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Methods in Prostaglandin Research

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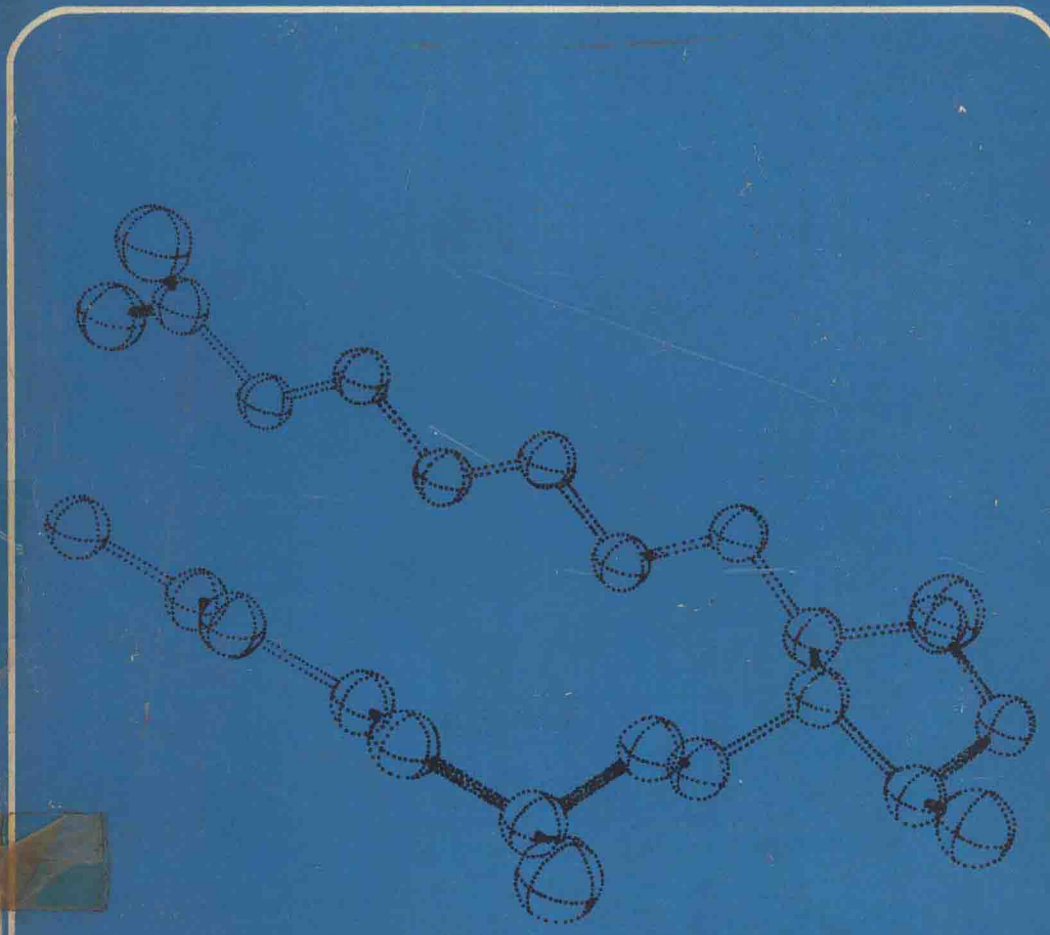
J.C. Frölich



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Methods in Prostaglandin Research

Advances in Prostaglandin and Thromboxane Research Volume 5

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Preface

This volume was written to meet the widespread need for a single source of the most commonly used methods utilized in the field of prostaglandin research. Methods are described in the detail necessary for accurate reproduction in any laboratory. The methods emphasized are those believed to provide the most reliable and accurate data for the purification and measurement of the prostaglandins, thromboxanes, prostacyclin and their metabolites.

