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CRITICAL EVALUATION OF THE USE AND ANALYSIS OF STABLE ISOTOPES

(Technical Report)

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Critical evaluation of the use and analysis of stable isotopes (Technical Report)

Abstract: In this paper the methodology of isotopic analysis is discussed. In the first instance because a choice of analytical methods often conditions the success of research carried out with stable isotopes. The methodology of use is treated in two different chapters according to their resting or not on measurements of isotope effects. In the second case, stable isotopes are specially useful for isotope dilution analysis, studies of transport phenomena, medical diagnoses. Advantages and drawbacks of their use with respect to that of radioactive isotopes are described and examples are given. Usage of isotope effects is a specificity of stable isotope work. Methods of measuring equilibrium and kinetic effects are explained. A discussion is given of tenets of applications in research in pharmacology, geochemical processes, and of kinetic effects in studies of reaction mechanisms, especially in biology. Utilisations of nuclear properties, particularly in medicine, are mentioned. This report does not cover techniques of isotope separation, preparation of labeled compounds, uses of isotopes in dating methods or in nuclear industries.

CONTENTS

I. INTRODUCTION AND OVERVIEW

Introduction

Following the discovery of "heavy hydrogen" by H.C. Urey, in 1931, uses of stable isotopes in biochemistry started with the pioneering work of Rudolf Schoenheimer, after his meeting with Urey (ref. 1). He was soon followed by David Rittenberg. Their researches were almost simultaneous with the work by G.C. Hevesy on radioactive tracers that earned him a Nobel prize.

The volume of applications of radioactive isotopes expanded rapidly when cyclotrons, and later experimental nuclear reactors, made artificial radioactive elements available. The pace at which stable isotopes uses have grown has been much slower, in spite of the development of methods to prepare separated stable isotopes useful for tracer work, and of considerable improvements in analytical techniques.

Though both stable and radioactive isotopes can be employed for many applications, there are fields specific for stable isotope work as well as others of radioactive ones. In some cases a simultaneous use of both kinds is rewarding.

Overview of uses of isotopes

Isotopes can be used as tags turning elements, or molecules which contain the elements, into tracers when their abundances are different from their normal values. This is always the case when artificially produced radioisotopes are employed; stable isotopes must either be partially or totally separated to be used, or else belong to elements having naturally a different isotopic composition from that of the material under study.

When there is no isotope effect, i.e. when, leaving out nuclear properties, no change of chemical or physical property is induced by the substitution of an isotope for another, isotopes may be used:

a) as tracers of the fate of elements or molecules in any process, in the laboratory, in industry or in nature. In the latter case 'natural' tracers are often used.

b) as tools for quantitative measurements by isotope dilution analysis, which leads to applications in many fields. For these uses both stable and radioactive isotopes are eligible.

Isotope effects can be exploited for:

a) measurements of equilibrium effects that have most applications in life sciences and in the study of natural processes

b) measurements of kinetic effects which serve to establish reaction mechanisms, specially in the field of enzyme reactions.

Studies of isotope effects are specific of stable isotope work. The only notable exceptions are uses of tritium, and, to a lesser extent, of carbon 14.

Differences of nuclear properties lead either to applications in the field of nuclear energy, or of production of radionuclides, or to use in medicine and various fields where either capture or induced emission of radiation are utilized.

In addition to uses mentioned above, we refer the reader to the literature for the following, which are outside the scope of this report: Studies of isotope separation (ref. 2), Utilisation of separated isotopes for the nuclear industry (ref. 3), Synthesis of labeled molecules, and uses of separated stable isotopes to produce purer radioactive ones (refs. 4, 5, 6) and Uses of stable isotopes for dating methods (ref. 7).

Comparison of uses of stable and radioactive tracers

(i) Advantages of radioactive isotopes

a) For tracer work, radioactive isotopes are often preferred to stable isotopes because they are easier and faster to measure.

b) They lend themselves to detection in situ, in vivo, and to imaging, which can be achieved with stable isotopes by NMR, but almost exclusively with carbon and hydrogen isotopes, and by SIMS mass spectrometry with all isotopes, but only to some degree.

c) except in cases of nuclides having a half life of more than a few years, and when special techniques are used as with tritium or ^{14}C (ref. 8), detection limits are lower for radioactive isotopes, when using counting techniques, than those permitted by stable isotope detection procedures

d) Radioactive isotopes often appear cheaper to employ, even when adding costs of waste disposal or radioactive protection, for which users sometimes do not pay directly.

e) Radioactive tracers are followed by detecting a single isotope. Only NMR enables one to measure usefully individual stable isotopes, and thus to make use of monoisotopic elements, like fluorine, as tracers.

f) Stable isotope analysis, except by NMR, rely on measuring abundance ratios and therefore applies only to the sixty three polyisotopic elements.

(ii) Advantages of stable isotopes

Applications to tracer work

In addition to advantages that are unnecessary to point out when handling non radioactive substances:

a) Drugs labeled with stable isotopes, e.g. deuterium at very high concentrations, have no reported toxic effect. Even heavy water can be ingested in large quantities without causing any harm, (ref. 9) and Halliday in (ref 5a). However when drugs are administered their level of detection may be higher when labeled with stable isotopes than with radioactive ones, and the necessary quantity of drug, that may be toxic per se, is correspondingly larger.

b) Molecules labeled with stable isotopes do not undergo radiolysis, that compells to periodic purification, and radiation from tracers might, in some cases, induce disturbing intra, or inter molecular exchanges.

c) Stable isotopes are the only ones that can be employed for most of the work with hydrogen, carbon, nitrogen and oxygen. Radioactive isotopes of nitrogen, oxygen and ^{11}C are too short lived to carry out studies of processes longer than a few minutes. Tritium and ^{14}C are so long lived, and enter so many compounds that for low level work special precautions are required to avoid contaminations. In biology they are now restricted almost exclusively to in vitro research.

d) Analysis of stable isotopes by NMR and, less easily, by mass spectrometry enables the observation and following of the position of an isotope in a molecule along a reaction path. This is only obtained, with radioactive tracers by the painstaking process of isolating the labeled part of the molecule, chemically or by enzymatic action.

e) The simultaneous use of several tracers, easily achieved with stable ones, is, at least, much more difficult, with radioactive tracers.

f) When studying metabolisms, dilution of a stable tracer by an endogeneous chemically identical compound is easily detected by the analytical process. Such mixing is almost impossible to detect when using radioactive tracers.

Applications resting on isotope effect measurements

a) Isotope effects can only be studied with accuracy with stable isotopes. With the exception of a few occasions where a stable and a sufficiently long lived radioactive isotope of the same element are used, because stable isotope techniques, and principally mass spectrometry, provide better precision, greater accuracy than radioactive techniques. Exact comparisons of detection limits must take into account specific counting equipment and mass spectrometers used (ref. 10).

b) Research on reaction mechanisms in chemistry and biology and their applications in pharmacology are based on isotopic effect measurements. They are therefore specific of stable isotope work - so are studies utilizing isotopic fractionations in nature.

c) Changes in isotopic composition, due to isotopic effects occuring in natural processes, are used for geological temperarure determinations, studies in climatology, agricultural researches, and many other applications.

d) Isotope effects on isotopes of hydrogen can be larger, by an order of magnitude or more, than those on isotopes of other elements. Working with hydrogen isotopes thus offers special possibilities; but, a contrario, precautions are required in order that experiments where they are used as tracers are not perturbed by spurious isotope effects.

Contents of this report

This report distinguishes uses of isotopes which do not rest on isotope effects, which can even introduce errors, from uses whose foundations are evaluations of such effects.

a) Part II discusses methods of isotopic analysis, an essential part of isotope work, because analytical techniques must be chosen before starting any application of stable isotopes.

b) Part III deals with uses in the absence of isotope effects.

c) Part IV deals with the use of isotope effects, and, briefly, with uses of nuclear properties.

d) A conclusion attempts to outline conditions for the expansion of the uses of stable isotopes.

e) Appendix A gives fundamental rules of the theory of equilibrium and kinetic effects.

f) Appendix B reports values of isotopic effects arising from different thermodynamic and kinetic origins and shows possible differences in orders of magnitude between them.

