FR8903323

COMMISSARIAT A L'ENERGIE ATOMIQUE

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CEA-CONF -9774

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PROGRESS IN STABLE ISOTOPE ANALYSIS AND NEW POSSIBILITIES OF CLINICAL INVESTIGATIONS

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Communication présentée à : 2. International Symposium o,. new trends in chemistry the role of analytical chemistry in national development

Le Caire (EG)
2-7 Jan 1989

THE SECOND INTERNATIONAL SYMPOSIUM ON

NEW TRENDS IN CHEMISTRY THE ROLE OF ANALYTICAL CHEMISTRY IN NATIONAL DEVELOPMENT

PROGRESS IN STABLE ISOTOPE ANALYSIS AND NEW POSSIBILITIES IN CLINICAL INVESTIGATIONS

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I - INTRODUCTION : ADVANTAGES AND LIMITATIONS IN THE CLINICAL USES OF STABLE TRACERS

The use of stable isotopes in medecine rests on three possibilities offered by labelling / 1 /.

- Identification of an element, a molecule, or a fragment of a molecule along its biological pathway.
- Quantification of biological pools by isotopic dilution.
- Measurement of metabolisation rates, and more generally of clearances.

Whenever a corporal function experiences a disregulation reflected either by changes in metabolic activity or modifications of the importance of pools of certain molecules the possibility exists of making use of isotopes in diagnosis.

Generally speaking radioactive isotopes lend themselves to the same uses as stables ones, however establishing molecular structures requires the use of mass spectrometry or magnetic nuclear resonance and concentrations necessary for that particular purpose are so large that labelling by stable isotopes is more practical, because such concentrations would lead to excessive radioactivity with the use of radioactive isotopes in a case where counting is not the analytical method. Hazard to users, radiolysis of the tracers would result.

Conversely stable isotopes do not, except by use of R.M.N. open the field of visualisation techniques, nor of direct therapy like radioactive ones. They could however be used as targets for neutron irradiation in order to induce a nuclear reaction at the place where they are located.

Light stable isotopes would seem a priori to be of special interest in medecine as neither hydrogen, carbon, oxygen or nitrogen, the most abundant elements of organic matter, have convenient radioactive isotopes.

- Tritium is only a weak β^- emitter.
- Carbon offers either a long lived isotope, also difficult to detect, or the short lived ^{11}C, β^+ emitter.
- Oxygen and nitrogen do no possess isotopes whose half life enables them to be used for the assessment of most physiological functions.

In addition the use of radioactive isotopes requires to exercice caution in order not to expose patients, or medical personnel , to injustified amount of radiations.

Even when all possible care is taken the fact remains that a fraction of any labelled element introduced in a living organisms is unretrievable, because it gets fixed on a very long term basis in the body.

This adds to the hazard of administering T or ^{14}C, with half lifes of 12 and 5700 years, just as diagnosis aids.

Why then are not stable isotopes more widely used than they are now, in spite of the fact that they served as tools in biological research almost as soon as deuterium was discovered by UREY in the early thirties ?

The answer is certainly to be found for a large past in the difficulty of the analysis of stable isotopes and the comparative lack of sensitivity of methods for their detection compared to that for tadioactive isotopes.

For instance Tritium with a half life of 12,3 years provides 37 desintegrations per second, that are easily countable, per 2.10^{10} atoms.

But 2.10^{10} atoms is the number of hydrogen atoms used to build $1,5.10^{-13}$ grams of water only. Measuring that amount of deuterium labelled water in a few micrograms of body fluid requires methods not available a few years ago.

A correlative aspect of lack sensitivity is that whenever a drug has to be administered it must be in larger quantities, and in cases where the drug itself has dangerous p"operties this may be a drawback to it's use. In all cases it makes the amount of labelled molecule to be used more costly and therefore less attractive.

Finally radioactive isotopes are very often measured within body fluids without any chemical separation. On the contrary when deuterium has to be put under gaseous form in order to be introduced in a mass spectrometer, this necessitates a chemical operation that may lengthen the analytical process.

For these reasons the use of stable isotopes in medicine for diagnostics or for in vivo studies of drugs is closely dependent on analytical possibilities.

Measurements should be easy if they are to be performed in an hospital ward. Response should be fast for practical reasons and costs should be low. Those three requirements are met when radioactive isotopes are being used.

On the other hand stable isotopes can only be detcted *in situ* by RMN but this does not prevent *in vivo* investigations. Samples to analyse are most frequently blood samples or urine, feces, expired air samples. Part of the analytical problems to solve rest in the treatment of samples.

Mass spectrometry the most versatile method of isotopic analysis requires samples free from mass interference. This means for example that CO_2 has to be introduced as a dry gas in the mass spectrometer if it is used as supporting molecule for the determination of ^{13}C, because most instruments will not resolve $^{13}C\ ^{16}O_2^{+}$ from $^{12}C^{16}O_2H^{+}$ formed in the presence of water. In a parallel way deuterated water has to be separated from body fluids to be analysed.

However stable isotopes offer a possibility that compensates the difficulty mentioned above. Their detection by mass spectrometry or RMN often enables a localisation of the isotopes on a specific site of the labelled molecule. It is thus possible to investigate which site is responsible for a given behaviour or to study mechanisms of metabolisms. And this possibility is enhanced by the fact that simultaneous labelling by different isotopes is possible.

We give examples of practical applications of stable isotopes and underline analytical problems that had to be solved.