Few areas of biological research have ever expanded as rapidly as that relating to the prostaglandins and their related compounds. A large number of publications have appeared that include quantitative data on these substances, but such reports have been widely conflicting, due primarily to their generally low levels in most tissues and the presence of several different compounds having similar structures, as well as numerous metabolites of these primary substances.

The often disconcertingly contradictory information on prostaglandin synthesis and metabolism will disappear only through the use of accurate and reproducible methods. It is our hope that this volume will provide the necessary critical evaluation of presently available methodology and thus aid in defining the role of prostaglandins and related compounds in biological systems. In this way we will be following the then revolutionary idea expressed by Galileo Galilei that signaled the beginning of scientific enlightenment:

> Measure what is measurable and make measurable what cannot now be measured.

This volume will be of interest to all biochemists, physiologists, and other scientists and clinicians interested in these substances and their wide range of effects in biological systems.

J. C. Frölich
Stuttgart, July, 1978

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1

Quantitative Measurement of Prostaglandins and Thromboxanes: General Considerations

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In the early 1960s, when prostaglandins were isolated and characterized, bioassay was the main method employed for their detection due to their strong smooth muscle stimulating properties. Within a few years, however, other detection methods were developed, such as spectrophotometric methods for the PGE, A, and B compounds, and gas-liquid chromatography of PGF compounds using flame ionization detectors (Bygdeman and Samuelsson, 1966; Samuelsson et al., 1971). Later it was discovered that PGE compounds could also be analyzed by gas–liquid chromatography after their conversion into stable O-methyl oximes (Gréen, 1969). However, although the bioassay methods were sensitive, they lacked specificity; conversely, the spectrophotometric and gas chromatographic methods, although considerably more specific, were too insensitive for use in most biological studies. Generally, 0.2 to 20 μg of the compound was required for detection using these latter methods. In 1969 Änggård et al. published an enzymatic technique capable of detecting approximately 500 pg of prostaglandin E or F. Unfortunately, the problems encountered when applying this technique to biological experiments precluded its use. In 1970 a new mass spectrometric technique was introduced. This was based on multiple ion de-

tection and the use of a deuterated carrier (Samuelsson et al., 1970). The method provided a sensitive technique for the quantitative analysis of prostaglandins, and was applied to a vast number of biological studies. Other methods developed at about this time include gas–liquid chromatography with electron capture detectors, bioassays of the cascade type and other types combined with antagonists of other biologically active substances, and various radioimmunological methods. The gas chromatographic methods, particularly in combination with mass spectrometry, are extremely specific, and misinterpretation of data or confusion with other compounds is highly unlikely. However, these methods require fairly large initial sample volumes and subsequent time-consuming purifications prior to analysis, and thus they have a low sample capacity. The bioassays are sensitive and require only minute samples. The specificity can be increased considerably by using a combination of organs having different reactions to the analyzed compounds, and also by blocking reactions to nonprostanoate substances by the addition of various antagonists. The specificity of a bioassay systems can, however, never be absolute, which is both a drawback and an advantage. When one is dealing with substances of unknown nature and looking for new biologically active compounds, bioassay cannot be replaced by any other quantitative method in this field. Unfortunately, like the gas chromatographic methods, the bioassays also have low sample capacities. Various radioimmunological methods are at present the most widely employed quantitative methods in this area of research since they offer the major advantages of a high sample capacity as well as in many cases extreme sensitivity. Their specificity on the other hand seems to be questionable and may in certain cases even be very low.

The characteristics of these different types of methods are described in the following chapters of this book. In this Introduction, the general approach to the development of an assay will be described, and various common problems with the determination of prostaglandins in biological material discussed.

Quantitative assays of various types have been developed for a large number of prostaglandins and related compounds, some of which are illustrated in Fig. 1.

The first step in a quantitative experiment is to decide which body fluid or tissue to study. To date, most quantitative measurements of prostaglandins have been carried out with blood (plasma or serum) or with urine (Samuelsson et al., 1975).