II. ISOTOPIC ANALYSIS

Main options

Choice of one of two categories of measurements

The first category includes methods that serve to identify, to follow the fate, or to measure the concentration of a single isotope. For some flow measurements or studies of biological pathways, identification only is required. In this first category, stable isotopes are in competition with radioactive ones. The second category comprizes methods that measure ratios of isotopic abundances. They are used for applications of isotope dilution analysis and isotope effects. In this category radioactive isotopes can only exceptionally be employed.

Isotopic ratios may be expressed either in absolute values, for instance for dating, or for isotope dilution analysis, or by differences with standard reference samples as in many climatic researches.
When accurate absolute values are required calibration of analytical instruments on an absolute scale is mandatory. Certified reference samples for that purpose can be procured (ref. 17).

For comparisons of relative isotopic abundances calibrations are not necessary; but in intralaboratory work one should always utilize the same method, the same instrument, and analytes under the same chemical form; for interlaboratory comparisons, common reference samples must be used.

Choice of analytical techniques. This choice may be critical to insure the success of stable isotope applications; a review of their possibilities starts, for this reason, this report. A method of isotopic analysis has to take into account of:

a) the importance of accuracy, precision, limits of detection, amount of sample

b) the nature of the molecule to which belongs the element whose isotopes are to be analysed, its physical state.

Reviews of modern isotopic analytical methods are numerous (ref. 18). Table 1 shows preferential fields of applications of analytical techniques.

Expressing relative isotopic abundances, the δ notation

Relative abundances have become the usual way of reporting data on isotopes of hydrogen, oxygen and carbon that have one main light isotope and one heavier isotope of low abundance. ^{17}O, having only an absolute abundance of 0.04%, is usually not taken into account (ref. 19).

One measures the ratios R of abundance of the heavy to the light isotope, the subscript s pertaining to samples, and st to standards. And δ is defined by (1)

$$\delta = (R_s/R_{st}-1)\cdot 1000 \qquad (1)$$

Caveat: δs are not additive. Consider two samples, 1 and 2

$$\delta_1 = (R_{s1}/R_{st}-1)\cdot 1000,$$

and

$$\delta_2 = (R_{s2}/R_{st}-1)\cdot 1000,$$

to compare directly sample 1 and sample 2, one defines δ_{12}

$$\delta_{12} = (R_{s1}/R_{s2}-1)\cdot 1000,$$

it is then seen that δ_1 is not equal to $\delta_{12}+\delta_2$, but is given by

$$\delta_1 = \delta_{12} + \delta_2 + (\delta_{12}.\delta_2)/1000 \qquad (2)$$

The difference is significant. In the case of carbon for instance, where precisions on δs of 0.1 are often required, as δs of 40 may be observed the last term in (2) could induce corrections of 1.6. When hydrogen isotopes are concerned, δs of several hundreds are encountered, either due to natural abundance variations of deuterium or to fractionations in the laboratory, inducing even larger corrections.

TABLE 1. Analytical Methods of Isotopic Analysis

Method	Advantages	Drawbacks	Principal uses
Mass Spectrometry	Usable for all isotopic ratios. Precise for sensitive analysis of minute samples and heavy organic molecules.	Memory effects. Continuous maintenance of a good vacuum. Cost of instruments.	Accurate isotopic abundance determinations. Isotope dilution analysis. Trace analysis. Metrology
Nuclear Magnetic Resonance (NMR)	Follows a single nuclide. Identifies chemical bonds. Imaging possible. Especially sensitive for hydrogen and ^{13}C	Observes only nuclides with uneven nuclear spins. Not suited for isotopic abundance measurements.	Studies of biological reactions. Measurements of intramolecular isotope effects. Product identification
Optical Methods	Cheap instrumentation. Adapts easily to routine work. Adaptable to field work.	Each analysis requires a special study. Often less precise than mass spectrometry.	D & ^{15}N analysis. Studies of reaction kinetics. Work in situ
Density Nuclear & Other Methods	Enable to solve specific problems. May be easier of cheaper.	Each method requires a special study usually for a single type of analysis	D_2O analysis. Allowing in situ analysis, e.g. neutron activation.

Use of reference samples

Reference samples mentioned in the preceding paragraphs are of two kinds. The first type are those whose absolute isotopic composition is not certified and are employed only to enable valid intercomparisons between laboratories. As they are used mostly in geochemical studies, they are often natural samples, such as PDB, a belemnite, a fossil, essentially a carbonate, used as standard for ^{13}C

determinations, or the Standard Mean Ocean Water, (SMOW), initially proposed by Craig, for deuterium and ^{18}O, as having an isotope composition as close as possible to the average of ocean waters from which deviations are small (ref. 20). Reference samples of the second kind are prepared by mixing known quantities of isotopes as completely separated as possible. They have an accurate isotopic composition, within calculable limits of error. Accuracy and precision of such samples are found in the reports of the Commission of atomic weights and isotopic abundances, CAWIA, of IUPAC, regularly published in *Pure & Appl. Chem.* They are used to establish absolute isotopic contents.

SMOW was linked to a standard water no longer distributed by the National Bureau of Standards (NBS), and PDB also is no more available and referred to only through secondary standards. To overcome those difficulties the International Atomic Energy Agency, (IAEA), defines a V-PDB close to the former, and tied to an available NBS 19 standard through specified δ values of ^{13}C and ^{18}O, and distributes two reference waters for deuterium, and oxygen isotope analysis: V-SMOW, (Vienna, Standard Mean Ocean Water), which is distilled ocean water, and a standard low in deuterium and ^{18}O, that was prepared by mixing melted antarctic ice to ocean water, V-SLAP, (Vienna Standard Low Antarctic Product,).

The reason why it was considered necessary, by IAEA, to prepare two reference waters was to enable laboratories to check the validity of their scale of responses. As for V-PDB, it is important not to drop the V of V-SMOW, as SMOW may stand for slightly different laboratory reference samples, and the use of V-SMOW should imply also that measurements have been normalised to the V-SMOW, V-SLAP scale. Values of δs to use with the Vienna standards are given by Coplen (ref. 21).

To insure the possibility of reproducing those samples in deuterium their absolute content was determined before distribution began (ref. 22). A difficulty lies in establishing a scale starting from a true zero deuterium value, because contamination of samples and apparatus by minute quantities of ordinary water, which has about 150 ppm of deuterium in temperate regions, is very difficult to avoid. A combined technique of preparing deuterium free water, isotope dilution, and analysis of two phases in equilibrium was developed (ref. 23). Absolute ^{18}O contents are more dificult to work out since natural abundance is much higher (2000 ppm), and it is more difficult to make water ^{18}O free than deuterium free.

Treatment of samples

Stable isotope analysis often requires, especially when mass spectrometry is the analytical technique, that the analyte be introduced into the analytical instrument under a chemical form different from that of the material sampled.

When determinations of isotope effects, or isotope dilution analysis, are made, no isotopic fractionations should take place, either during sampling, or when pepreparing the analyte.
Absence of isotopic fractionation may be difficult to verify, but risks can be minimized. For instance chemical conversions, such as dissolutions of solids to avoid the risk inhomogeneities, should be made as complete as possible to exclude possible fractionations.

To compare isotopic abundances of elements belonging to chemically different species one must put the samples to be analysed under the same chemical form, valence state included. Biases due to differences between chemical forms of samples would occur, for instance, if one were to compare the H/D ratio of hydrogen gas, measured with a mass spectrometer on peaks at mass 2 for H_2, and 3 for HD, with the ratio of peaks of the mass spectrum of water at mass 16 for H_2O, and mass 17 for HDO. Neither would it be sound, in order to compare those ratios, to measure them in hydrogen by mass spectrometry, and in water by infrared absorption, though, theoretically, an absolute calibration of analytical instruments of both types could be a valid procedure. Thus only one technique is to be used: if it is mass spectrometry water should be reduced to form hydrogen, if it is infrared hydrogen must be burnt to form water.