II - CHOICE OF THE TRACER FOR WATER

Ruling out tritium, for medical diagnosis in man, leaves open the choice between ^{18}O and D as tracers for water. $H_2{}^{18}O$ a priori offers the advantage of being less liable to exchange, with other molecules than H_2O, than D_2O. For example amino groups have hydrogen atoms and can exchange this hydrogen water. Also if, along a metabolic pathway, isotopic fractionation occurs the study of the behaviour of labelled atoms is more difficult, though in some cases this fractionation provides useful information. As equilibrium isotopic effects are smaller with ^{18}O than D, though kinetic

effects are LARGER, there is no intrinsic reason to rule out ^{18}O, as a tracer for water '-? on the contrary.

Analytical pos bilities are a factor of choice.

^{18}O is said to be analysed by mass spectrometry with better precision than deuterium, but usually samples of the order of a milliliter of water are required, and equilibration between water and CO_2, the gas usually fed to the mass spectrometer for ^{18}O analysis, introduces a time lag in the analystical process.

However in our laboratories we have developped a method for ^{18}O analysis that overcomes those disadvantages : it enables the determination of ^{18}O in the mass spectrometer directly on the H_2O molecule, in microgram samples / 2 /. Instruments on that principle have been operated on a routine basis in Saclay for many years with a sensitivity comparable to that of the conventional method.

However a precision of 1 % is not good enough, because to induce a visible change in the ^{18}O measured on total body water, circa 50 kilograms for an average man, one gram of pure $H_2{}^{18}O$ should be used (as it contains 1 % of the amount of ^{18}O present in 50 Kg of water) and to perform an experiment probably 10 to 20 grams ; and $H_2{}^{18}O$, costs about 150 to 200 $ per gram.

For deuterium analysis also a completely automatic mass spectrometer is available in our laboratory. In fact it is the same machine as the oxygen one. As accuracy for both isotopes is comparable (about 1°/₀₀ of natural abundance) the amount of tracer needed for a deuterium experiment with a natural abundance of .15°/₀₀, is about ten times less that needed for ^{18}O with a natural abundance of about 2°/₀₀.

This, added to a ratio of costs of $\sim$ 1000 in favor of heavy water versus $H_2{}^{18}O$, settles the problem of the choice of the tracer except in cases where specific exchanges have to be avoided.

III - IMPROVEMENT IN ANALYTICAL TECHNIQUES, CHOICE OF INFRARED ABSORPTION

With M.S. converting samples to water is necessary. We wanted to avoid this conversion, and analyse directly body fluids for clinical purposes.

We then turned to infrared spectrometry, the only other techniques that could have a comparable precision to mass spectrometry, at least with comparatively simple machines. RMN usually requires much larger samples than the two previous techniques, accuracy for isotope ratio measurements is less and instrumentation much more costly. The infrared absorption spectrum of blood presents a "window" around 2500 cm^{-1}. It allows the measurement of the band due to the OD vibrator when D_2O is injected in the blood.

When blood is sampled, samples must be analysed rapidly because absorption ar 2500 cm^{-1} varies with time, and usually the result sought involves a comparison of the deuterium content of blood over a few hours period. Up to now the techniques involved for analysis of discrete samples are, for blood, two fold.

In the case of mass spectrometric analysis : deproteinisation by zinc sulfate dissolved in water of measured deuterium content, followed by a double distillation in a flow of helium gas / 3 / is necessary.

In the case of infrared analysis the serum is separated by centrifugation and kept in a refrigerator up to the time it is lyophilised just before analysis / 8 /.

But direct on line analysis of blood can readily be performed by infrared absorption using a special, infrared analyser, designed and developped in our laboratories in close conjunction with several medical units / 4, 5 /.

This kind of analysis can be performed for research on animals or in the hospital at the patients bed and has been applied to the measurement of extravascular water in the lung.

IV - APPLICATIONS

IV.1 - Total body water measurement

In order to measure pools of fluids, when straight isotopic dilution can be used, discrete sampling is sufficient.

Feeding heavy water to a patient and analysing the blood or urine after three hours, when isotopic equilibrium is completed, provides a measurement of total body water if one corrects for heavy water eliminated during the time to reach equilibrium.

$$\text{Total body water} = \frac{D_2O \text{ fed to the patient} - D_2O \text{ eliminated}}{\text{concentration of blood, or urine, at equilibrium}}$$

This equation simply expresses the balance of deuterium.

Samples of urine can neither be analysed directly nor be kept without purification, because a marked evolution of absorbance at 2500 cm^{-1} takes place under such conditions. Filtration on activated carbon or simple vacuum distillation is not good enough, lyophilisation again is the preferred technique / 6 /.

Special modifications of available infrared spectrometers enable to detect 50 ppm excess deuterium concentration in waters resulting from lyophilisation.

IV.2 - Measurement of extravascular water in the lungs

In such a case the only practical way to inject the tracer is in the blood, labelled water gets immediately diluted by the water content of the blood and rapidly by the total lung water, into which it diffuses.

To discriminate between intra and extravascular water the technique is to inject, simultaneously with D_2O, a dye that gets only diluted by the

intravascular blood volume. Cardiogreen (indocyanine-green, ICG) is the usual choice. To overcome the difficulty of dilution of D_2O by the total body water, one has to measure the dilution of D_2O in the blood after this fluid has been pumped through the lungs, but before it has had a chance to irrigate other tissues, i.e. one has to sample it in an artery.

One will final in / 5 / and / 7 / a complete description of the special apparatus built for the simultaneous measure of D_2O and ICG concentrations.