Blood measurements offer certain advantages. First, it is possible to follow rapid changes in prostaglandin concentration. It is known that prostaglandin production and release often occur in a pulsative way as peaks of short duration (e.g., Barcikowski et al., 1974). This can sometimes easily be seen if a major PG metabolite, for instance, a 15-keto-13,14-dihydro compound, is monitored in peripheral blood (Kindahl et al., 1976) (Fig. 2, *upper panel*). If samples are instead obtained from both the arterial supply and the venous drainage of an organ, then it is

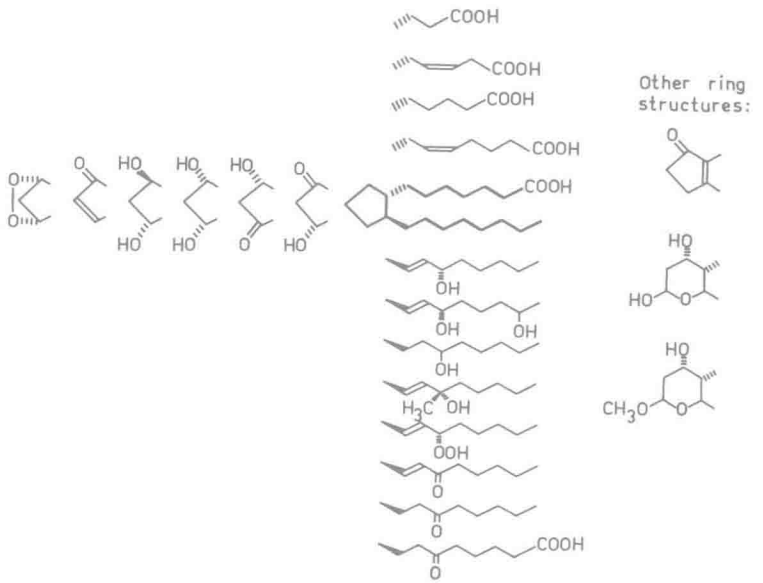

FIG. 1. Examples of prostaglandins and related compounds for which quantitative methods exist.