Review of techniques

(i) Mass spectrometry

Mass spectrometry measures isotopic ratios. In spite of the development of other techniques it remains the only universal method of isotopic abundance analysis. It is also the most precise, sensitive and accurate one. When, instead of isotopic ratios, concentrations of one isotope are required either isotope dilution is used or mass spectrometry is coupled with another method. Accuracy is obtained more easily than with other methods because yields of production, and collection of all isotopic ions of an element are very close to one another, and mass discrimination is comparatively well understood. Corrections of raw data are therefore comparatively small, and calibration with separated isotopes need not be too costly, due to the small amount of analyte required. Efforts are made also to measure absolute ratios without employing calibrated reference samples, especially at the Institute for Reference Materials and Measurements at Geel. Table 2 illustrates performances of conventional mass spectrometry.

Processing of samples before analysis. Mass spectrometry always requires processing of samples before they can be introduced into a mass spectrometer. Necessary processes may be complex. For instance analysing oxygen isotopes from solids, specially rocks, involves liberating it as gas almost necessarily by fluorination. To accomplish this under mild conditions decomposition under the action of solid cobalt difluoride can be used. Recently chlorine trifluorine has been employed, the reaction being started by heating the sample with a laser radiation. Milligram samples can be analysed with a precision of 0.1 δ, allowing for corrections to be made for bias introduced by the process (ref. 24).

Special treatments. Some treatments may simplify the analysis, as shown by the following example. A gas of molecules possessing several atoms of the same polyisotopic element consists, at equilibrium, of molecules containing isotopes randomly distributed, therefore of several isotopic compositions. In order to measure the overall isotopic composition one may avoid to measure the amount of each kind of molecule by establishing a calculable equilibrium repartition of isotopes.

For example di-nitrogen has two isotopes ^{14}N, and ^{15}N whose natural abundance is only 0.36%. At equilibrium, at natural abundance of ^{15}N, it is sufficient to measure the ratio of the peak at mass 29, corresponding to the $^{14}N^{15}N$ molecule to the peak at mass 28 of the $^{14}N_2$ molecule to calculate the ratio of isotopes, because the probability of occurence of $^{15}N_2$ ions at mass 30 is about $1.6\ x10^{-5}$. When the gas is very enriched in ^{15}N the peak at mass 30 is no longer negligible, but if equilibrium prevails it can be calculated and one may still limit measurements to peaks at mass 29 and 28. If necessary one establishes the equilibrium distribution; an electrodeless discharge is described for that purpose (ref. 25).

Different kinds of mass spectrometers. There exists several kinds of mass spectometers to meet specialized needs, all operate under two constraints. Firstly, mass spectrometers must be kept under vacuum which necessitates continuous pumping; instruments can not be switched on and off at will, nor are they easily adaptable to field work. Secondly, samples are introduced inside the vacuum chamber, this induces the possibility of memory effects, it is not advisable to run without careful precautions samples of widely different isotopic compositions one after the other on the same instrument. It is only possible here to mention a few arrangements used for isotopic analysis, and to refer to a review of techniques (ref. 26).

(TABLE 2. Isotopic composition of elements determined by mass spectrometry)

Atomic Number	Element	Mass Number	Evaluated Range of Natural Variations (Atom %)	Anno-tations	Best Measurement from a Single Terrestrial Source (Atom %)
1	H	1	99.9978 - 99.9816	r, g	99.984426(5) 2s C
		2	0.0184 - 0.0032	m	0.015574(5)
2	He	3	0.0041 - $4.6\text{x}10^{-8}$	r, g	0.0001343(13) 1s C
		4	100 - 99.9959		99.9998657(13)
3	Li	6	7.68 - 7.30	r, g	7.525(29) 2s C
		7	92.70 - 92.32	m	92.475(29)
4	Be	9			100
5	B	10	20.316 - 19.055	r, g	19.82(2) 2s C
		11	80.945 - 79.684	m	80.18(2)
6	C	12	99.01 - 98.85	r	98.8922(28) P C
		13	1.15 - 0.99		1.1078(28)
7	N	14	99.652 - 99.615	r, g	99.6337(4) P C
		15			0.3663(4)
8	O	16	99.7780 - 99.7447	r	99.7628(5) 1s N
		17	0.0407 - 0.0350		0.0372(4)
		18	0.2147 - 0-1879		0.20004(5)
9	F	19			100
10	Ne	20	90.514 - 88.47	r, g	90.484(9) 1s C
		21	1.71 - 0.266	m	0.270(1)
		22	9.96 - 9.20		9.246(9)
11	Na	23			100

In this excerpt from the 1995 report by CAWIA, *Pure & Appl. Chem.* (in press), column 4 covers the extreme abundances published from evaluated measurements when a range of natural variations has been established. Published data may exceed that range. In column 5, r indicates that there is a range in isotopic composition of normal terrestrial material, g that geologically exceptional specimens are known with isotopic compositions outside the preceding range; m indicates that modified isotopic compositions, due to isotopic fractionation, may be found in commercially available material. Column 6 gives the best published measurements from a single terrestrial source, that may come from any part of a natural range. Numbers in parenthesis are uncertainties on the last digit, as published; 1, 2, 3s indicate 1, 2, or 3 standard deviations, P is some other type of error, mentioned by the author. C is appended when calibrated mixtures have been used to correct mass spectrometer biases; the measurement is often said 'absolute'. N is appended when none of the above requirements are met.

Gas source instruments. Such instruments, either of the magnetic sector type or quadrupoles, whose range continually extends toward higher masses, can analyse without special modification any non corrosive gas.

Isotope Ratio Mass Spectrometers (IRMS). Such instruments compare directly the intensity of isotopic peaks, and are not always capable of scanning spectra. They are adapted to routine isotopic analysis especially for H, C, N, O analysis. Double inlet systems enable to compare reference and unknown samples. They are used for drug research, breath tests, and the like, (ref. 27). Quadrupole instruments are equally employed. Continuous flow isotope ratio mass spectrometry (CF IRMS) is well adapted to routine work, (ref. 27). They are often low mass resolution, magnetic sector, multicollector instruments, completely automatized and computerized. Computerization, with all its advantages, has slowed down reductions in prices linked to economies made by using small permanent magnets, or simple quadrupole devices. It sometimes also induces a loss of flexibility; for instance instruments set to measure ^{13}C or ^{18}O at mass 45 or 46 in the CO_2 mass spectrum can rarely measure the doubly labeled gas at mass 47.

Inductively coupled plasma mass spectrometry (ICPMS). This arrangement is increasingly used for multielement analysis and for obtaining low detection limits. ICPMS, combined with flow injection, enabled to analyse, at the three times background level, in natural waters, approximately 3 pg of Re, 6 pg of Ir, and 14 pg of Pt (ref. 28). Detection limits are compared to those permitted by six other methods. In sea water, the same technique was applied for Ni, Cu, Zn, Ag, Cd, Pb (ref. 29). In most cases ICPMS is a good solution to obtain high sensitivities, as shown by studies of heavy metals in sediments that have established the size of extractible and exchangeable fractions of Pb, using aqua regia as solvent, and of traces of Cd by isotope dilution analysis (ref. 30). It also offers the possibility of multielement analysis (ref. 31).

Mass spectrometers coupled to gas chromatographs (GCMS). GCMS, and more recently coupling of mass spectrometers to high performance liquid chromatographs, takes an ever increasing importance in the analysis of heavy organic molecules.It enables to analyse, in an isotopically selective way, metabolites of drugs for instance. As isotopic species overlap in the chromatograms reaction vessels can be placed between the chromatographs and the mass spectrometers. In these vessel complex molecules can be converted into simpler ones: CO_2, for the analysis of carbon or oxygen isotopes, $(CN)_2$ for nitrogen isotopes, H_2O for deuterium. Sensitivities of detection of a few picograms of isotope per second are reported (ref. 32), and instrumentation is described (ref. 33)

Thermal ionization source mass spectrometers (TIMS). They are used to produce positive ions, and analyse isotopically all but a few elements, when these are deposited, usually as oxides, on a heated filament. Negative thermal ionization (NTI) can also be used in many cases. It is shown that precision is improved by an order of magnitude (ref. 34), and a higher yield than by positive emission is obtained for some elements. P, As and Sb are reported in (ref. 35), other elements, including halogens, Se, Te, B, Mo, W and Os in (ref. 36). The relative standard deviation obtained on 100 pg to 1 ng amounts of osmium is 0.004% on the $^{189}Os/^{192}Os$ ratio (ref. 37).

