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THE LANGUAGE OF BIOLOGICAL FLUIDS

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Abstract - Glass capillary gas chromatography in combination with mass spectrometry is an efficient tool for the investigation of steroids and acids occurring in biological fluids. Many of the compounds indicated in the gas chromatograms are still unknown. Their structure determination can be done with a combination of microchemical reactions and mass spectrometry.

A few examples demonstrate the usefulness of "profiling": Emotional stress cause a 10 - 30 fold increase in the excretion of dehydroepiandrosterone (DHEA). An even higher increase in the DHEA excretion is observed in some cases of hirsutism. Physical stress is manifested by an increase in the excretion of citric acid and a decrease in conjugates of amino acids.

The main steroid in blood plasma is DHEA. In patients suffering from uremia the main steroid was found to be 3β,17β-dihydroxy-5-androstene.

INTRODUCTION

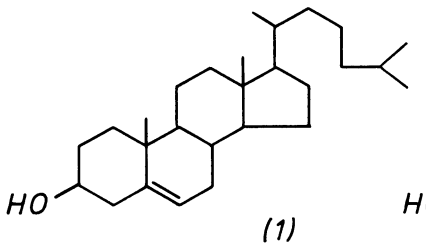
The human body is a perfect working chemical factory, utilizing food and drink as fuel, and rendering poisonous substances innocuous. The pipeline fluids are blood and CSF, the waste water is urine.

Just as a defect in the function of a factory can be spotted by a change in the composition of pipeline fluids and waste water, the qualitative and quantitative composition of the compounds in blood and urine offers us information about the present state of our body. Such an analysis offers a possibility of recognizing malfunctions of the body in an early stage. In consequence it may provide the basis for repairing the defect.

At the moment the language of the metabolites is still obscure, because until quite recently we have not had any methods to analyse such extremely complex mixtures as biological fluids. Only in very few cases were connections between an abnormal excretion of body-produced compounds and malfunctions recognized and used for diagnostic purposes, for instance, by colour tests to diagnose diabetes or phenylketonuria. Nowadays the introduction of powerful separation techniques like glass capillary gas-chromatography in combination with sensitive and specific identification methods like mass spectrometry, offers the possibility of investigating low molecular weight metabolites and thus enables us to learn more about the language of body fluids.

WORK-UP PROCEDURE

Biological fluids are extremely complex mixtures containing compounds in very different relations. For instance in blood the amount of cholesterol (1) and its esters is about 1000-times higher than that of dehydroepiandrosterone (2), the next most abundant steroid. The total amount of all other steroids is only 2/3 of that of dehydroepiandrosterone - and these are the interesting ones.



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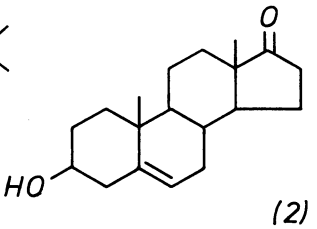

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The dynamic range of the analytical tools currently available is inadequate for determining minor components in biological fluids in the presence of large amounts of major components. Removal of the latter by various chromatographic techniques is thus essential.

A direct analysis of biological fluids is not possible for other reasons, too. A great number of organic compounds occur as conjugates; for instance hydroxysteroids are often conjugated either with sulfuric acid or glucuronic acid. These conjugates are so highly polar that they can not be extracted by organic solvents, requiring either their cleavage before extraction or the application of the elegant method of adsorption on XAD resins, as suggested first by Bradlow (Ref. 1).

Bradlow observed that conjugates and nearly all small organic molecules are adsorbed on styrene polymers, so-called XAD resins. Usually the organic fluid is poured on a column filled with resin. After washing with water, the organic molecules can easily be desorbed by rinsing the column with methanol. Extremely polar compounds like disulfates of steroids are partly lost during the washing of the XAD column with water needed to remove inorganic salts. Consequently these compounds are extracted better and more quantitatively by ion exchange resins.

After the enzymatic cleavage, the compounds are separated into neutral, basic and acidic fractions and if necessary they can be further chromatographed either on Sephadex or SiO_2 columns to remove the interfering main compounds.

SEPARATION BY GLASS CAPILLARY CHROMATOGRAPHY

After derivatisation to block polar OH and NH groups the extracts are ready for gas-chromatographic separation. Owing to the fact that some losses during the working up procedure are inevitable, the resulting gas-chromatograms give only a semi-quantitative picture of a group of body compounds. But this is sufficient for most purposes, since metabolic abnormalities are recognized by such drastic changes (factors vary between 3 to 1000) that small losses can be accepted. Such "profiles", - using the expression of Horning who pioneered in this field (Ref. 2) - are mainly available today for steroids and acids, but exist also for other groups of body compounds.

IDENTIFICATION

The first profiles were obtained from steroid fractions (Ref. 2). The identification was realized by retention-time measurements and in comparison with authentic material. Meanwhile it was recognized from mass spectra that this procedure is ambiguous, because two or more components are often hidden under one gas-chromatographic peak. Profiles of acid derivatives show a much higher complexity than those of steroids, so enhancing the identification problem.