It is characterised by a non dispersive selection of the wave lengths (use of filters), the use of a single source for both waves lengths. Sensitivity achieved corresponds to a detection limit of 3 mg of D_2O per liter of blood (3 ppm) i.e. 1/50 of natural concentration or .08 mg of ICG.

The necessary volume of blood (in the cell) is a few tens of microliters. The flow of blood through the cell is a few ml per minute. The total measurement takes three or four minutes, but could be reduced as the transit time is less them half a minute with a difference between ICG and D_2O transit time of circa 3 seconds (Of course the instrument described can also used for total body water).

The difference in the transit times of the tracers is very small, and continuous analysis is necessary firstly for that reason. The extracellular volume of lung water is evaluated from the following equation :

$$v = Q \times (\bar{t}_1 - \bar{t}_2) f$$

where :

v is the volume irrigated
Q the flow of blood
f is the fraction of water in the blood
$\bar{t}_1$ is the transit time of D_2O between the injection point and the analytical point
$\bar{t}_2$ is the corresponding time for cardiogreen

$$Q = q \,/ \int_0^{\infty} c(t)\, dt$$

$$\bar{t} = \int_0^{\infty} t\, c(t)\, dt \,/ \int_0^{\infty} c(t)\, dt$$

where :

q is the quantitify of tracer injected

c(t) is the exces concentration of the tracer over it's natural level as a function of time

Units of concentration are chosen to match units in which q is expressed.

IV.3 - Experimental conditions

- Total body water

Heavy water fed to the patient is .15 g per kg of corporal weight, precision of the results is about 2 %.

- Lung water

For each measurement a sterile solution containing 2 g of D_2O and 5 mg of ICG is injected. Three measurements are performed at 15 minutes interval. The flow in the analytical circuit is about 15 ml per minute.

V - RESULTS

V.1 - D_2O used alone

a) Provides measurements of total body water. This application is routinely used to follow hemodialysis procedures / 8 /. In normal young subjects total body water has been estimated to represent 55 $\pm$ 4 % of total body weight.

b) Can compete with ICG, for cardiac flow rate measurements, as it necessitates the same injection and sampling techniques and is less costly, less toxic, does not induce colour in patients.

V.2 - D_2O used in conjunction with ICG

Provides a tool for the measurement of extravascular water in the lungs that has proved more sensitive than conventional X-ray measurement.

VI - THE $^2H_2{}^{18}O$ METHOD FOR ENERGY EXPENDITURE MEASUREMENTS

When water labelled with deuterium is given to a subject isotope dissapearance is exponential. If water is lebelled with ^{18}O dissapearance is also exponential but with a shorter biological half life. The difference is due to the fact that deuterium is lost only as water whereas ^{18}O is lost both as water and carbon dioxide.

Within assumptions that have been validated by careful investigations / 9, 10 / the difference in decay rates of deuterium and ^{18}O, followed for instance by the analysis of urine samples, provides a measure of energy expenditure.

The advantage of this method over the classical determination by heat evolution measurements during which the subject has to remain in a calorimeter, is precisely that it can be carried out under normal conditions.

VI.1 - Analytical problems

They are numerous :

a) a low detection limit is required to minimise the cost of ^{18}O by authorizing the use of water only 10 % enriched.

b) a fast detection method is desirable. This is more conviently the mass spectrometer described earlier / 8 /. One can also use improved chromatographic equilibrium methods for the ^{18}O exchange between water and CO_2, which is finally analysed by mass spectrometry / 12 /.

These analytical developpements are responsible for the increasing interest in energy expenditure measurements.

VII - BREATH TESTS UTILIZING CARBON 13

Started with ^{14}C they consist in measuring labelled carbon, under the form of CO_2, in respiratory gases following the absorption of a drug incorporating this tracer.

This technique apparently simple gives access to rates of metabolisms and therefore to malfunction of the liver / 13, 14 / or of the gastro-intestinal organs. It also enables to follow the recovery of a normal metabolic rate in a patient having undergone surgery.

^{14}C can only be used in extreme cases, and never when there is a risk of permanent retention of metabolites.

^{13}C would be ideal if direct analysis in respiratory gases was cheap and easy, as it enables to diagnose the origin of mal absorption of food, for instance to detect whether the problem rests with glucides lipides or protides, without modifying the diet of the patient.

To overcome the difficulties mentioned at the beginning of this paper on line processing of respiratory gases has been described / 14 /.

It could also be interesting to make use of infrared absorption on very narrow lines which would eliminate the need of to treat the gases. Such a technique has been used to analyse isotopically atmospheric CO_2 / 15 /.

A very low detection limit for isotopic variations around the natural abundance of ^{13}C (circa $1^{o}/_{oo}$) enables to use natural tracers ; i.e. for instance glucose from cane sugar that has a δ of -10.6, compared to -25.8 the ^{13}C basal δ value (in normal respiratory CO_2 in the middle latitudes of the northern hemisphere / 16 /).

VIII - THE USE OF NITROGEN 15 TO GAUGE THE EFFECTS OF SEVERITY OF SURGICAL TRAUMA ON WHOLE BODY PROTEIN TURNOVER

It has been shown by classical means that protein catabolic response to injury may be large, specially to surgery.

This difficult measurement is made simpler by administrating ^{15}N labelled glycine to patients and using urinary nitrogen 15 analysis to measure the nitrogen turnover.

An interesting information that can be obtained is that of the recovery of the turnover rate of nitrogen of a patient to it's preoperational level / 17 /.