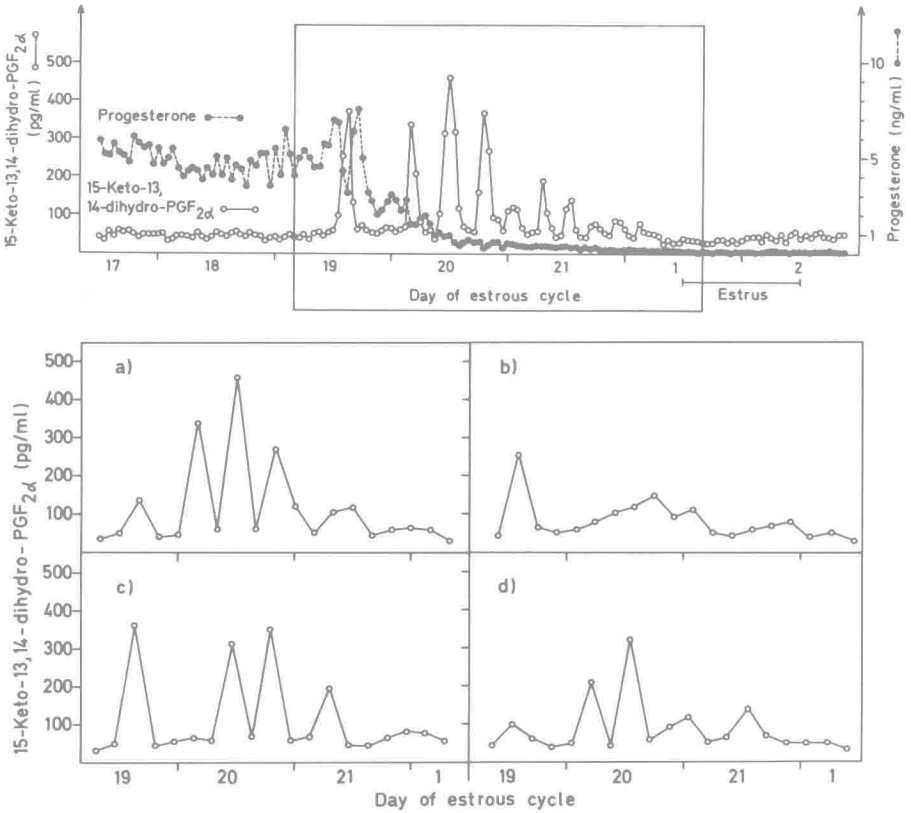

FIG. 2. Peripheral plasma levels of 15-keto-13,14-dihydro $PGF_{2\alpha}$ (○—— ○) and progesterone (●--- ●) in a heifer during luteolysis and estrus. The upper panel shows the pattern obtained when hourly samples were analyzed (from Kindahl et al., 1976). The lower panels show the four different possibilities that would have been obtained if the PG metabolite had been measured only every fourth hour.

possible to obtain information about metabolism of the PG in the organ, or to establish the exact source of PG production.

However, the drawbacks of blood measurements are numerous. Due to the possibility of rapid fluctuations in the PG levels, little if any reliable information is provided by a single PG value in blood. Since the half-lives of even the comparatively stable 15-keto-13,14-dihydro compounds are short (around 8 min in

the human), even a large PG release may be completely missed, unless a considerable number of samples are collected frequently enough. This problem is illustrated in Fig. 2, which shows a study on the release of $PGF_{2\alpha}$ in connection with luteolysis in the cow. This release was monitored by sequential measurements of the major circulating metabolite, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, in blood withdrawn from the jugular vein. The upper panel demonstrates the pattern obtained when hourly samples were collected (Kindahl et al., 1976). The lower panels illustrate the four different possibilities that would have been obtained if samples had instead been taken only every fourth hour during this particular luteolysis. From panel b it can be seen that even with a sampling frequency of 6/24 hr, the prostaglandin release can be almost completely missed. It is thus easily realized how little, and perhaps how erroneous, information would have been obtained from blood samples collected only once or twice daily, a protocol that in fact has been followed in many published studies. Thus when PGs are measured in the circulation very frequent sampling may be necessary to acquire meaningful information. This may not be possible in a small animal such as the guinea pig or the rat, or for ethical reasons, in the human. However, even if it is possible to obtain a number of samples in a short time, it is well known that certain commonly used laboratory animals (rabbit, cat, mink) may deviate from their normal physiology if they are handled too much, and may for instance ovulate spontaneously, thus adding problems to the interpretation of results.

If for any of these aforementioned reasons it is difficult to perform reliable PG measurements in blood samples, it is possible instead to assay urine. This approach removes the ethical problems in human studies. In both human and animal experiments, it is possible to perform long-term studies, when collection of urine is all that is needed, and even sensitive animals are not likely to deviate from normal physiology under these conditions. A further advantage is that the quantitative analysis of a single 24-hr portion of urine contains considerably more infor-

mation than that of one blood sample taken some time during this period; there is no risk of missing even a short surge of PG production, since most of it will eventually be excreted into the urine as degradation products. On the other hand, an obvious drawback with urine measurements is that no rapid changes in PG production can be seen, and of course it is impossible to establish the source of the PG production without further studies. However, it is not always necessary to know the site of this biosynthesis, and a general increase or decrease in the PG production may provide considerable information about normal physiology, pathological conditions, effects of drug therapy, and other factors.