The separation problem was more or less solved by the introduction of glass capillary columns (Ref. 3). Nevertheless the problems of identification remained. Although ambiguous identification by retention-time measurement with only one standard compound was substituted by the more reliable admixture of several standard compounds (saturated straight chain hydrocarbons) with Kovats index determination (Ref. 4), some uncertainty could nevertheless not be excluded. Kovats index determinations (Ref. 4) are only sufficiently accurate if the gas-chromatographic columns are run isothermally.

By taking profiles of biological fluids, compounds in an extremely broad range of volatilities are investigated. Therefore gas-chromatograms must be

run with temperature-programming up to very high temperatures, rather than isothermally. Consequently, the retention indices can only be reproduced up to three units (Ref. 5). This reproducibility is too inexact for an unambiguous identification of a compound.

An additional characterization procedure is therefore required, and for that purpose mass spectrometry is almost ideally suited.

It must be emphazised, that neither mass spectrometry nor the Kovats indices alone are sufficient for an exact identification. Very often the isomers present in biological fluids show almost identical mass spectra. But as a rule these isomers have quite different Kovats indices. Hence the combination of both methods is necessary for unambiguous identification.

STRUCTURE DETERMINATION OF UNKNOWN COMPOUNDS

The mass spectra of the peaks in the profiles of biological fluids revealed that a large number of all the compounds detected is still unknown.

If the compounds are related to known compounds the mass spectra usually give enough hints of their structures, even if molecular ions are absent. For instance an investigation of the acid fraction of blood plasma (Ref. 6), obtained by extraction with ethyl acetate (Fig. 1) revealed the presence of a series of minor acidic compounds besides the saturated and unsaturated monocarboxylic acids already known.

The acid function could be recognized in the mass spectra (one of these is reproduced in Fig. 2) by a key fragment of mass 59, corresponding to the $COOCH_3^+$ ion. Additional key fragments of mass 74 and 87 pointed to the presence of an aliphatic methylester.

Obviously the fragment of highest mass at m/e 185 was a fragment ion, produced by the very common loss of $\cdot OCH_3$ (31 mass units) from the $-COOCH_3$ group in a methyl ester, indicating a mass of 216 for the molecular ion.

If this assumption is correct, the peak at mass 152 should correspond to the loss of two molecules of methanol. This points to the presence of a saturated dicarboxylic acid. Methyl esters of dicarboxylic acids are reported to show no molecular ions, but rather peaks at M-31 and M-64 (Ref. 7), so we were probably dealing with such a compound.

As can be concluded from the presence of the key ions of mass 74 and 87, there are no substituents in the α and β positions, but no conclusions can be drawn as to a methyl branch at another position.
Two structural possibilities remain:

$H_3COOC-CH_2-CH_2-(CH_2)_3-CH_2-CH_2-COOCH_3$ (3a)

$H_3COOC-(CH_2)_2-CH_2-CH(CH_3)-(CH_2)_2-COOCH_3$ (3b)

The Kovats index was determined for the most likely compound, dimethyl azelaate and found to be identical, within experimental error, with that of the dicarboxylic acid derivative from blood. To confirm the structure absolutely, the authentic material was coinjected for comparison. Both dicarboxylic acid-esters were eluted with the same indices. Therefore the dicarboxylic acid found in blood must have been azelaic acid.

By this method of index measurement, coinjection and mass spectrometric determination, the following mono- and dicarboxylic acids were identified in blood for the first time.

Monocarboxylic acids $CH_3-(CH_2)_n-COOH$ n = 6, 7, 8, 9, 11

Dicarboxylic acids $HOOC-(CH_2)_n-COOH$ n = 3, 4, 5, 6, 7, 8

Quite often we obtain mass spectra which unfortunately give little indication of structure. Thus we found in blood and urine a series of acids, characterized by a key fragment of mass 179 (Ref. 8). The spectrum of one of the most abundant compounds of this series is reproduced in Fig. 3

