously impossible in view of the minute amounts of the concentrates *U* and *V* available. Accordingly, thin-layer chromatography comparisons were made between the concentrates *U* and *V* and authentic samples of PGE_1, $PGF_{1\alpha}$ and $PGF_{1\beta}$ kindly supplied by Prof. Bergström.

Concentrate *U* showed a single major spot by thin-layer chromatography which was readily detectable by the characteristic red-brown fluorescence in ultra-violet light which developed after its exposure to acid ceric sulphate, followed by moderate heat; this spot contained the physiologically active substance. Both $PGF_{1\alpha}$ and $PGF_{1\beta}$ gave this colour reaction and the former gave a thin-layer chromatography spot close to that given by this menstrual stimulant of concentrate *U* (Fig. 5*a*).

Treatment of concentrate *U* with diazomethane gave a relatively inactive product which most significantly showed the characteristic spot (thin-layer chromatography) at a slightly greater R_F value than that of authentic $PGF_{1\alpha}$ methyl ester (Fig. 5*b*). Location of this spot by an iodine spray[11] facilitated extraction of the active substance without damage. This product was then exhaustively methylated with diazomethane–boron trifluoride and examined by thin-layer chromatography. The major discrete spot behaved chromatographically in a manner exactly analogous to that shown by $PGF_{1\alpha}$ trimethyl ether methyl ester using the hexane–ether system.

At this stage, both the sample and comparison spots of the completely methylated products were extracted, after location by the iodine technique, and the minute amounts of product (~50 μg) examined separately by gas liquid chromatography. As may be seen (Fig. 6), the product derived from concentrate *U* and that derived from $PGF_{1\alpha}$ behaved very similarly, although not identically. By comparison of the relative retention volumes with those for standard fatty acids on this non-polar column the observed difference strongly suggested that the menstrual stimulant present in concentrate *U* was in fact $PGF_{2\alpha}$ which contains one more double bond than $PGF_{1\alpha}$ in the carboxylated side-chain.

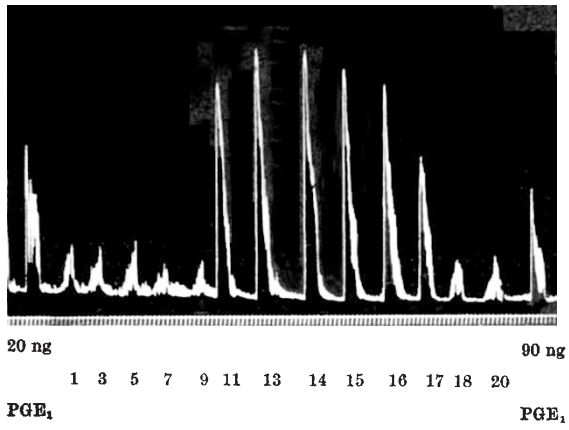

Fig. 4. Rabbit jejunum responses (bath-volume 4 ml.; time marker 1 min) to 1/1,000th aliquot of fractions 1–20 of reversed-phase chromatogram of concentrate *Q* showing PGF-type in fractions 11–17 (concentrate *S*)