Secondary Ion Mass Spectrometers (SIMS). This technique (ref. 38) enables to scan surfaces, and some arrangements offer imaging possibilities. In mineralogy, SIMS has been used for isotope determinations of uranium, of lead in uranium oxides and other minerals to understand mechanisms which control their genesis (ref. 39).

Fast atom bombardment sources (FAB). Such sources are well suited for organic and biological material.e.g. for simple, sensitive and precise measurements of Zn isotopes in biological samples (ref. 40).

Optical ionization sources. They are adapted to difficult analysis, and to the need for high sensitivities. But for optical excitation methods, a case by case study must be made. Isotopic discrimination, though possibly large, may be avoided (ref. 41). Saturation laser ionization techniques are described in the preceding reference. Photon burst mass spectrometry tries to rival AMS (ref. 42). Laser resonance ionization techniques (RIT) enable to measure isotopic ratios, e,g. of Os, on micro to picogram quantities.

Accelerator mass spectroscopy (AMS). It has been developed for very high sensitivity in detecting low abundance, long lived radioisotopes; it can be applied to stable isotope analysis for minute samples and trace elements (ref. 43). Reviews of the subject are found in ref. 26 and principally ref. 8, where its detection limits, for 13 elements, that range from 10^{-9} to 10^{-12} gram per gram, are compared with those of atomic absorption, instrumental neutron activation, PIXE, ICP-MS, SIMS.

Specially designed mass spectrometers. They are built in laboratories having precise requirements. Some working under static vacuum enable to analyse very small samples; for example as little as 10^5 atoms of helium (ref. 44), or carbon isotope ratios, on 10^{-9} moles of CO_2 (ref. 45).

Mass spectrometers connected in series. They belong to what is described as tandem mass spectrometry (ref. 46), or MS-MS. Their use may simplify. the analysis of complex mixtures, for example that of labeled drugs excreted in urine when monitoring the exposure of patients to carcinogenic agents.

(ii) Nuclear magnetic resonance (NMR)

NMR detects directly only nuclides having an uneven nuclear spin. The intensity of NMR signals is proportional to the number of nuclides detected and to their relative sensitivities. Positions of signals depend on the chemical bonding of the atom and the nature of its neighbours. Examples of sensitivities, which may differ by orders of magnitude even between isotopes of the same element, are given in table 3 (ref. 47). Recent developments of usages of isotopic studies with NMR are found in ref. 48.

NMR offers the possibility of studying monoisotopic elements, which is not feasible by mass spectrometry. But measurements of isotopic ratios are not straightforward by NMR because it can not, in most cases, detect all the isotopes of an element and, when it can, detection limits may, like sensitivities, differ by orders of magnitude. As ratios of isotopic abundances are not directly measurable by ratios of signals heights or surfaces, calibration of signals for measuring abundances is painstaking because nuclides to be compared should be in equal numbers, or in known ratios. Nevertheless, NMR is used for deuterium abundance determinations, see NMR 20 in the series quoted (ref. 48). An advantage over mass spectrometry is that converting a sample to water is never necessary

TABLE 3. Comparison of nuclides used in NMR. From NMR 22 Isotope effects in NMR spectra, Diehl et al. eds., by permission of Springer verlag, Berlin, (1990).

Nuclide	**Nuclear Spin, I, Multiples of h/2π**	**Natural Abundance (%)**	**Realtive sensitivity constant of nuclei at constant field**	**Sensitivity corrected to the natural abundance**	**NMR Frequency at 1 kG (MHz)**	**NMR Frequency at 23.5 kG (MHz)**
^{1}H	1/2	100.0	1	1	4.257	100.0
^{2}H	1	0.015	$9.65x10^{-3}$	$1.4x10^{-6}$	0.653	15.3
^{14}C	1/2	1.11	$1.59x10^{-2}$	$1.8x10^{-4}$	1.070	25.1
^{14}N	1	99.63	$1.01x10^{-3}$	$1.0x10^{-3}$	0.308	7.24
^{15}N	1/2	0.37	$1.04x10^{-3}$	$3.8x10^{-6}$	0.431	10.1
^{17}O	5/2	0.04	$2.91x10^{-2}$	$1.2x10^{-5}$	0.577	13.6
^{19}F	1/2	100.0	$5.33x10^{-1}$	$8.3x10^{-1}$	4.005	94.0
^{23}Na	3/2	100.0	$9.25x10^{-2}$	$9.3x10^{-2}$	1.126	26.5
^{31}P	1/2	100.0	$6.63x10^{-2}$	$6.6x10^{-2}$	1.723	40.5

and that site-specific contents may be obtained. Precision quoted around the natural abundance in deuterium, which is about 0.015%, is 0.2%. Absolute abundances can even be obtained after going through delicate calibration procedures. When a low abundance isotope is studied variations in the intensity of signals provide a relative evaluation of changes of abundance.

Characteristics of NMR measurements:

a) sensitivity increases with the magnetic field. At 12.5 T it is possible to study molecules up to 100 kilodaltons

b) whereas sensitivity depends on the strength of the field, ratios of sensitivities of isotopes are constant. For instance, sensitivities of pure H and D are 1 and 9.6 $\times 10^{-3}$. Thus at normal abundance (D/H around 1.5 $\times 10^{-4}$), the ratio of signals is 1/1.45 $\times 10^{-6}$. The precision on this ratio, even using instruments of only 2.35 T could be 0.25%.

c) sample volumes in "ordinary chemistry" are in the cubic centimeter range, but one can sometimes employ as little as a few tenths of cubic millimeters. NMR also enables one to investigate very large sizes, for instance to follow an isotope in vivo in the liver of man.

d) NMR instruments can provide imaging, as practiced in medicine.

e) The time required for an analysis depends on many factors. For instance, while it takes 6.8 s only, with a given spectrometer, to register one spectrum of ethyl alcohol, when measuring the deuterium abundance in ethyl alcohol it is necessary to carry out 10 runs of 300 spectra each to obtain the precision quoted above, cf. Martin and Martin in NMR 23 (ref. 48).

f) Structural investigations of heavy molecules requiring four dimensional analysis may take several months.

Summary

a) The invaluable advantage of NMR is that it enables one to follow the fate of a given isotope in a reaction pathway, and to establish the position it occupies in a molecule. It has thus become the standard method to follow successive steps of organic molecule synthesis.Uses of NMR in these fields have developed considerably with ^{13}C NMR in biochemistry because ^{13}C chemical shift range is so large that chemically distinct atoms in a molecule exhibit distinct resonances that can be studied separately using isotopic enrichment when necessary.

b) NMR is a tool for the study of molecular structures. Multi-dimensional analysis is a powerful technique for this purpose. It consists in following the relaxation of NMR signals, subsequent to pulses inflicted on the sample, according to a strict protocol. Up to 4D analysis are reported.

c) NMR is the easiest technique of observing intramolecular isotope effects and of site specific identification. Deuterium site specific concentrations are extensively studied at natural abundances of deuterium (ref. 49). Carbon isotopes site specific ratios, at natural abundances, can be studied by NMR, with more limited applications than those allowed by the use of mass spectrometry.

d) The shape of NMR signals can be used to measure isotopic exchange rates.

(iii) Optical methods

Specifics of optical methods. Optical methods rest on a choice of optical lines, or bands, in absorption or emission that must be established for each individual isotopic analysis. Calibration of isotopic ratios is needed, because different isotopes are measured on different lines. Thus optical methods are preferentially used to study series of similar samples. They may be employed in absorbtion, as with Fourier transform infrared techniques (FT-IR), in emission with atomic emission detection (AED).

Advantages of optical methods:

a) To make possible a better analysis (faster, more precise, etc..), for instance the saturation technique enables to detect as little as 5×10^{10} atoms of an isotope of osmium in a 50 ng sample (ref. 50).

b) To avoid having to purify samples, when it is possible to work on such narrow lines that interferences of impurities can not occur. Also memory effects are less to be feared than in mass spectrometry because samples are introduced in cleanable cells.

c) To make analysis from a distance possible, particularly in space (ref. 51).

d) To enable to work in the field.

e) To be cheaper than other methods.