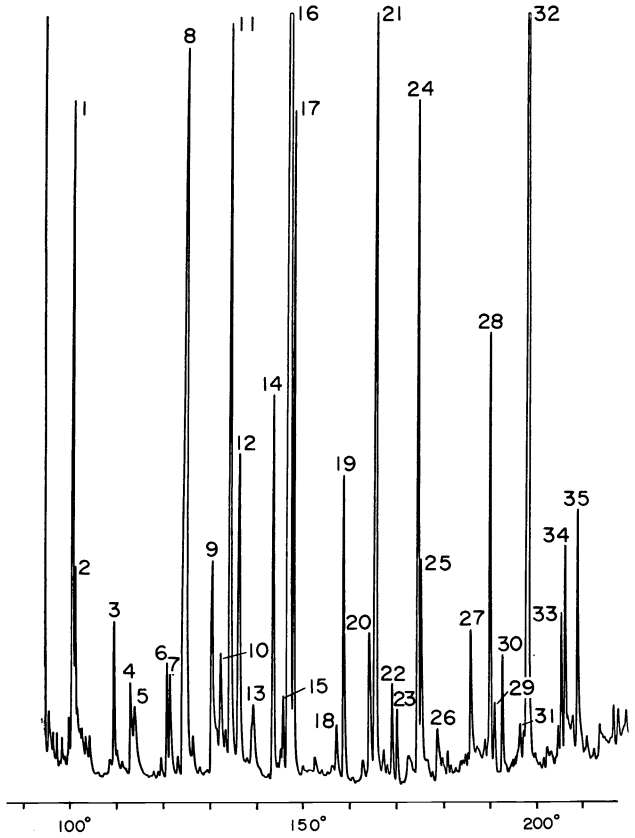


Fig. 1. WCOT column (coated with SE-30) chromatogram of methylesters of acids isolated from plasma. G.C. conditions: Carlo Erba gas chromatograph 2300; FID, injector temperature 275° C; detector temperature 280° C; Temperature program 80° C - 230° C, 4° C/min. The following acids were found: 2= caprylic, 3= benzoic, 4= pelargonic, 5= adipic, 6= capric, 7= pimelic, 8= 1,3,5-trimethyl-2,4,6-trioxo-perhydrotriazine, 10= suberic, 11= hippuric, 12= phthalic, 14= lauric, 15= azelaic, 16= citric, 17= 2,6-di-tert-butyl-4-methyl-phenol, 18= tridecanoic, 19= sebacic, 21= myristic, 26= pentadecanoic, 27= palmitoleic, 28= palmitic, 31= margaric, 32= phthalic, 33= linoleic, 34= oleic, 35= stearic, 30= same compound as isolated from urine, mass spectrum see Fig. 4, 24= lower homologue of compound 30.

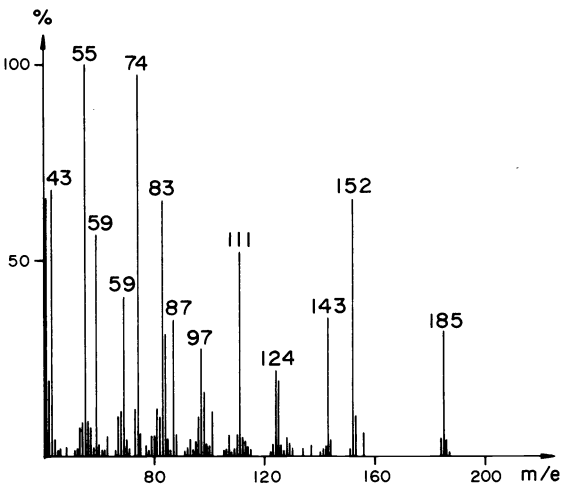


Fig. 2. Mass spectrum of peak 15 in the G.C. reproduced in Fig. 1.

The presence of at least one methoxycarbonyl group was indicated by loss of 31 mass units from the molecular ion and an ion at M-60, corresponding to the expulsion of CH_3OH and CO.

In such cases the determination of the molecular formula, important for a literature search and for the determination of the number of double bond equivalents is extremely difficult.

When working with glass capillary columns and a high resolution mass spectrometer, insufficient ions for an unambiguous result are collected, even at 5000 resolution, when slits are wide open to maintain sensitivity. Instead, the fraction containing the unknown compound must first be enriched by preparative G.C. on packed columns, and is then subjected to high resolution gc-ms, again on a packed column. The mass spectrometer is adjusted to the key ions found at the low resolution. The ion-intensity is now enhanced by the enrichment procedure and the peak broadened by the use of the packed column, giving sufficient time for peak matching. This is more accurate than exact mass determination with a computer.

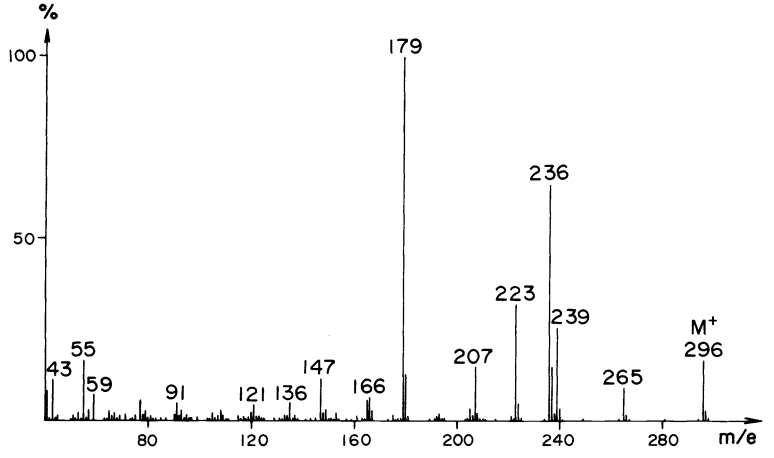
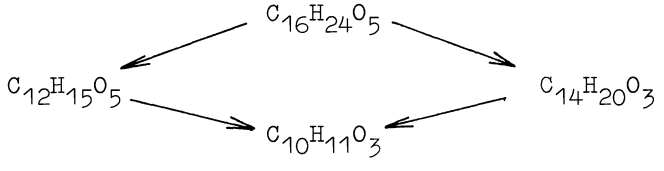


Fig. 3. Mass spectrum of an apparently unknown acidic compound found in urine

By this procedure the masses of the main peaks in the compounds with the key fragment at mass 179 were determined. The mass determinations revealed that the compound with the molecular weight 296 contained 5 oxygen atoms and 5 double bond equivalents.