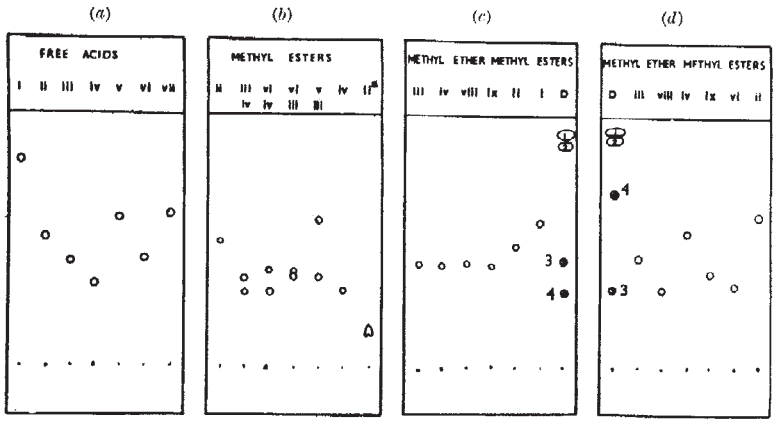

Fig. 5. Characteristic thin layer chromatograms on 0·2 mm layers of 'Kieselgel G' of prostaglandins and allied compounds. *a*, Chloroform/methanol/water, 60 : 10 : 0·5 v/v. Layers made up with 0·2 N oxalic acid pre-washed with developing solvent and developed in saturated atmosphere. *b*, As in (*a*) except that layers were made up normally and not pre-washed. Note tailing of phloionolic acid (ii*); *c*, Hexane/ether, 1 : 1; saturated atmosphere development. *d*, AnalaR chloroform in non-saturated atmosphere. Substances: (i) 15-hydroxypentadecanoic acid; (ii) 9,10,18-trihydroxyoctadecanoic (phloionolic acid); (iii) $PGF_{1\alpha}$; (iv) $PGF_{1\beta}$; (v) PGE_1; (vi) concentrate *U*; (vii) concentrate *V*; (viii) least polar borohydride reduction product from concentrate *V*; (ix) most polar borohydride reduction product from concentrate *V*. *D*, Dye marker mixture of (1) azobenzene, (2) sudan red, (3) *p*-hydroxyazobenzene, (4) *p*-aminoazobenzene

This was substantiated by the thin-layer chromatography behaviour of the trimethyl ether methyl ester derivatives of $PGF_{1\alpha}$ and of the menstrual stimulant using silver nitrate complex formation on the plates[12]. The relative change in R_F value on formation of the complex indicated that the menstrual stimulant contained an additional double bond as compared with $PGF_{1\alpha}$ and that this double bond was probably of the *cis* configuration.

Final confirmation of this identity with $PGF_{2\alpha}$ was obtained by mass spectrometric comparison of the trimethyl ether methyl ester derivatives of $PGF_{1\alpha}$ and of the menstrual stimulant.

As only small quantities of each compound were available ($\sim$ 10 μg), a special technique was utilized by which the compounds were introduced directly into the ion source. The spectra were obtained by an A.E.I. *M.S.*9 double-focusing mass spectrometer and were run and analysed by Dr. M. Barber and Dr. M. Elliott of A.E.I., Ltd., Manchester, and Dr. R. I. Reed of this Department, who report as follows.

"The spectrum of $PGF_{1\alpha}$ trimethyl ether methyl ester has already been reported[13] and our spectrum agreed closely (Fig. 7*B*). One interesting difference observed by us was the parent molecular ion M/e = 412 which was not previously reported.

"The spectrum of the concentrate *U* derivative was very similar (Fig. 7*A*). Up to M/e = 179 the two spectra are the same whereas above M/e = 249 the corresponding ions have a mass two units smaller in the menstrual derivative. The two compounds are therefore of similar structure, but the concentrate *U* derivative contains one more double bond. The cracking pattern is entirely consistent with the assignment of the structure $PGF_{2\alpha}$ trimethyl ether methyl ester to this concentrate *U* derivative."

Concentrate *V* was similarly examined by thin layer chromatography (Fig. 5*a*) and showed, after destructive spraying and heating, a pale-blue spot under ultra-violet light (a characteristic of the PGE family) at a position closely similar to that simultaneously obtained for an authentic sample of PGE_1; the activity of this spot was also shown to be of the PGE type.

Sodium borohydride reduction of an aliquot of *V* (equivalent to 6 μg PGE_1) and examination of the products by thin-layer chromatography revealed that the pale blue spot moving in the PGE region had been converted into two spots closely comparable in R_F value and colour to $PGF_{1\alpha}$ and $PGF_{1\beta}$. This chromatographic change was paralleled by the corresponding change in the type of physiological activity which was now only present in the PGF region of the plate.