More specialized quantitative studies of PGs have been carried out in a large number of tissue fluids other than blood plasma/serum or urine. These include joint fluid (Robinson et al., 1975*a;* Herman and Vane, 1975), cerebrospinal fluid (Wolfe and Mamer, 1975; Philipp-Dormston and Siegert, 1975), follicular fluid (Lemaire et al., 1973; Ainsworth et al., 1975), amniotic fluid (Gustavii and Gréen, 1972; Dray and Frydman, 1976), inflammatory exudates (Heap and Poyser, 1975, Ohuchi et al., 1976), seminal fluid (Cooper and Kelly, 1975; Clarke et al., 1974), gastrointestinal secretions (Peskar et al., 1974), fluid from pleural or abdominal cavities (Velo et al., 1973), sweat (Förström et al., 1974*a*), menstrual fluid (Pickles et al., 1965), lymph (Änggård and Johnson, 1971), milk (Manns, 1975), aqueous humor (Paterson and Pfister, 1975), and several others.

In the last few years, increasing interest has been focused on PG analysis of tissues, and a large number of reports have been published concerning PG content in either biopsy specimens, for example, endometrium (Gréen and Hagenfeldt, 1975) or gastric mucosa (Peskar and Peskar, 1976), or from organs removed post mortem. However, the extreme rapidity of the biosynthesis of prostaglandins makes it doubtful whether it is possible to determine the *in situ* levels of these compounds in the tissue. It seems that the mere excision of a tissue from an

anesthetized animal quickly leads to initiation of biosynthesis of PGs from endogenous precursor acids, thus giving falsely high levels of the *in situ* concentration. Even if a biopsy specimen is deep-frozen in, for example, liquid nitrogen within a few seconds after removal, it is not certain that the later measured levels are the true *in situ* levels (Gréen and Hagenfeldt, 1975). If the PG levels are determined in an organ removed after death of an animal, it is essential that any PG biosynthesis in that organ was interrupted at the same moment that the animal was killed (e.g., by the microwave technique, Bosisio et al., 1976). When processing the tissue sample, it is necessary to homogenize it either in an organic solvent or in a medium containing prostaglandin synthetase inhibitors; the deep-frozen organ or tissue must not be allowed to thaw before this procedure (Gréen et al., 1973). If these precautions are not taken, then the measured "levels" of PGs are more likely to reflect the capacity for prostaglandin synthesis and metabolism by the tissue in question, rather than the actual *in situ* levels (Jouvenaz et al., 1970, 1973). Moreover, if fluctuations in the blood levels of PGs and related products are in part a reflection of fluctuations in tissue production, then it becomes readily apparent that a single measurement from a given tissue may provide, as with blood, little and in some instances misleading information if cautious interpretation of the results is not exercised.

Finally, quantitative PG measurements may also be carried out using a single cell type, such as platelets. The measurements in these cases are generally not carried out on intracellular prostaglandins but instead on the cell culture fluid. Cell types that have been studied in this way are, for example, fibroblasts (Levine, 1972; Lindgren et al., 1974), synovial membrane (Robinson et al., 1975*b*), and epidermal cells (Förström et al., 1974*b*). Platelet PGs have been measured either in platelet-rich plasma or in platelet suspensions after induction of the so-called release reaction (Hamberg et al., 1974; Malmsten et al., 1975; Granström et al., 1976).

After having decided on the most suitable organ or body fluid

for a given study, the scientist must next decide what compound to monitor. In earlier days, the primary prostaglandins were the only compounds considered. Subsequently, however, a large body of knowledge has accumulated about the metabolic transformations of these substances, and it is now obvious that in many cases measurements of primary prostaglandins alone will provide directly misleading information. For example, it is known that prostaglandins are completely degraded in the body before being excreted into the urine (Samuelsson et al., 1975). However, minute amounts of primary prostaglandins have, in fact, been detected in urine, and studies have established that local synthesis by the kidney accounts for their presence (Frölich et al., 1975*b*). Therefore, measurement of the primary prostaglandins in urine cannot be expected to yield any valuable information about the release of these substances from other organs, or about the total body production. The compounds of choice for studies of urinary prostaglandin levels for this purpose are the major urinary metabolites, for example, in the human, 7α-hydroxy-5,11-diketotetranorprosta-1,16-dioic acid (main metabolite of PGE_2) or 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid (main metabolite of $PGF_{2\alpha}$). In other species, other tetranor compounds may be more suitable.