Applications, comparisons with other methods:

Because of their specificity that balances their advantages, optical methods are mainly used for the analysis of isotopes of hydrogen and nitrogen and actively studied for those of carbon.

a) *Hydrogen isotopes.* The analysis made on the Balmer lines of electroytic hydrogen, by Urey and Brickwedde, proved the existence of deuterium in 1931. The analysis of deuterated waters, in the near infrared, has developed consistently since 1951. Its main advantages are that water can be analysed without being reduced to hydrogen, as is the almost universal practice with mass spectrometry, and that for nearly pure heavy waters contamination risks are minimized. Its principal uses are in the nuclear industry, to monitor heavy water in production plants and nuclear reactors (ref. 52), and for biological or medical purposes at tracer doses, for instance to measure total body water, or fat mass (ref. 53). Fourier transform analysis offers, among others, advantages of flexibility (ref. 54). Infrared techniques enable to make measurements of deuterium in vivo and in situ, on continuous flows, even in blood (ref. 55), without chemical treatment. It has been used on animals (ref. 57), and also on patients in hospitals (ref. 56).

Assay of D_2O in body fluids has also been reported by measures of refractive index (ref. 57). The detection limit of D_2O in H_2O is 10^{-6}. Deuterium abundances in planetary atmospheres can be measured from the earth on HD or CH_3D lines (ref. 51).

b) *Nitrogen isotopes.* Optical methods were first devised to enable determinations useful for agricultural research to be made inexpensively, and possibly in the field (ref. 58), because analysis of ^{15}N is central in many agricultural, and also biological or environmental studies. Special spectrometers were first commercialized using the emission lines of N_2 excited by a high frequency discharge. Recent mass spectrometry and optical emission procedures and instruments are discussed (ref. 12c). Most measurements are made at concentrations below 5% ^{15}N. Mass spectrometry is able to give results with a relative precision of one per mil; the precision of optical emission results is rather of one percent. High precision techniques might enable to use nitrogen depleted in ^{15}N as tracer, which is much less expensive (ref. 59).

Advantages of mass spectrometry are precision, the small amount of sample required, down to the microgram range, instead of the milligram. Low cost and simplicity are the assets of optical measurements. But small benchtop mass spectrometers are in wide use and tend to be competitive in price, whereas optical instruments become more sophisticated and precise.

c) *Carbon isotopes.* CO_2 is the usual molecule handled when analysing carbon isotopes whether it results from the combustion of a sample or is the gas analysed in breath tests. The standard method for ^{13}C analysis is mass spectrometry, well described in many texts and (ref. 60). It provides precisions better than one per mil (circa 0.1δ) around natural abundances. However in recent years an increasing effort has been put in developing optical methods, to take advantage of a simpler treatment of samples. By using rotational lines to absorb the emission of a diode laser, signals from other molecules do not interfere, in particular CO_2H that in mass spectrometers appears when CO_2 is not dry enough, or mixed with H_2S or H_2, (ref. 61). The concept, initially proposed by Oeschger (ref. 62), is discussed (ref. 63 and 64). An application to breath tests is described (ref. 65). Sensitive non dispersive infrared spectrometers have been described for the same purpose (ref. 66) and (ref. 67).

d) *Inorganic molecules including H, C, N, O. Noble gases.* Possibilities of isotopic spectral analysis of those molecules are found in the work of Funtov (ref. 68).

e) *Other elements and techniques.* Laser spectroscopy can be used to obtain very high sensitivities by the saturation technique. Isotopes of osmium were analysed (ref. 69) in samples where the lowest abundance was 0.007% of ^{192}Os. Atomic emission with a cold hollow cathode has been proposed for the determination of isotopes of elements in dielectric powders (ref. 70). Fabry-Perot interferometers have also been used for uranium isotopic analysis (ref. 71), or as simple teaching tools (ref. 72). Raman scattering has been demonstrated to be able of high precision measurements of abundances of ^{15}N, ^{18}O, ^{13}C, and D, and to permit analysis of fluid inclusions in minerals (ref. 73).

(iv) Chromatography, Coupled methods

Chromatography may enable to separate isotopically differing species,in which case detection after separation does not need to be isotopically selective. For instance, high resolution HPLC columns enable to separate deuterated drugs; conventional, inexpensive, ultraviolet detectors permit simultaneous determination of labeled and unlabeled drugs (ref. 74). Catharometers, by measuring thermal conductivity, coupled with gas chromatography, are used to analyse mixtures of hydrogen isotopes (ref. 75), and even of the ortho and para varieties also separated by the chromatograph (ref. 76).

When chromatography does not enable to separate isotopic molecules, as when analysing heavier isotopes, it is nevertheless used to isolate the species of interest in a mixture, and is then coupled to an isotopically selective detector. For this purpose coupling with mass spectrometers is often made as described above. Optical devices can also be employed. As in the case of coupling with mass spectrometry a post column reactor may be set up, in order e.g. to produce CO_2 and to work on the CO molecular emission in the vacuum ultra violet for ^{13}C contents (ref. 77). Detection limits for C, H, N, are in the nanogram per second range (ref. 78).

Applications of chromatography are often found in paragraphs concerning detectors with which it is coupled. Performances of GC-MS, GC-IRMS, GC-AED for ^{13}C isotopic enrichment measurements from ^{13}C progesterone have been compared (ref. 79). Detectable limits of enrichment are found to be: 0.6% for GC-MS, 1.8% for GC-AED, and 4.8×10^{-3}% for GC-IRMS. and Quantifiable limits of enrichments, 2.07%, 6.03%, 1.6×10^{-3}%. However quantities to be injected in the gas chromatograph were respectively: 10ng, 10ng, 628ng. The latter amount is necessary to generate the minimum 10nmoles of CO_2 needed for the isotope ratio measurement.

(v) Nuclear techniques

The principle is to activate stable isotopes (uses of naturally radioactive isotopes are not considered here) or have them enter a nuclear reaction, and to measure the corresponding radioactivity, whether prompt or delayed. Nuclear methods give access to the absolute number of atoms in a sample. When isotope ratios are required two determinations are necessary that may require using two independent methods because nuclear properties of isotopes may differ as much as nuclear properties of different elements.

Two main families of nuclear methods exist: neutron activation (NA) and charged particle bombardment; γ irradiation is sometimes also used. NA possibilities are evaluated in ref. 86. NA analysis can make use of neutrons of different energies. and of various detection techniques. As a rule it almost always gives results of isotopic contents of the bulk of samples. Recently, however, depth profiling of ^{6}Li in LiO_2 was achieved by detecting tritium produced, and measuring the energy of recoiling nuclei from (n,p) or (n,alpha) reactions. Probe depths may be a few tens of nanometers (ref. 80).

Particle bombardments are multifarious (ref. 81). A given isotope can be analysed by several different reactions, and to measure isotope ratios one may sometimes have to use different particles. Particle bombardment enables to make isotope concentration profiles.

Advantages of nuclear methods:

They enable to measure monoisotopic elements, and provide concentrations of atoms. They may enable one to analyse samples without previous chemical treatment, though often requiring a post irradiation treatment.

Neutron activation enables one to analyse simultaneously a large number of samples. NA can furnish multiisotope analysis.

Nuclear microprobes provide both low detection limits and high spatial resolution.

Nuclear reactions may be the best way to look at ratios of isotopes of different elements, e.g. ratios of ^{18}O to ^{15}N.

Disadvantages of nuclear reactions:

A special study must be made for each sample.

Isotopic abundances of elements are not readily measured, and precision is usually orders of magnitude lower than by mass spectrometry.

Except in the less frequent instances of γ irradiations, samples after irradiation become radioactive.

Except when the user has his own neutron source or accelerator, he must have access to collective equipments and accept formalities, and sometimes delays. Disposing of radioactive wastes must be considered.

Applications:

Nuclear methods are best adapted to analyse series of similar samples. Their application to stable isotope dilution analysis is reviewed (ref. 82). Neutron activation is the nuclear method most frequently used for stable tracers. ^{50}Cr, which serves to measure blood volume by isotope dilution, is analysed as ^{51}Cr after irradiation with predominantly thermal neutrons (ref. 83). Total body oxygen has been determined in vivo by 14 MeV neutron activation (ref. 84). Other examples are quoted in (ref. 85). A chinese paper reports the analysis of more than 20 isotopes to study silt motion (ref. 86). Enriched ^{58}Fe is used for the study of iron absorption in humans, and dysprosium as a stable tracer in the environment (ref. 87). Neutron activation can be used to detect elements in closed packages. It has been employed for Pu (ref. 88).