The remainder of the concentrate *V* was similarly reduced and the PGF-type products purified using Bergström's second system. After conversion to the trimethyl ether methyl ester derivatives the two isomers were resolved by thin-layer chromatography on 'Kieselgel *G*' plates by means of chloroform (Fig. 5). In this system the $PGF_{2\beta}$ derivative migrates faster than the corresponding $PGF_{2\alpha}$ compound and, although the conditions are difficult to control, rather remarkable separations are possible. Final purification of each trimethyl ether methyl ester using the more consistent, but less selective, hexane ether system was followed by gas liquid chromatograph analysis of the products. One of the isomers was found to be identical with that obtained from concentrate *U* (that is, $GPF_{2\alpha}$) and the other possessed an R_F value in accordance with that predicted for $PGF_{2\beta}$. Thus the identity of the menstrual stimulant in concentrate *V* was confirmed as PGE_2.

The known occurrence of prostaglandins in human semen[9] made it highly desirable to exclude the possibility that this might be the source of the prostaglandins in our menstrual fluid samples. Accordingly, a number of specimens were examined which were known to be free from this possible adulteration, and which had been collected in menstrual cups in order to avoid any contamination from the usual absorbent materials. They showed physiological, chromatographic and chemical behaviour identical with those exhibited by the main collection.

The identification procedures described in this article were carried out on amounts of material ranging from

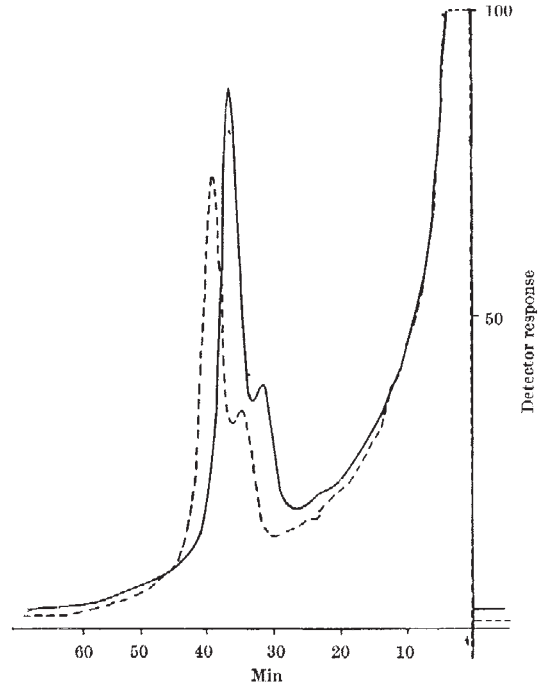

Fig. 6. Gas-liquid chromatograms of the trimethyl ether methyl esters derived from (*a*) $PGF_{1\alpha}$ isolated from menstrual fluid (——) and (*b*) $PGF_1\alpha$ (– – – –). Column, 6 ft. × ¼ in. of 10 per cent 'Apiezon *L*' on siliconized 'Embacel' 80–100 mesh; column temperature 233° C; preheater 240° C; inlet pressure 15 lb./in.^{2}; gas flow 30 ml. of argon per min; detector voltage 1,750; attenuation × 1; load 0·5 μl., of 0·2 per cent solution in chloroform. Retention time for hexacosane 66·5 min and eicosane 8·4 min