Neither, in most cases, should the primary PGs be monitored in the peripheral circulation. It is now well known that the measured concentrations do not reflect the true endogenous levels (Samuelsson, 1973). The half-lives of these compounds are extremely short, probably less than 30 sec, and it has been calculated that the endogenous level of, for example, $PGF_{2\alpha}$ does not exceed 2 pg/ml plasma (Samuelsson, 1973). Instead, the greater part of PGE or PGF that reaches the bloodstream occurs as the corresponding 15-keto-13,14-dihydro metabolite, which has a considerably longer half-life. The levels of these metabolites are approximately 50-fold higher than those of the parent prostaglandins. It is also well known that primary prostaglandins are synthesized and released artifactually during the collection of the blood samples: from platelets or white blood

cells, from nonenzymatic cyclization of precursor fatty acids, and so forth. This biosynthesis is extremely rapid and cannot be entirely prevented by including, for example, indomethacin and heparin in the collecting tube, or by chilling and rapid centrifugation of the sample. The problem of artifactual biosynthesis during collection and handling of samples does not arise with the 15-keto-13,14-dihydro metabolites. Measured amounts of these compounds are likely to reliably reflect the endogenous levels.

A different situation arises if the venous drainage of an organ is cannulated, and if it is known that the synthesized prostaglandin is released as such and not as a metabolite. Such is the case with the production of the luteolytic $PGF_{2\alpha}$ in the uterus of many species. In this type of study it is of course proper to monitor the primary PG; however, the problem of artifactual production during blood sampling remains. This problem is greatly reduced when a large anteriovenous difference can be demonstrated.

Another situation where it is proper to focus on the primary PG is in clinical studies, where the compound is administered, and the scientist wants to study the correlation between a certain blood level of the PG and, for example, the frequency of side effects.

It has been proposed that prostaglandins of the A type are circulating hormones, because the biological activity of these compounds survives passage through the lung (Horton and Jones, 1969; Piper et al., 1970). However, it has been demonstrated that PGA is a substrate for the 15-hydroxy prostanoate dehydrogenase (Nakano et al., 1969), that PGA_1 is extensively degraded *in vitro* in rabbit kidney, and that a metabolite, 13,14-dihydro PGA_1, has about the same biological activity as PGA_1 itself (Attallah et al., 1975). Thus it is far from certain whether PGA really occurs as such in the bloodstream. One recent study in the rat (Anderson and Eling, 1976) showed that PGA was not a substrate for the uptake system in the lung, in contrast to being a substrate for the degrading enzymes. This finding was