The compound appears to fragment in two different ways: Elimination of $C_4H_9^{\bullet}$ can be followed by loss of methanol and CO to form the fragment of mass 179 ($C_{10}H_{11}O_3$), or the carbomethoxy group can be lost together with a hydrogen atom, followed by expulsion of $C_4H_9^{\bullet}$.



Related compounds, shorter by two CH_2-groups, or bearing a $-CH_2-CH(OH)-$group showed corresponding fragmentations, and we conclude that the loss of $C_4H_9^{\bullet}$ corresponds to the structural element $-CH_2CH_2CH_2CH_3$.

In such cases the next problem is the determination of functional groups. For this purpose we have to collect some samples in order to be able to carry out chemical reactions on a microscale with about 0.1-0.5 mg.

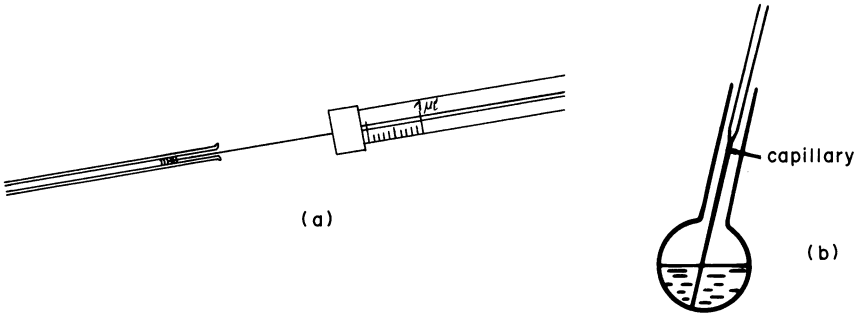


Fig. 4a. Solvent addition to a capillary tube with a syringe.
Fig. 4b. Ball tube as reaction vessel.

Usually we collect the samples in small glass capillary tubes. Then we add a few drops of solvent into the capillary tube with a syringe. By moving the capillary tube slightly the collected sample will be dissolved. Then the solution is transfered from the tube with a syringe to the reaction vessel - usually a small ball tube (Fig. 4b) - in which the chemical reaction is carried out.

Much additional care is required in microscale work. No unextracted filters should be used. A filter 10 cm in diameter contains about 0.1 mg of organic compounds (Ref. 9) which will be easily extracted by a solvent. Consequently filters must have been cleaned by extraction with a solvent before use. One must be extremely careful not to touch the filter with ones fingers, especially if the filter is wet with solvent. Organic compounds are transfered from the fingers to the filter causing the accummulation of impurities in the residue after filtration and evaporation of the solvent.

Furthermore silicon grease for tightening the connection of joints should be strictly avoided, for it is dissolved by solvent. All solvents, as well as chromatographic materials should be checked for purity.

Even if the work-up procedure is done with the greatest care, the inclusion of impurities cannot be avoided completely. Therefore in any case a gas-chromatographic separation of the reaction products is indicated.

If oxygen atoms are present we start the chemical reactions by trying to determine the number of OH groups by trimethylsilylation or acetylation. After removal of solvent (not always necessary) the reaction product is analysed again in the combination instrument. The change in the molecular weight, if any, allows us to determine the number of functional groups. Thus, an increase of 72 mass units after trimethylsilylation indicates the presence of one OH group. The compound of molecular weight 296 did not react, and the starting material was recovered unchanged - indicating that the compound contained no OH groups.

Other valuable microreactions for the determination of functional groups are hydrogenation, $LiAlH_4$-reduction, and exchange of OH and active CH groups by deuterium. The compound of molecular weight 296 reacted with $LiAlH_4$ but so far we have failed to isolate the reaction product.

The determination of the skeleton of really unknown compounds by the above procedures is very difficult. We must try to change the electron density in different parts of the molecule to obtain specific fragments from each part in turn.

Hydrogenation and oxydation reactions are again most useful for this purpose as well as the introduction of groups with high electron density, such as the conversion of an oxofunction into an ethyleneketal function.

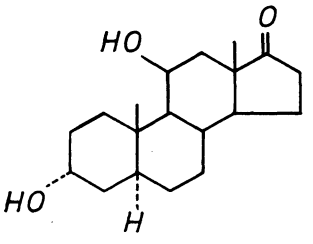
Sometimes even these procedures do not lead to success. We then have to collect and purify an amount of material which will permit the application of other spectroscopic methods (especially C-13-NMR spectroscopy).

The structure elucidation of the huge number of compounds still occurring in biological fluids is only one aspect of the research in this field. It obviously will improve our knowledge of metabolism. But this is only a necessary first step in the processof learning the language of profiles.