Photon activation has been used to analyse ^{16}O (ref. 89), and also enriched ^{86}Sr and another isotope for isotope dilution (ref. 90). Proton irradiation enables to analyse ^{18}O by a (p, alpha) reaction producing ^{15}N. Numerous other examples of analysis and spatial distribution determination of elements in biological samples are described (ref. 86).

Resonance scanning is often a useful complement to Rutherford back scattering for depth profiling of low Z elements in high Z substrates. Alpha alpha resonance signals of ^{16}O enable to establish the profile of a 204 ångstroms SiO_2 film deposited on Si (ref. 91).

(vi) Miscellaneous techniques

Analytical techniques

Any property that enables one to characterize isotopes may in principle be used for analytical work.

Density measurements, are the most often encountered, and are set up in various forms for the assessment of deuterium compounds, nitrogen samples in heavily labeled biological compounds (proteins), and, to a lesser extent, of lithium compounds. Advantages of density measurements are simplicity and low cost of equipment. High precision may be obtained by pycnometry if samples are purified and large enough. The falling drop method was used in the past, to analyse small samples of deuteriated water. Disadvantages are the necessity of purifying samples very carefully, the time consuming character of some techniques, and the fact that an independent evaluation of errors must be made for each sample. Disadvantages are such that, except for biological samples analysed by the density gradient technique, and perhaps in production plants, where large samples can be taken, density methods are almost abandonned.

Freezing point elevation, falling drop, infra-red absorption and gas chromatography, have all been used for measurements of D_2O in plasma water (ref. 92).

A temperature-float method has been described for the determination of distribution constants of deuterium between H_2S and H_2O (ref. 93) .

Special combinations of techniques are used for isotopic analysis of trace gases in the atmosphere (ref. 94), and of main and trace gases in air (ref. 95).

Treatments of samples prior to analysis:

Deuterium oxide at tracer levels in biological fluids can be extracted by sublimation before infrared analysis (ref. 96). Hydrogen isotope analysis of waters can be made on hydrogen gas brought to equibrium with the sample. The method seems specially attractive for brines (ref. 97). Carbon isotopic analysis of proteins can use the reaction of ninhydrin with amino acids to extract peptide bonded carbons in proteinaceous material as CO_2 (ref. 98).

III. USES OF STABLE ISOTOPES IN THE ABSENCE OF ISOTOPE EFFECTS

Overview of applications; rules of employing stable isotopes as tracers

Stable isotopes have possible uses in every tracer application, with special advantages in isotope dilution analysis. However, for an efficient use of stable tracers, the following precautions should be taken. Firstly one must verify that no parasitic exchange simulates the disparition or, especially in biology, the introduction of the tracer through a process other than the one investigated. Secondly, when the intensity of the isotopic signal is important, as for applications of isotope dilution, for various studies of metabolisms or reaction kinetics, care must be taken not to be misled by either equilibrium or kinetic isotope effects, according to the case. Both may be very large in the case of hydrogen (see Appendix B). However isotope effects do not affect results of experiments when, for instance, just the time at which a signal is observed matters. Thirdly, in order to use stable tracers economically, the minimum amount of isotopically enriched material, at the lowest degree of enrichment, should be used. An estimation of the necessary amount and enrichment of the tracer must be made.

A detailed example of such an evaluation, given in (ref. 12c), is the study of ^{15}N as tracer of the incorporation of nitrogen in sojaplants. Starting from the minimum signal desired from the analytical instrument, given the size of samples that an analysis requires, the minimum excess ^{15}N in the sample is evaluated. The volume of the pool to be analysed, which is at the same concentration as the sample, determines its content in^{15}N. Finally, the quantity of tracer needed at the start is calculated by taking into account the yield of the transfer process from injection to the pool. The number of determinations necessary determines the amount of tracer required.

A choice can be made between spikes in larger volumes, at lower enrichments, or the reverse, provided that the necessary minimum concentration of tracer in the sample to analyse is present. It should be cheaper to use as low a starting isotopic concentration as possible, when commercially available, as obtaining low isotopic concentrations by dilution of highly enriched material should be costly.

Finally, in some cases, particularly in biological studies, natural tracers, can be used, i.e. elements found in nature with an isotopic abundance differing sufficiently from that in the system under study. The most frequently employed are carbonaceous ones, because ^{13}C is generally enriched in C4 versus C3 plants, in cane sugar versus beetroot sugar, for instance.

Isotope Dilution Analysis

Basic assumptions

Many uses of stable isotopes are direct applications of isotope dilution analysis, hence its importance. Dilution methods were first used to evaluate populations of rare bird species living on islands, i.e. in a closed system. Rings were put on the legs of a given number of birds which were released, and sufficent time was allowed to elapse before recapturing another flock of birds, so that ringed birds could mix randomly with unringed ones. The proportion of unringed to ringed specimens gave the ratio of the total population to the number of ringed one. Hevesy was the first to use dilution methods with isotopes, radioactive ones at the time.

A basic assumption of dilution methods already appears: perfect mixing of the two kind of birds. And an advantage of dilution is clear: it was not necessary to capture all the birds in order to count them. The precision of the result was limited by statistical errors. Fields of applications of isotope dilution analysis, that will be summarized later in table 4, are mainly:

a) the analysis of traces of elements, or molecules,

b) speciation,

c) the measurement of volumes or surfaces, by the determination of the amount of matter within volumes, or on surfaces.

General equations

Definitions:

The isotopic abundance of an isotope is the the ratio of the number of atoms of that isotope to the total number of atoms of the element.

N_x is the unknown number of atoms of an element to be determined in a sample.
N_y is the known number of atoms of the elment in the added spike.
A_x is the isotopic abundance of the isotope which is used as label in the sample.
A_y is the isotopic abundance of the labeling isotope in the spike.
A_m is the isotopic abundance of the labeling isotope in the mixture of spike and sample.

Applying a general method to the case of elements having more than two isotopes, one writes the conservation of the number of atoms of the label after mixing spike and sample; it is expressed by (3)

$$N_x A_x + N_y A_y = (N_x + N_y) A_m \tag{3}$$

therefore

$$N_x = N_y \cdot (A_y - A_m)/(A_m - A_x) \tag{4}$$

Solving (4) requires the measurement of three isotopic abundances; each necessitates to determine several isotopic ratios. If radioactive isotopes were used, the As would represent specific activities.

In the case of elements having only two isotopes, isotopic abundances can be expressed in terms of isotopic ratios that are directly measured by mass spectroscopy. Let N_1 and N_2 be the numbers of isotopes 1 and 2,

$$R = N_1/N_2$$

and $$A = N_1/(N_1 + N_2) = R/(1 + R) \tag{5}$$

Equation (4) becomes

$$N_x = N_y \cdot [R_y/(1 + R_y) - R_m/(1 + R_m)] / [R_m/(1 + R_m) - R_x/(1 + R_x)] \tag{6}$$

Equation (6), which replaces (4) in this case, can be solved knowing only three isotopic ratios. Usually a spike is used for the analysis of many samples, and sometimes unknowns are of natural origin and have a sufficiently well known isotopic composition. Then, analysing each new sample requires only one isotopic ratio measurement.

When elements, specially hydrogen, carbon, nitrogen and oxygen, possess an isotope overhelmingly more abundant than the others, in most cases, even in the spike, the Rs are small with respect to 1; therefore with no measurable error, or within an approximation of a few percent, (7) can be substituted for (6):

$$N_x = N_y \cdot [R_y - R_m]/[R_m - R_x] \tag{7}$$

To obtain the ratio of the weights of elements in the sample to that in the spike, conservation of matter, and of atoms, requires that the ratio N_x/N_y of numbers of atoms should be multiplied by the ratio of atomic weights, M_x/M_y, of the element in sample and spike, which differ from unity because of their different isotopic composition (ref. 99).