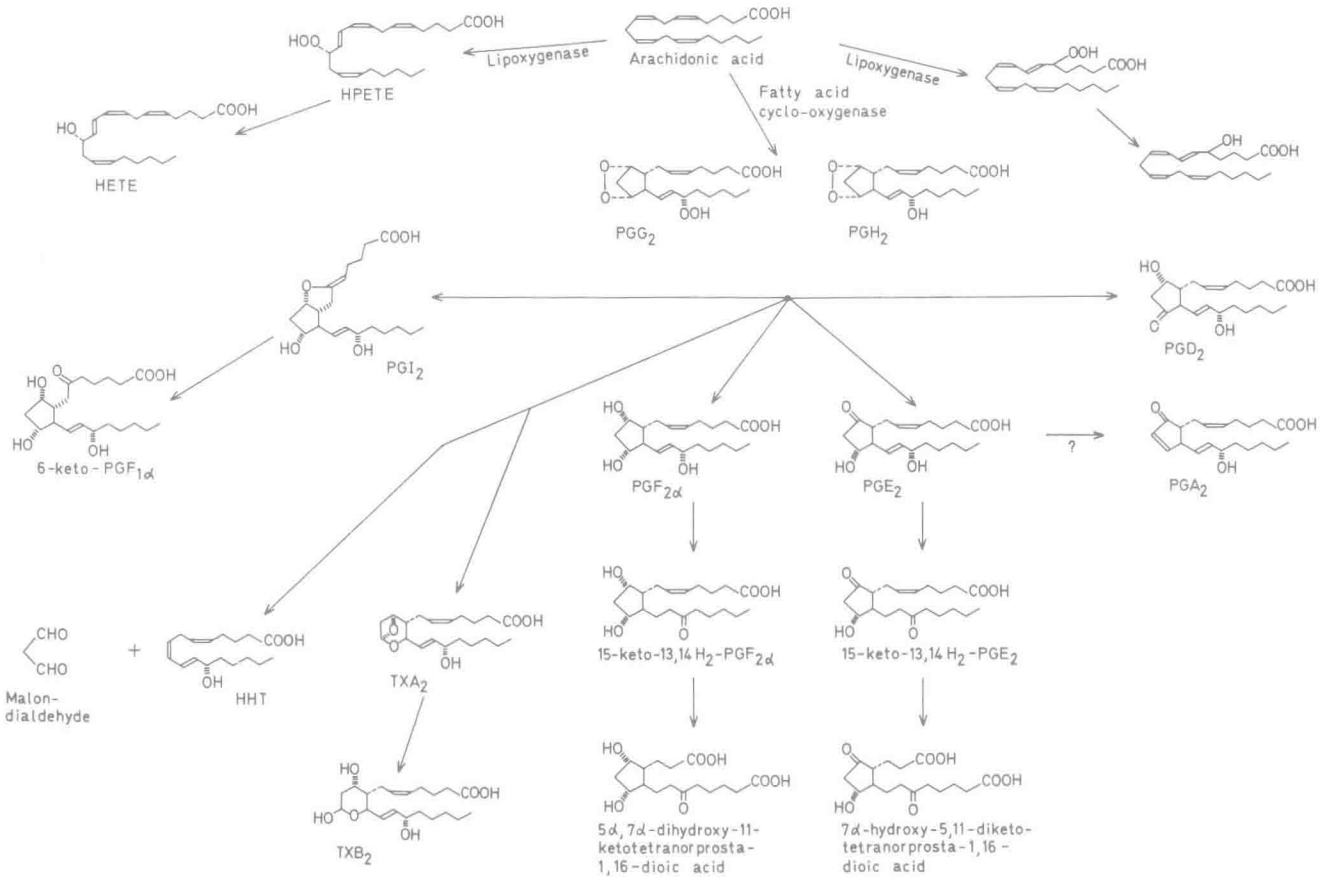

FIG. 3. Major metabolic pathways in the conversion of arachidonic acid in the human.

regarded as supportive of the theory that PGA is a circulating hormone. However, the lack of uptake has not been demonstrated for other species or other organs (see Attallah et al., 1974). On the contrary, several mass spectrometric studies indicate that PGA can hardly be detected at all in human plasma (Frölich et al., 1975*a;* Gréen and Steffenrud, 1976). In spite of this most uncertain situation, many laboratories have developed radioimmunological methods for measuring PGA compounds in plasma, and reported "basal levels" of these compounds in human plasma ranging from a few hundred pg/ml to several ng/ml.

Certain cells or tissues, such as blood platelets and lung tissue, are known to metabolize arachidonic acid almost exclusively to compounds other than prostaglandins (Hamberg and Samuelsson, 1974; Hamberg et al., 1975). In studying these systems it may be misleading to measure prostaglandins at all, whereas the monitoring of thromboxanes or their metabolites is more likely to give a reliable picture of the metabolic events.

Figure 3 summarizes today's knowledge of the more important metabolic pathways in the conversion of arachidonic acid in the human, and provides guidelines for the choice of a suitable compound for quantitative measurements in different types of studies.

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