RESULTS OBTAINED BY INVESTIGATION OF PROFILES

First investigations of profiles were restricted to those physiological and pathological cases where a strong deviation of the normal steroid metabolism was already known, as for instance in pregnancy, or in the post menopausal period. Furthermore, tumors in adrenals and ovaries (Ref. 10) cause a tremendous increase in the production of steroids such as 11β-hydroxyandrosterone (4), 11β-hydroxyetiocholanolone (5), pregnanetriol (6) and also dehydroepiandrosterone (2).

Profiling has also been used to determine small amounts of α-tocopherol and free fatty acids in blood plasma samples and to attempt the correlation of this phenomenon with stroke (Ref. 11).



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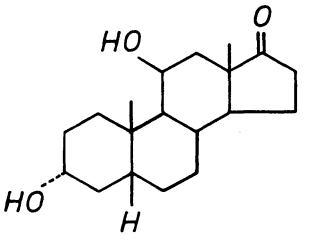

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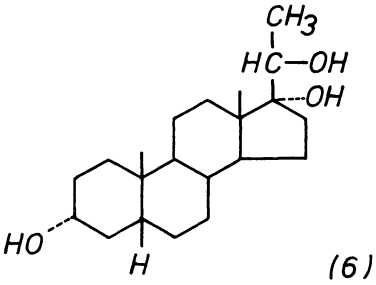

(6)

Jellum (Ref. 12,13) used the technique of preparation of acid profiles to detect metabolic diseases in retarded children. He has succeeded so far in finding two unknown metabolic diseases which are characterized by a tremendous overproduction of pyroglutamic acid (Ref. 14) and methylmalonic acid (Ref. 15) respectively. However drastic changes in metabolism are observed not only in pathological cases or pregnancy. Profiles taken from different healthy individuals, or from a single person at different times are often rather different. At first sight this seems to exclude any correlations. However we felt that in reality these deviations should tell us something about the state of our body. Unfortunately we have so far not been able to listen to them, since we do not understand their language.

Therefore we initiated a program to study the physiological response of the composition of body fluids to external changes. In trying to establish such correlations we did not restrict ourselves to a study of one group of compounds, but examined changes in the profiles of each of the acidic, neutral and basic fractions.

In order to carry out a systematic study we first investigated profiles of single persons at different times. These profiles indicated the possibility that the excretion of dehydroepiandrosterone (2) in urine could be dependent on the emotional state.

This assumption was proved by running urine profiles of candidates entering their final examination (Ref. 16). Before the examination, and immediately after it the profiles showed a 10-30 fold increase over the normal in the excretion of dehydroepiandrosterone (Fig. 5a), returning to normal 2-3 days after the examination (Fig. 5b).

This close correlation of the increased dehydroepiandrosterone excretion with emotional stress was demonstrated by the investigation of profiles of examination candidates over a period of 6 or 7 days. Although the last urine sample taken a day before the examination showed the same values as those of the examination day (20-30 fold increase) the dehydroepiandrosterone excretion on the morning of the examination day was only slightly higher than normal. Within 2 hours of his getting up however, the values increased 10-15 fold. Thus it is obvious that the production of dehydroepiandrosterone is immediately related to emotional stress. Physical exertion does not alter the steroid pattern at all (Ref. 16) but is manifest in a pronounced change of the acid profile. So for instance, about 1 1/2 hours after a cross country run (Fig. 6b) by a person not in training, the excretion of citric acid in urine had increased 3-5 fold compared to excretion before the exertion (Fig. 6a). At the same time the level of hippuric acid and other aminoacid conjugates decreased considerably.