Conditions to be fullfilled in isotope dilution procedures

Conditions to be fullfilled result from the preceding equations

a) An isotope dilution measurement involves normally three isotopic determinations. It has been shown that, in favorable cases, only R_m needs to be measured for analysis of natural materials, when utilising a given spike. In every case, R_m may be measured on any aliquot of the mixture. Isotope dilution analysis does not require quantitative extraction of the analysed species.

b) N_x, number to be determined of atoms of an element in a sample, can only be obtained, at best, with the same degree of accuracy and precision as N_y which is obtained by taking a given volume, or weight, from the stock of the spike. A volumetry, or gravimetry, is needed for that, and also for determining the size of the sample to obtain the concentration of the element. However, as shown below, when looking only for the ratio of the amount of an unknown to a known constituant, instead of looking for its concentration, the use of a multiple spike enables one to avoid either gravimetry or volumetry.

c) Mixing of the spike and the analyte must be perfectly homogeneous. After the mixing, the spike should be under exactly the same chemical form and valency as the species to analyse. In many cases this leads to performing 'valency cycles' after adding the spike to the sample. No isotopic or chemical exchange between the form investigated and other forms present in the sample should occur, and no other form should be co-extracted.

Solid samples should in general be dissolved, and the spike added to the solution. No simplifying assumption is safe; e.g. it has been shown (ref. 100) that, after dissolution, chemical conditions for a satisfactory exchange of a Zr spike with U-Zr-Al alloys are substantially different from those for the determination of zirconium in Pu-Zr-Al. alloys, in spite of an anticipated similarity of behaviour. When samples can not easily be dissolved, dissolution may, in some cases, be avoided by heterogeneous isotopic equilibration, if conditions for complete equilibrium and absence of isotopic discrimination are met. For instance to determine traces of boron in solid samples of alloys of Fe, Ni, Cr, Mo and Cu, without dissolving them, the choice was made of a gaseous carrier of enriched boron that was stable at a temperature suitable for isotope exchange, and did not chemically react with the metal analysed (ref. 101).

Causes of errors

In addition to errors due to experimental procedures that do not respect the preceding conditions, the following causes may induce errors:

a) Discrimination occuring during chemical separations. Discrimination is mostly to be feared in the case of hydrogen isotopes.

b) Introduction of significant amounts of the analysed species in the system by reagents, water, contaminants. To avoid this cause of error, one may have to work in "white" laboratories.

Special techniques

Use of a long lived radioactive isotope as spike. When an isotope is long lived, whether it is natural, for example, ^{230}Th, ($T_{1/2} = 8\times10^4$a), or artificial, for example, ^{129}I ($T_{1/2} = 1.7\times10^7$a), it can be analysed in the same way as stable isotopes and used as a spike. Normal materials contain no radioactive spiking isotope, and $A_x = 0$ in equation (4), which becomes

$$N_x = N_y[(A_y/A_m) - 1] \qquad (8)$$

If counting techniques were used the same equation would apply, A_y and A_m would then represent specific activities, and would represent quantities instead of dimensionless numbers. In that case, substoechiometric extraction (ref. 102), that enables to avoid weighing, could be employed.

Multielement analysis. By using several spikes simultaneously, it is possible to analyse several elements at the same time (ref. 103). Simultaneous determination of seven elements by surface ionisation (thermoionisation) has been achieved in standard materials (ref. 104). Interferences should of course be avoided. For instance, when mass spectrometry is the chosen analytical method, one can not use ^{138}Ce as spike simultaneously with ^{138}La, unless one goes through difficult chemical separation procedures.

Avoiding gravimetry and volumetry. Avoiding volume or mass determinations during the course of an analysis is achieved by measuring ratios of elements and using multiples spikes. A classical example, given here to explain the procedure, is the analysis of plutonium generated in nuclear fuel elements, that is commonly made by measuring the ratio of plutonium to uranium rather than its concentration.

The desired ratio is that of the main isotopes of uranium and plutonium, i.e. ^{238}U and ^{239}Pu; the spike consists of a solution of pure ^{233}U and ^{242}Pu, (^{244}Pu would be even better), isotopes which are absent, or present with a negligible abundance, in the samples. Following the general rule, one writes that the amount of each isotope remains constant after mixing sample and spike, and the following equations are derived:

$$(V_x + V_y).[^{239}\mathrm{Pu}]_m = V_x{\cdot}[^{239}\mathrm{Pu}]_x \qquad (9a)$$

$$(V_x + V_y).[^{242}\mathrm{Pu}]_m = V_y{\cdot}[^{242}\mathrm{Pu}]_y \qquad (9b)$$

$$(V_x + V_y).[^{238}\mathrm{U}]_m = V_x{\cdot}[^{238}\mathrm{U}]_x \qquad (9c)$$

$$(V_x + V_y).[^{233}\mathrm{U}]_m = V_y{\cdot}[^{233}\mathrm{U}]_y \qquad (9d)$$

Subscripts *x*, *y*, and *m*, are for sample spike and mixture. V_x and V_y represent volumes of the sample and of the spike solutions, respectively. Brackets represent concentrations (in moles per liter or atoms per liter) of isotopes. The ratio of concentrations $[^{242}\mathrm{Pu}]_y/[^{233}\mathrm{U}]_y$ is known from the preparation of the spike, and isotopic ratios in the mixture are measured. Combining equations (9a) to (9d) one obtains

$$[^{242}\mathrm{Pu}]_m/[^{239}\mathrm{Pu}]_m = (V_y/V_x){\cdot}[^{242}\mathrm{Pu}]_y/[^{239}\mathrm{Pu}]_x \qquad (10a)$$

$$[^{233}\mathrm{U}]_m/[^{238}\mathrm{U}]_m = (V_y/V_x){\cdot}[^{233}\mathrm{U}]_y/[^{238}\mathrm{U}]_x \qquad (10b)$$

Combining (10a) and (10b) one solves for the ratio of ^{239}Pu to ^{238}U in the sample.

$$[^{239}\mathrm{Pu}]_x/[^{238}\mathrm{U}]_x = [^{242}\mathrm{Pu}]_y/[^{233}\mathrm{U}]_y{\cdot}([^{239}\mathrm{Pu}]/[^{242}\mathrm{Pu}])_m{\cdot}([^{233}\mathrm{U}]/[^{238}\mathrm{U}])_m \qquad (11)$$

Each ratio on the right hand of (11) is known or measurable. It can be seen from (11) that sample and spike can be mixed in unkown proportions, that volumes need not be measured and that the analysis is performed exclusively from isotopic ratio measurements. In the general case, the spike may contain non negligible amounts of the main isotopes.The corresponding equations are lengthier, but easily derived. Finally, microgram amounts of spikes enable to carry out hundreds of determinations, because mass spectrometric analysis can sometimes be made by using nanograms, or even lesser amounts of mixture.

Reverse isotope dilution analysis. Measurement of microgram amounts of elements, for instance of spikes such as ^{242}Pu of which larger amounts are not available, can be made by using a weighable amount of a more abundant isotope, in this case ^{239}Pu. Then, in equation (7) R_m is close to R_y, and very different from R_x, N_y is large with respect to N_x. The expression reverse isotope dilution analysis originates from the use of isotope dilution when the spike is a stable isotope and the unknown a radioactive species.

Reverse isotope dilution can be used to measure dissolved amounts of compounds of low solubility, e.g. determination of water in CCl_4 has been demonstrated using heavy water in a modified reverse dilution technique (ref. 105). Results showed that: 1) isotope dilution is faster, more precise, and more accurate than conventional (Karl- Fischer) methods, in this sub ppm range. 2) The protium (^{1}H) content of heavy water was analysed more easily by infrared absorption than by mass spectrometry

Analytical techniques

The acronym IDMS is used when mass spectrometry, in one of its many versions, is employed for analysis together with isotope dilution. Nuclear activation, optical methods, are used also but more seldom.

Isotope Dilution Mass spectrometry (IDMS)

Precision and accuracy. Precision and accuracy in trace analysis are unrivalled (refs. 106 and 107). An example of precision and accuracy obtained with IDMS is given by the certification of Cd in polyethylene even at concentrations of 0.363 mmol/kg (40.9 mg/kg) (ref. 108).

Detection limits. IDMS detection limits are given for various elements and ionic species in (ref. 101). They range from fractions of a picogram per gram in the case of cadmium in an aquatic system ($2\text{x}10^{-4}$ ng/g), and comparable values for Cr, Ni, Mo, Tl, Pb, Th, U, to 120 ng/g for boron in steel. Quantities of a few 10^{-16} g (ref. 10) and concentrations in the ppt, or even femtogram per gram ($fg*g^{-1}$) range (ref. 34) can be measured, when mass spectrometry is used for isotopic analysis.