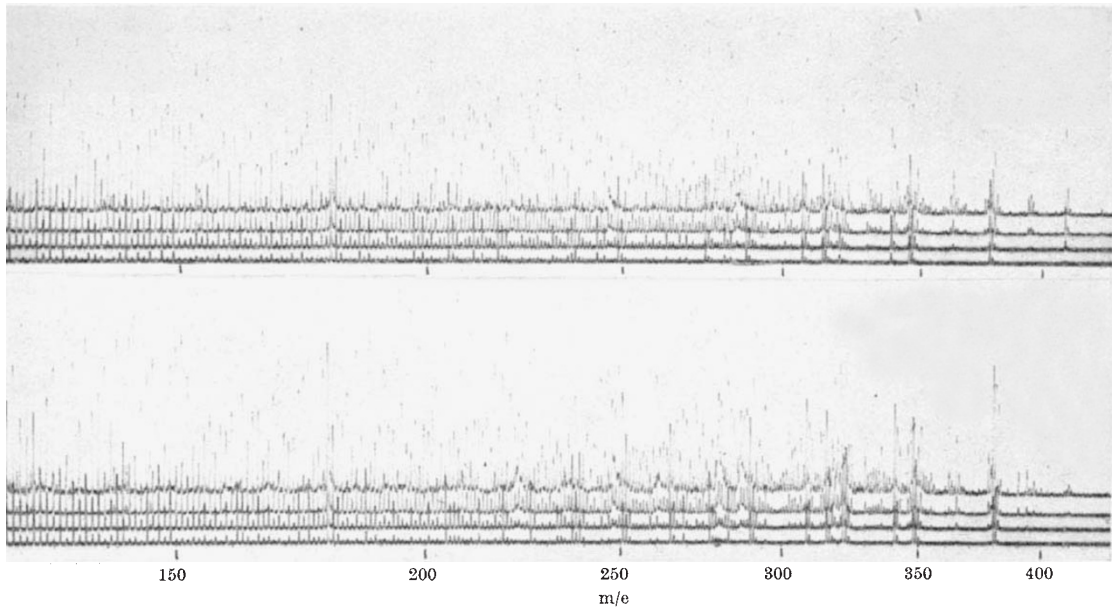

Fig. 7. Mass spectra of the trimethyl ether methyl esters of *A*, $PGF_{2\alpha}$ from concentrate *U*; *B*, $PGF_{1\alpha}$

1 to 50 μg and demonstrate convincingly the power of modern separation and spectroscopic techniques when allied to physiological potency of such a high order.

The isolation of these two prostaglandins from human menstrual fluid adds this source to the several already described[9] for this class of compound. From our present results we cannot estimate their concentration in the secretory endometrium; but the yield of $PGF_{2\alpha}$ from menstrual fluid is comparable with that obtained by Bergström *et al.* from pig lung[14]. The quantity of PGE_2 is, however, much less than that of the PGE substances in human semen[9]. The 'menstrual stimulant' prostaglandins in the female may be thought of as homologous to the seminal prostaglandins in the male; however, the wide distribution of smaller amounts of these highly potent substances suggests that they may have some general type of action. For example, Vogt[15] has suggested that the smooth-muscle contracting long-chain hydroxy-acids may interact with calcium ions, and Clegg, Hopkinson and Pickles[16] have independently suggested a somewhat similar interaction with magnesium ions.

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ANOMALY OF ALDOLASE IN PRIMARY LIVER CANCER

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RESEARCH in enzymology has been more extensive in experimental cancer than in human cancer, quantitative investigations not always being valid in post-mortem cases and biopsies not always being possible.

The discovery of multiple molecular forms of the enzymes named by Markert and Moller "isozymes"[1] enabled us to re-orient this research on a new basis. In the animal, Angeletti and Moore[2,3] have shown three peaks for glucose-6-phosphate dehydrogenase of mouse rhabdomyosarcoma, by chromatography on substituted celluloses, instead of one peak in the normal muscle; they have obtained analogous results on the acid phosphatase of the rat hepatoma, in which the chromatographic pattern is different from that of normal liver.

In man, Starkweather and Schoch[4], removing cancers of diverse origins a few hours after death, found that the distribution of the five isozymes of lactic-dehydrogenase, very varied in normal tissue, tended to become uniform and to predominate in one peak in cancer tissue.

The mode of action on the two substrates, fructose-1,6-diphosphate and fructose-1-phosphate, of aldolase from different tissues, and more particularly of hepatic aldolase, has given us a new approach for the enzymological examination of hepatomas.

One of us, with Payet[5], had, in 1960, pointed out that hyperaldolasæmia in the course of a primary liver cancer was not of the hepatic type, in the sense that the ratio of the two aldolase activities, fructose-1,6-diphospho-aldolase and fructose-1-phosphoaldolase $\left(\frac{\text{F-1,6-P}}{\text{F-1-P}}\right)$ was in the range of 3, instead of 1 or nearly 1 as in the hepatic type; this ratio of 1 being that found in the normal liver. We had at that time suggested the following interpretation: hyperaldolasæmia comes from the