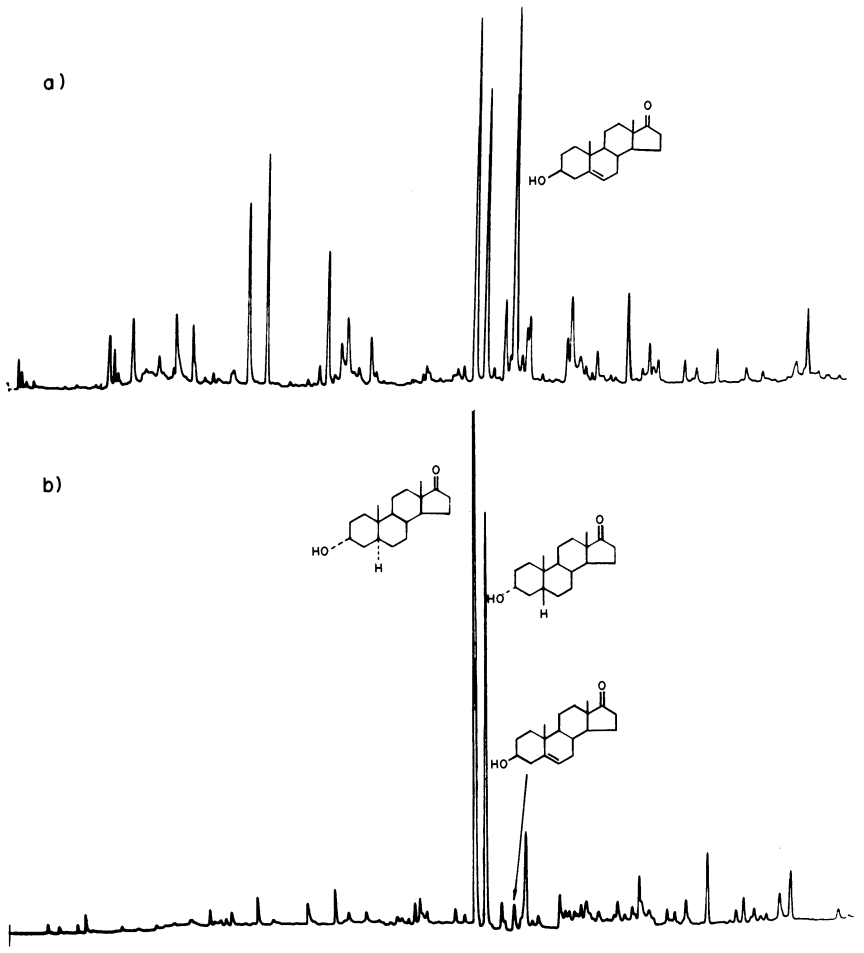

Fig. 5a. Steroid profile of a healthy man immediately after the stress of taking an examination.
Fig. 5b. Steroid profile of the same subject three days after the examination (corticoid steroids were partly lost during derivatisation).

This raises the question whether it is wise to pool 24 hour urine samples. Does this not result in a loss of information?

A further example of the importance of screening for specific steroids is illustrated by the analysis of steroid excretion by women suffering from ideopathic hirsutism. The 17-keto-steroid excretion of such women is usually at the upper limit of normal value.

Their steroid profiles revealed (Ref. 17) that about 50 % of them develop dehydroepiandrosterone levels which are 10-50 times higher than normal, thus showing the same picture as individuals suffering from a steady emotional stress (Fig. 7).

It is easily understood that this effect can not be recognized by a simple 17-ketosteroid determination, because even a hundred fold increase in the excretion of dehydroepiandrosterone would bring it to the same order of magnitude as the excretion of androsterone and etiocholanolone and consequently would cause an increase in 17-ketosteroids of about 30 % only which is within the normal limit.

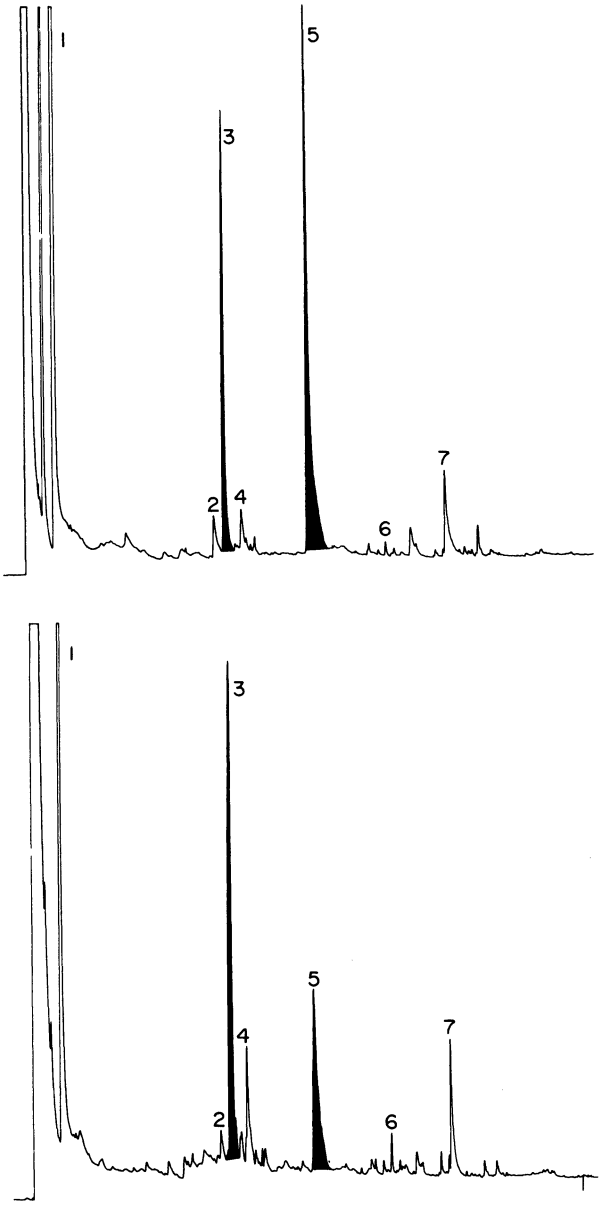

Fig. 6a. Profile of the acid fraction of a healthy male subject, aged 23 years, after a rest period.
Fig. 6b. Profile of the acid fraction of the same subject, 90 min after a 1/2 hour cross-country run.
1= phosphate, 2= adipic acid, 3= citric acid, 4= iso-citric acid, 5= hippuric acid, 6= palmitic acid, 7= phenylacetic acid-glutamic acid conjugate.