The instrument consists of a total nitrogen analyser and a mass spectrometer.

IX - NUTRITION PROBLEMS : Study of the absorption of iron, copper and zinc

Nutritional studies are developping around the role of oligoelements in food.

Tracer techniques can only be used with the help of stable isotope in this case, because of the high doses to which radioisotopes of these elements would expose subjects.

All the possibilities of tracers techniques are taken advantage of in these researches that enable to follow the variations of absorption (retenticn) and excretion rates of these elements with the patients age, physical condition, diet.

Special mass spectrometric techniques had to be worked out for the isotopic analysis of these difficult elements / 18, 19 /.

X - THE CASE OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY FOR NUCLIDES OTHER THAN THE PROTON / 20 /

Ten years ago NMR had not reached the point where clinical applications could be envisaged by using other then proton resonance, at least *in vivo*. Even so it alrealy had an immense potential for medical applications.

But progresses that can not be analysed within the scope of this paper have opened the field in particular to ^{13}C investigations, either directly or by observing the signal of a proton linked to a ^{13}C.
^{13}C resonance is used in a number of biological, pharmacological researches, impossible to summerize and, in clinical applications, notably in many *in vivo* studies.

We will content ourselves here to mention three investigations :

Using enriched glucose it has been possible to study glycogen storage diseases in children and adults / 21 /.

Natural abundance ^{13}C NMR investigation of the prostate has been carried out. Interestingly this study concludes : "in order for ^{13}C NMR spectroscopy to obtain clinical relevances we will need to seek methods for obtaining spectra from smaller volumes" / 22 /.

But already NMR by enabling to follow ^{13}C at its natural abundance in metabolites is used in a number of studies or even in diagnostics, inclu-

ding glucose metabolism in human brain and the search for ethanol in the Rabbit brain / 23, 24 /.

XI - CONCLUSION

Analytical progresses in mass spectrometry, gas chromatography, infrared analysis, nuclear magnetic resonance have opened the way to clinical investigations more detailed and precise.

The use of compounds whose isotopic composition differ from the usual one, whether by artificial enrichment or because of naturally induced fractionation enable in particular to investigate metabolic pathways, to study malabsorption of specific aliments provided the analytical techniques exist.

Improvements of the latter, both in separation processes and in detection or measuring instruments, have increased perhaps tenfold the number of diagnostics made possible *in vivo* and *in situ* by the use of enriched non radioactive isotopes.

And, in parallel with imaging techniques by NMR or echography, progresses in this direction help to develop more and more non invasive procedures.

Further developments have still to take place in order to take full advantage of the possibilities of breath tests, of the analysis of urine, of NMR. But there are few fields in which progress in analytical technique and savoir faire are more necessary and have proved more able to bring benefits to mankind than in that of clinical investigations.

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Figure captions

Fig. 1 Diagram of the Duplex sepctrophotometer.

Fig. 2 Principle of the Duplex spectrophotometer.

Fig. 3 Principle of the measurement of Cardiac output and of extravascular pulmonary water.

q is the amount of tracer injected

$\dot{Q}$ is the Cardiac output.

idQwI is the extravascular pulmonary water volume

Fwb is the fractional amount of water in the blood

C (t) is the concentration of the tracer at a given time t.

Fig. 4 Measurement of lung water in a rat

curve 1 indocyanine curve

2 D_2O curve

3 $H_2{}^{18}O$ curve that has been experimental as a check.

Fig. 5 Exploration of a liver function after absorption of ^{13}C labelled aminopyrine.

vertical scale = percentage of labelled coumpond expired at time t

Dots = control subjects. Triangles = patients suffering from the liver.

The rate of metabolization of patients is slower than that of controls.

Fig. 6 Effect of insuline injection to patients suffering from diabetes on the rate of metabolization of labelled glucose. The higher the dose the faster a normal rate is recovered.

Fig. 7 Changes in ^{13}C spectra of rat brain during hypoxid

- ^{13}C glucose in α form (peak 4)

and β form (peak 5)

appear after injection (curve B)

under hypoxia (reduction of oxygen concentration)

α form decreases more rapidly than β form

a lactate signal (peak 1) appears (curve C)

after reoxygenation (curve D) the lactate peak dissapears the α form of glucose reappears.

Fig. 8 Human hepatic and muscle glucogen labeling by ingested ^{13}C glucose.

<u>Fig. 1</u> - Glycogen signal in the liver 30 minutes after a first drink of a 20 % ^{13}C glucose drink (curve b) and after a further 60 minutes a second drink increases the signal (curve 1c). The base line (curve 1 a) is obtained after the subject has fasted for 30 hours.

<u>Fig. 2</u> - Shows the ^{13}C spectra in the gastrocnemius muscle 45 minutes after ingestion of the second ^{13}C drink (curve 2b). The reference signal is given in curve 2a.