This example demonstrates that with group determination methods as often applied in medicine, meaningful data can only be obtained if the level of main steroids is altered. Significant changes in the levels of minor compounds will be overlooked.

Profiling makes it possible to obtain an insight into the production of all compounds of a distinct class, and to determine changes in their relative composition. With this method we came across compounds which had not been anticipated.

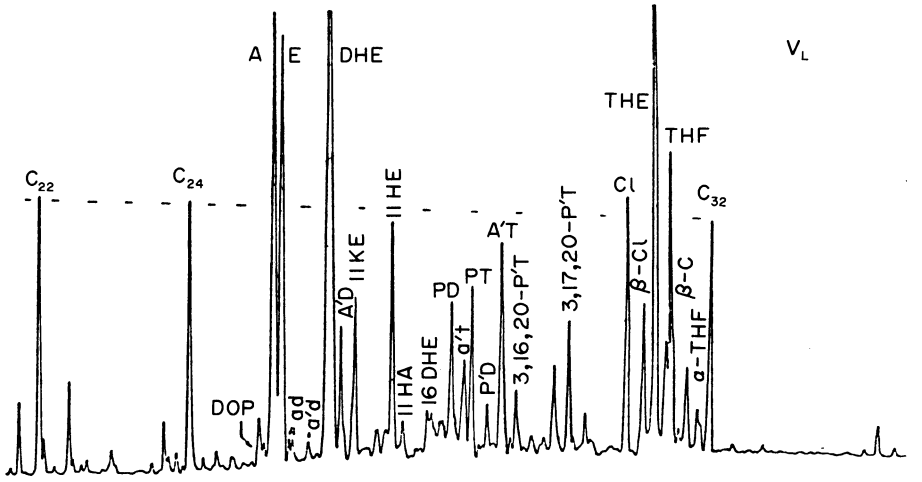

Fig. 7. Steroid profile of a hirsute woman, aged 22 years. DOP= dioctylphthalate, A= 3α-hydroxy-5α-androstan-17-one (androstane), E= 3α-hydroxy-5β-androstan-17-one (etiocholanolone), DHEA= 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone), 11KE= 3α-hydroxy-5β-androstane-11,17-dione, 11HA= 3α,11β-dihydroxy-5α-androstan-17-one, 11HE= 3α,11β-dihydroxy-5β-androstan-17-one, 16DHEA= 3β,16α-dihydroxy-5-androsten-17-one, PD= 3α,20α-dihydroxy-5β-pregnane, PT= 3α,17α,20α-trihydroxy-5β-pregnane, P'D= 3β,20β-dihydroxy-5-pregnene, A'T= 3β,16α,17β-trihydroxy-5-androstene, Cl= 3α,17α,20α,21-tetra-hydroxy-5β-pregnan-11-one (cortolone), β-Cl= β-cortolone, THE= 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione, C= 3α,11β,17α,20α,21-pentahydroxy-5β-pregnane (cortol), β-C= β-cortol, THF= 3α,11β,17α,21-tetra-hydroxy-5β-pregnan-20-one, α-THF= 3α,11β,17α,21-tetra-hydroxy-5α-pregnan-20-one.

The value of such screening may be demonstrated by a final example. The blood of patients suffering from uremia must be purified either by dialysis, or a new method recently developed by Prof. Scheler (University Göttingen) called "hemofiltration".

Since this too is a kind of "blood filtration" hemofiltrates are expected to be of similar steroid composition as blood plasma. Actually the profiles of uremia hemofiltrates (Fig. 8) differed greatly from normal plasma (Fig. 9) in containing major steroids oxidised at C-11.

Plasma profiles of these patients showed as main steroid 3β,17β-dihydroxy-5-androstene - a metabolite of dehydroepiandrosterone (DHEA) - while DHEA is the main steroid in normal blood plasma (Fig. 9).

Apparently in these cases the steroids have passed through the liver so often that they have undergone much enzymatic oxidation and reduction. In this respect it may be interesting to note that these patients also suffered from severe disturbances of their sexual life (impotence and disturbances of the menstrual cycle). With methods other than combined GC-MS, such deviations could not have been detected, owing to the lack of sensitivity.