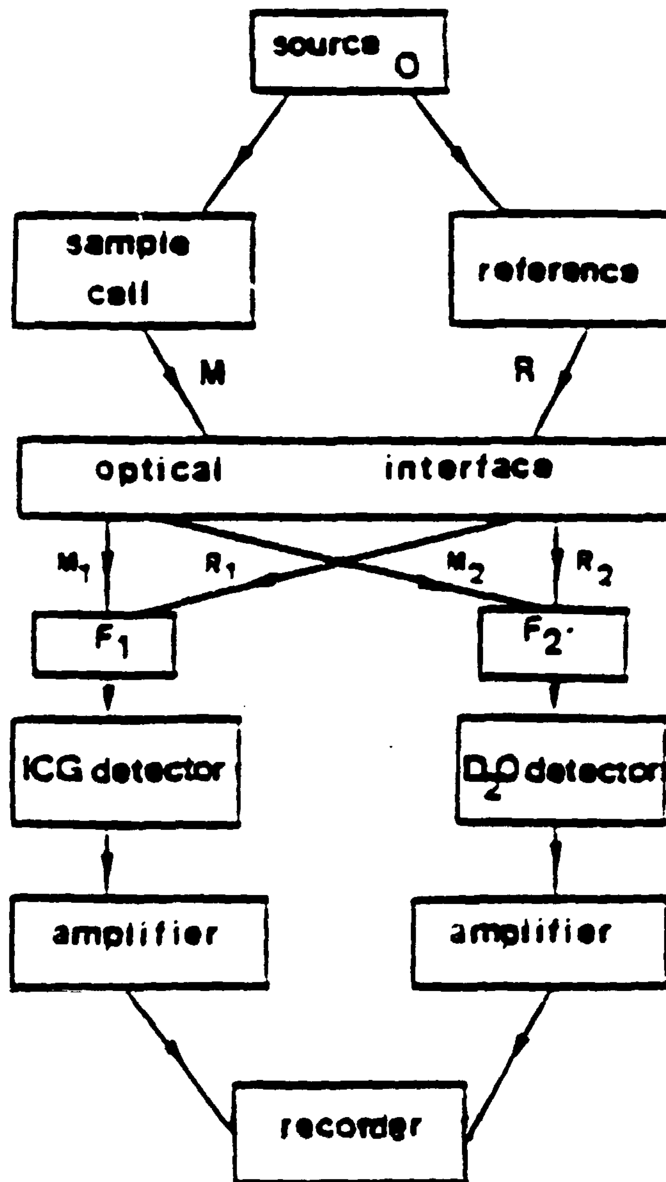


FIG. 1 Diagram of spectrophotometer for simultaneous measurement of DHO and ICG.

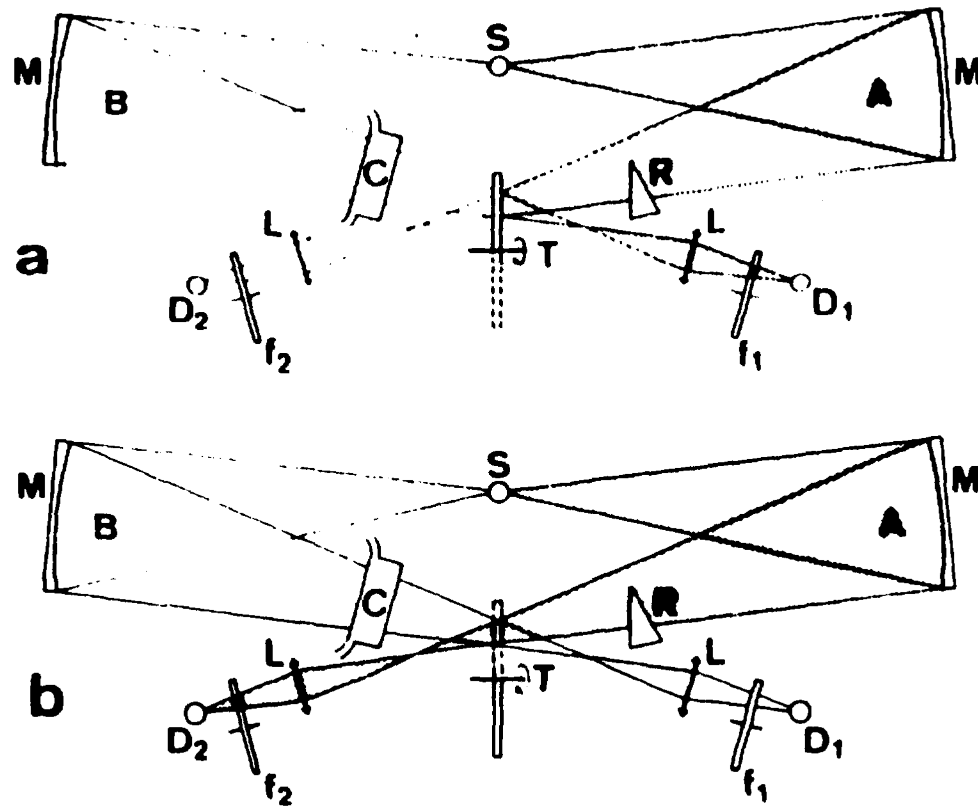
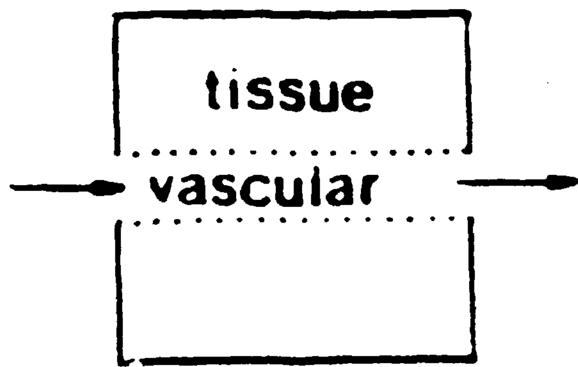
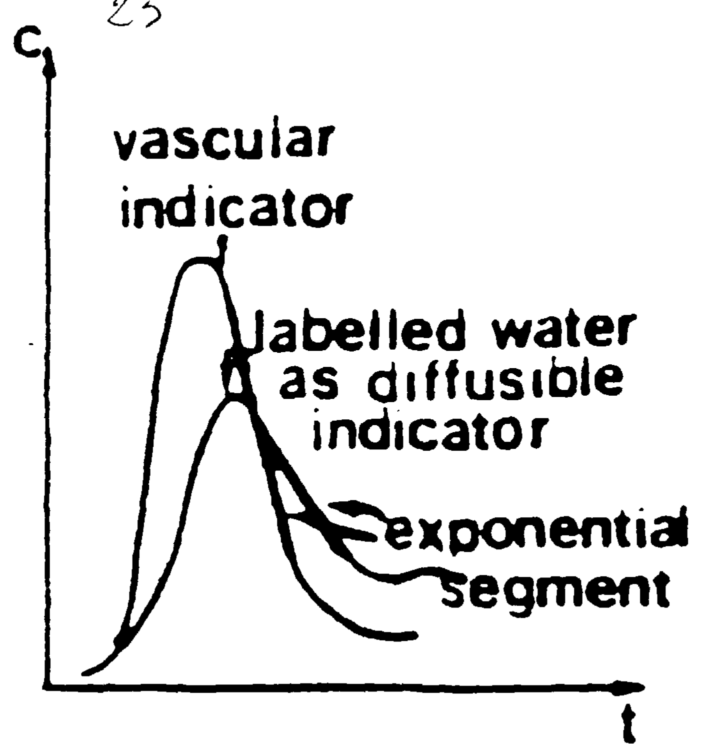


FIGURE 2. Principle of the Duplex Spectrodosimeter. a and b: two successive sequences. S: light source, M: concave mirrors, C: optical absorption cell, R: reference absorber, T: a rotating disc made of sectors alternately transparent or reflecting on both sides, L: lens, f_1 and f_2: interference filters, D_1 and D_2: (0.8 μ and 4 μ) detectors.

23





Cardiac Output

$$\dot{Q} = \frac{q}{\int_0^\infty c(t)\,dt} \qquad \dot{Q} = \dot{Q}_{vasc.} = \dot{Q}_{water}$$

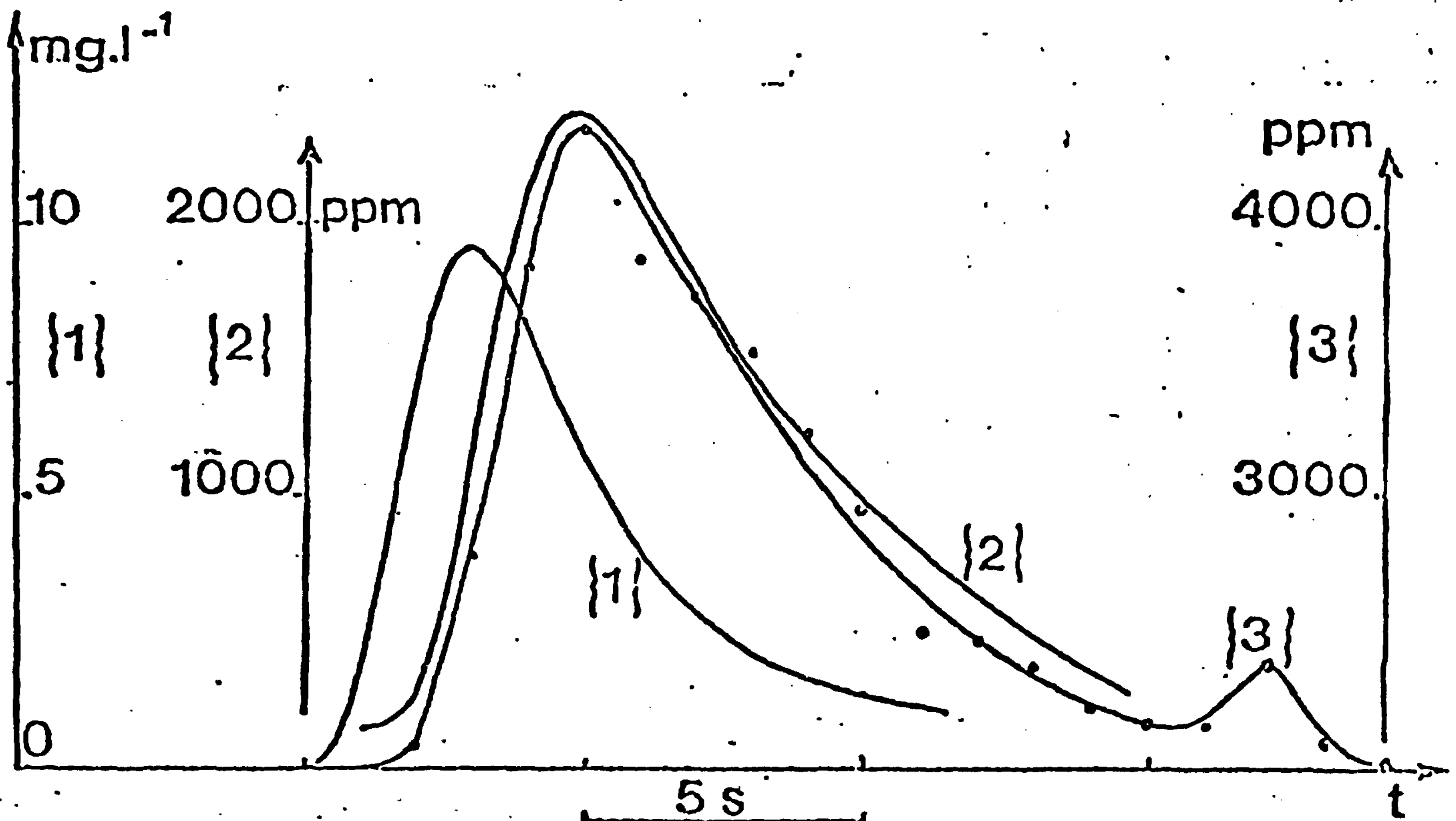
Mean Transit Time

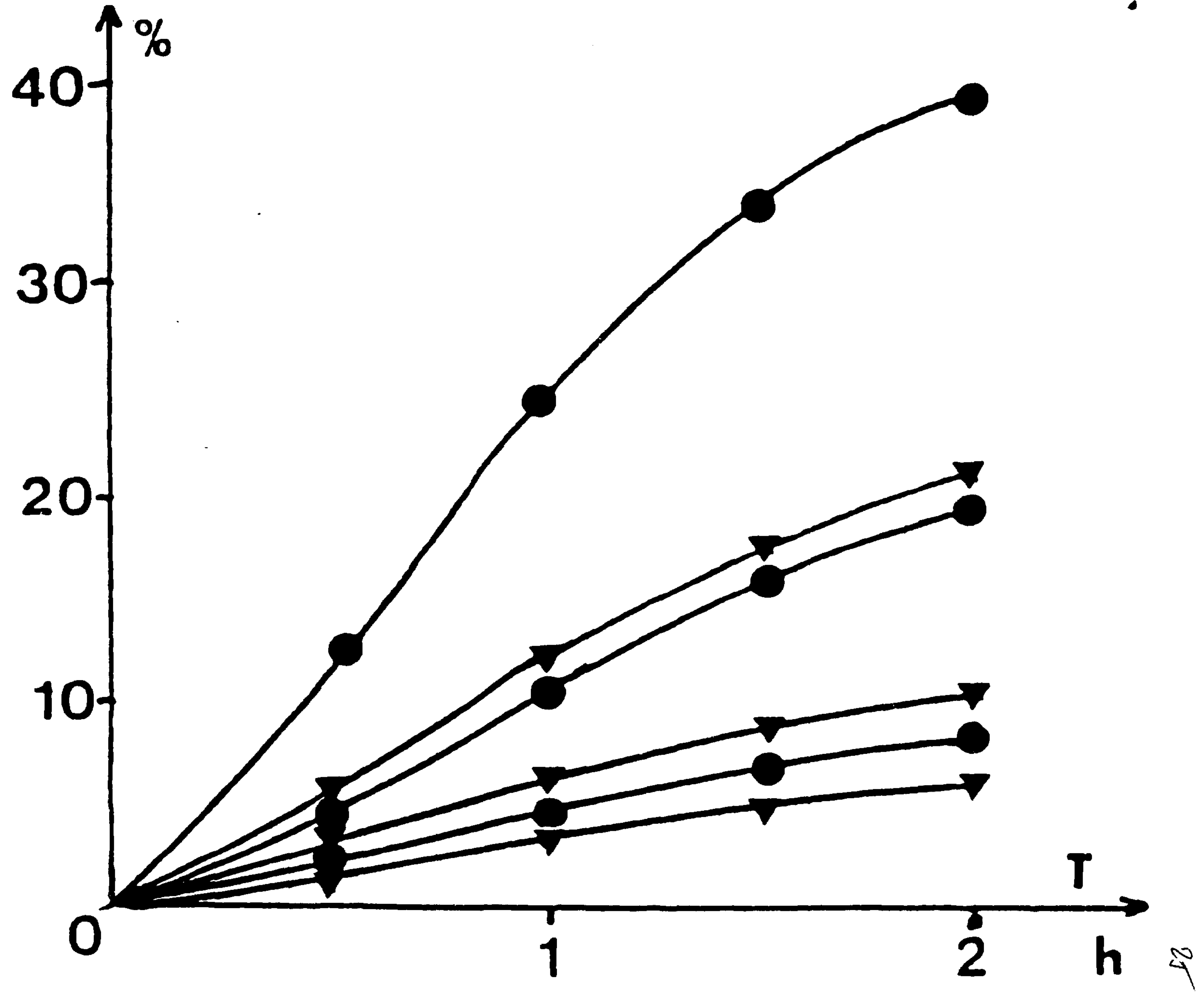
$$\bar{t} = \frac{\int_0^\infty t \cdot c(t)\,dt}{\int_0^\infty c(t)\,dt} \qquad \bar{t}_{water} > \bar{t}_{vasc.}$$