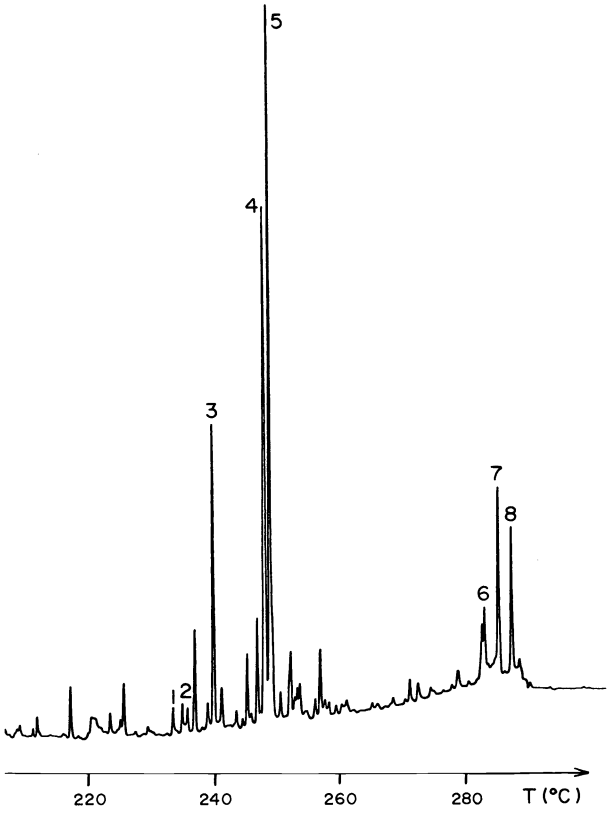

Fig. 8. Steroids isolated from hemo-filtrate. 1= 3α-hydroxy-5α-androstan-17-one (androsterone), 2= 3α-hydroxy-5β-androstan-17-one (etiocholanolone), 3= 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone), 4= 3α,11β-dihydroxy-5α-androstan-17-one, 5= 3α-hydroxy-5β-androstane-11,17-dione, 6= 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (tetrahydrocortisone), 7,8= 3,11,17,21-tetrahydroxy-pregnan-20-one.

CONCLUSION

Profiles show that a very large number of compounds produced in our body are still unknown. Structure determination of these compounds is achieved in many cases by mass spectrometry in combination with chemical degradation reactions. Profiles can clearly provide us with unique information about the state of the body. The better we learn to understand the language of body fluids the more we will be able to help physicians in establishing a diagnosis.

An important problem in medicine is the varying response of individuals to drug treatment. We have found that roughly every tenth person has an abnormal gas chromatographic profile. It may be that this can be correlated with abnormal response to drug treatment.

My collaborators Dr. Gerhard Remberg, Dr. Manfred Ende, Dr. Helga Ludwig, Dr. Hermann-Josef Egger, Josef Reiner, Michael Soiteller, Jürgen Pfordt and Wolfgang Gärtner were involved in different phases of the work which I have reported here. It is a great pleasure for me to express my thanks for their enthusiastic cooperation. This work was financially supported by DEUTSCHE FORSCHUNGSGEMEINSCHAFT and Fonds der Chemischen Industrie. Their help is gratefully acknowledged.

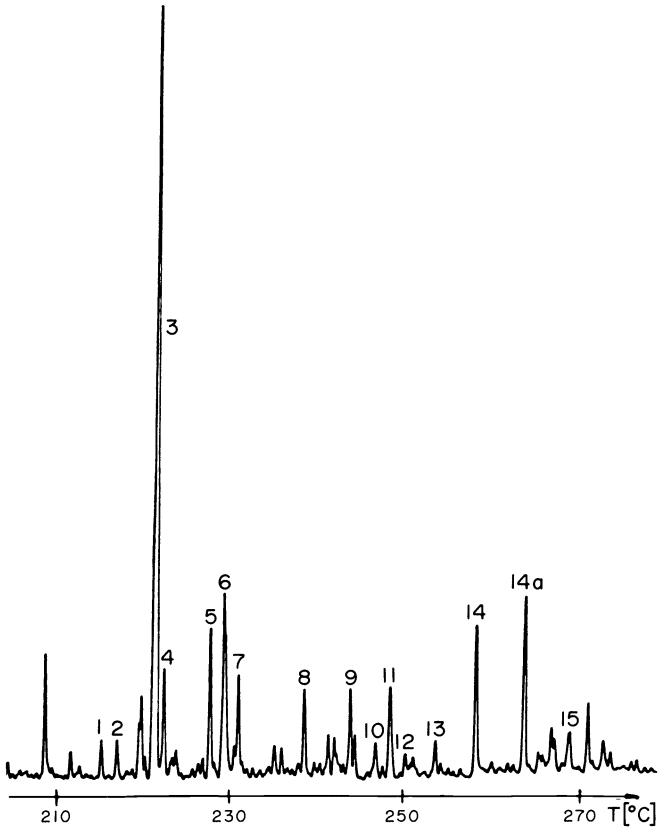

Fig. 9. Steroids isolated from plasma. 1= 3α-hydroxy-5α-androstan-17-one, 2= 3α-hydroxy-5β-androstan-17-one, 3= 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone), 4= 3β-hydroxy-5α-androstan-17-one, 5= trimethylsilyl derivative of the enol of compound 3, 6= 3β,17β-dihydroxy-5-androstene, 6a= 3β,17β-dihydroxy-5α-androstane, 7= 3β-hydroxy-5α-androstane-11,17-dione, 8= 3β,16α-dihydroxy-5-androsten-17-one, 9= 3,16,17-trihydroxy-5-androstene, 10= 3β,20α-dihydroxy-5-pregnene, 11= 3β, 16α,17β-trihydroxy-5-androstene, 12= 3,16,20-trihydroxy-5-pregnene, 13= 3β,17α,20α-trihydroxy-5-pregnene, 14= 3β, 17α,20α-trihydroxy-5-pregnene, 14a= unidentified pregnane, 15= 3β-hydroxy-5-cholesten (cholesterol).

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