Distribution Volume $= \dot{Q} \cdot \bar{t}$

Extravascular Pulmonary Water

$$idQwl = \dot{Q}\,(\bar{t}_{water} - \bar{t}_{vasc.})\,F_{wb}$$


mg.l -1
10
5
0
{1}
2000 ppm
1000
{2}
ppm
4000
3000
{3}
{1}
{2}
{3}
5 s
t



25

%. $^{13}CO_2$

140
130
120
110

6.2.76
7.2.76
13.2.76

16 U insuline

6
12 h
T

%. $^{13}CO_2$

140
130
120
110

27. 2.76
28.2.76
5.3.76

16 U insuline
+ 8 U

6
12 h
T

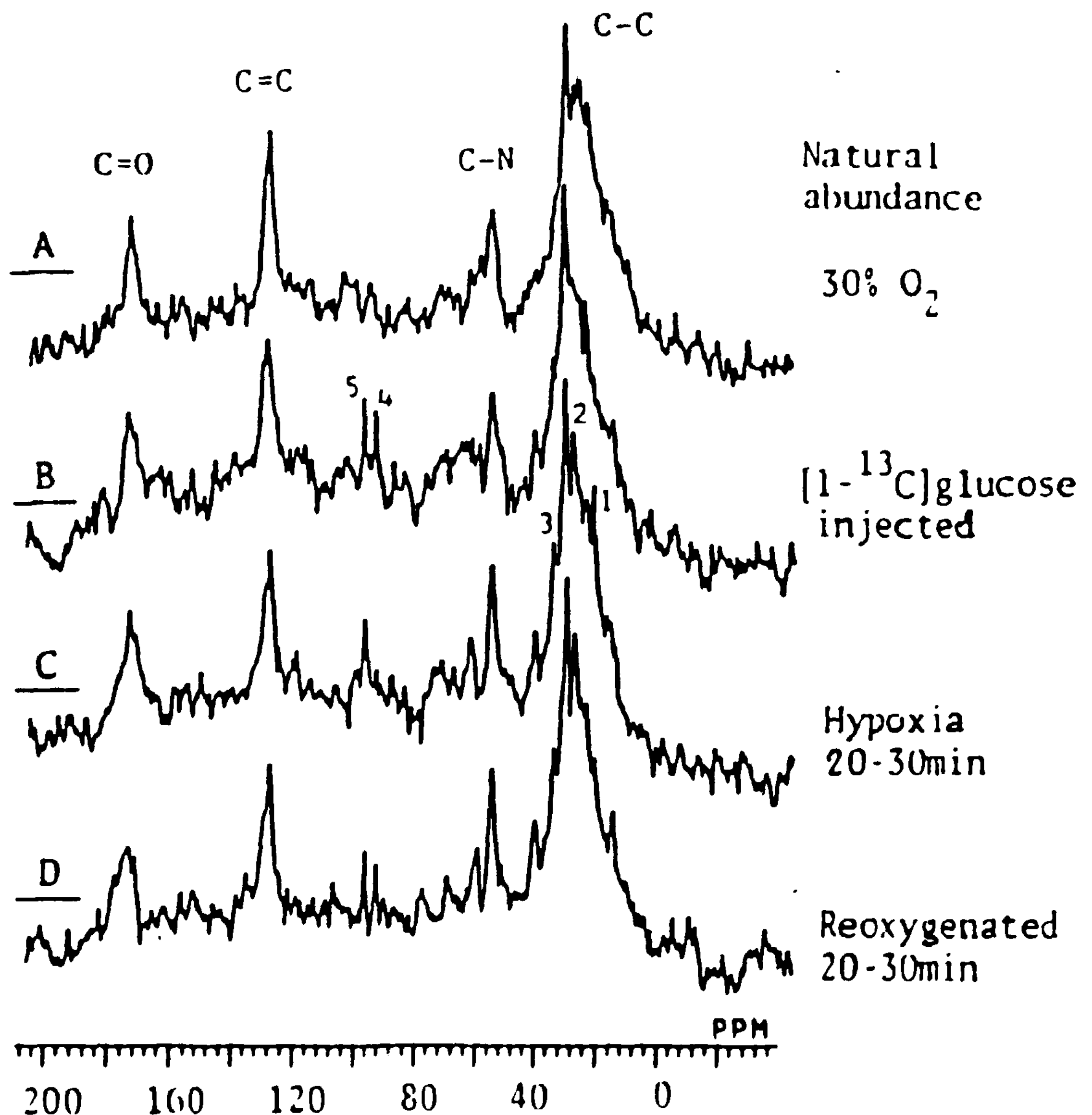


Fig.1. Changes in 13C spectra of rat brain during hypoxia. Numbered signals were assigned as follows; 1:lactate (C3), 2&3:glutamate/glutamine(C3&C4), 4:α-glucose(C1), 5:β-glucose(C1).

8.8

Fig. 1

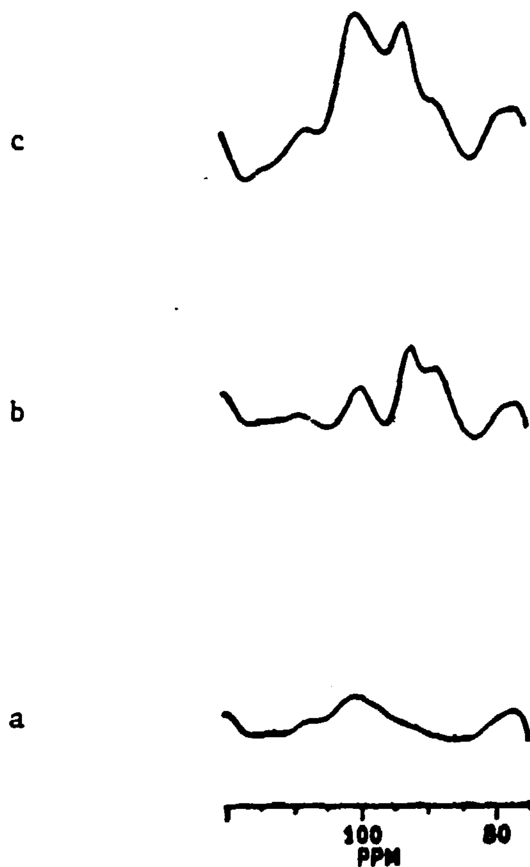


Fig. 2