Negative thermoionisation has been shown to be advantageous for isotopic dilution analysis of many elements (ref. 34).

Blank corrections in IDMS can be evaluated by a general equation derived in (ref. 109).

An 'average mass' treatment of mass spectral data for IDMS (ref. 110) is claimed to have the advantage of requiring none of the often nesessary assumptions, with regard to the isotopic purity of the labeled compound, to the distribution of naturally occuring isotopes in the sample and the spike, and to interfering fragment ions. It should produce a linear calibration plot.

Neutron activation analysis

Neutron activation analysis often enables to analyse samples without chemical treatment, or to perform multielement determinations and has low detection limits; but generally precision and accuracy are much less than those obtained IDMS. Advantages, and limitations, of the coupling are discussed (ref. 82).

Optical methods

Optical methods are commonly used when dilution of heavy water is practised because of the easy infrared method of analysis available, cf. Part II. Ref. 68 describes a combination of isotope dilution and spectral gas analysis, which may include physico-chemical transformations, for example isotope 'balancing', (the equivalent of equilibrating). Detection limits for instrumental techniques are 100ppb-10ppt, and for the combined techniques 1ppm-1ppb. H, C, N and O and some of their inorganic gaseous compounds were analysed in complex gas mixtures.

Examples of applications

Trace analysis

Isotope dilution analysis provides low detection limits. Equations (1) to (5) apply even when N_x, number of atoms in the sample, is smaller than N_y, number of atoms of spike, by a large factor. For instance, if R_y is close to 100, and R_x less than 5/1000, then, if the Rs are measured with enough precision to evaluate a difference of 1% to better than a few percent, N_x can be smaller than N_y by a factor of about one hundred. This factor may be larger when low detection limits are obtained. By mass spectrometric analysis femtogram of elements are determined, and concentrations of Ag less than 0.001 to 0.01 pg/g in pure HCl or HNO_3 were measured (ref. 111).

Precision and accuracy of trace analysis by isotope dilution analysis are features as important as low detection limits. Equation (3) shows that they are maximum for high enrichments of the spike, low natural abundance in the sample, and comparable values of N_x and N_y. In many cases, micrograms per gram of elements may be analysed with a precision of a few percent

Speciation

When speciation is sought, requirements on chemical and valency state of the spike are particularly stringent, but even speciation of several organic molecules simultaneously can be achieved.

IDMS has enabled one to determine four different iodine species in an aquatic system: iodide, iodate and two organoiodine compounds (ref. 112). This work illustrates:

1) the use of a long lived iodine isotope as "stable" spike (^{129}I, $T_{1/2} = 1.7x10^7a$)

2) that, in different natural waters, total iodine contents in the 2-7 mg/l range and detection limits, for iodide and iodate, of respectively 0.5 mg/l and 0.1 mg/l, can be obtained.

3) that the use of iodides and iodates spikes to measure inorganic iodine show that organic iodine separated, and that two forms could be identified: one that could be eluted from a chromatographic column, the other that remained fixed. Ignoring, a priori, the molecular nature of the species did not allow the preparation of spikes of precise composition.

4) the use of radioactive tracers to investigate the absence of isotope exchange between the two forms.

Measurement of volumes and masses

a) The mass of nuclear material in tanks of a reprocessing plant can be measured using lutecium as tracer (ref. 113).

b) D_2O dilution is a well established method for the measurement of body water, lean and fat mass (ref. 114). Total body water and body weight are closely correlated, fat mass varies with age (ref. 115). See Part I .

c) the volume of extravascular lung water is measured by the simultaneous use, as tracers, of heavy water, that diffuses into the total lung water, and of a dye that does not diffuse through the walls of capillary vessels and provides the volume of intravascular water which is subtracted from the total one. The tracers are injected in a vein and sampled in an artery. Heavy water and dye are analysed on line in the flowing blood by a special infrared, non dispersive, instrument (ref. 116).

d) Blood volumes of horses have been calculated from erythrocyte volumes because the ratio of the latter to the blood volume has a known value. The volume of erythrocytes could be determined by isotope dilution using ^{50}Cr to label erythrocytes used as spike. Erythrocytes were extracted after a time sufficient for equilibration, and analysis was made by neutron activation (ref. 117).

e) Isotope dilution can be used to determine concentrations of large molecules such as vitamins. The total body reserve and liver concentration of vitamin A, for instance, was estimated by measuring the dilution of a 45 mg oral dose of tetradeuteriated retinyl acetate, 99% pure (ref. 118).

<u>*Summary of characteristics of isotope dilution*</u>

TABLE 4. (Isotope Dilution Analysis)

Advantages	**Limitations**	**Possibilities**	**Fields of Applications**
Quantitative separations not required.	Isotope effects.	Quantitative trace analysis.	Measurements of biological pools.
	Isotope exchange.	Measurements of volumes, of low solubilities, etc.	Immunoassays
Gravimetry or volumetry may be avoided.	Poor mixing of spike and sample.		Volumes of reservoirs.
	Purity of reagents.	Speciation	Bioavailablility studies.
Precision and accuracy			

General characteristics of the method, summarized in table 4, are discussed in (ref. 119).

Characteristics common to the use of stable and radioactive isotopes

Common disadvantages. One can only measure by isotope dilution analysis elements, or species, that are looked for at the start. Impurities in reagents (blanks) are measured together with traces in the sample,

Common advantages. As quantitative analysis is performed by non-quantitative extraction from the sample, it enables, inter alia, one to analyse whole systems that can not be subjected to extraction in their entirety. For instance, trace elements of the human body are analysed by isotope dilution. Errors due to losses of analyte during the analytical procedure, which occur accidentally, or by adsorption on vessel walls, etc., are eliminated. Different valence states of the same element can be analysed separately if the proper spikes can be prepared. One can take advantage of the fact that a spike may exchange with species that, partly, may not be extractible to measure separately extractible, and non-extractible fractions of the same species. In some cases isotope dilution analysis is faster and simpler than conventional methods, and the smaller the amount to be measured, the less tracer is needed.

Characteristics specific of the use of stable isotopes.

Drawbacks. In some cases, no stable, or long lived, isotope is available, or even exists, for the required analysis. Isotope dilution analysis with stable isotopes is more difficult, more expensive, often slower, and requires more material than isotope dilution with radioactive isotopes. Analytical instruments, when not specific for a given analysis are expensive: mass spectrometers or NMR spectrometers.

Advantages. Isotope dilution, coupled with an analysis by mass spectrometry, is recognised as a 'definitive' method, meaning that it can furnish the best accurate as well as precise results.

Several elements, or species can be determined simultaneously. When the ratio of two elements or species is sought no volumetry or gravimetry is required. The general advantages of the use of stable over radioactive isotopes can be claimed. They are especially important in the life sciences in which low Z elements play a great role.

Perspectives of isotope dilution analysis

Trace analysis is of growing importance in environmental studies, especially to establish tolerance limits, and to verify whether they are reached in given samples. Isotopic dilution is probably the most accurate method for this purpose (cf. refs. 107 and 119).

Isotope dilution analysis is most likely to play an increasing role in pharmacology and clinical chemistry for diagnostic purposes. Easy, rapid and inexpensive methods of analysis should be developed whenever routine and fast determinations are essential.

Metrology, i.e. certification of reference materials, is also a privileged field of application of isotope dilution analysis (ref. 120) and should be of increasing importance as quality insurance practices develop.

Uses of Stable Isotopes as Tracers

We recall the particularities of the use of stable tracers: several can be employed simultaneously, the use of natural spikes may be advantageous, as sometimes is a combination of a stable and a radioactive tracer. When using appropriate techniques, (cf. also Part I), the position of a labeled atom in a molecule can be determined. Table 5 summarizes uses of tracers in the absence of isotope effects, other than isotope dilution.

TABLE 5. (Uses of Tracers in the Absence of Isotope Effects

Advantages	Limitations	Possibilities	Fields of application
Atoms, molecules are followed even under equilibrium between species.	Occurrence of isotope effects	Mechanisms, and kinetics of reactions.	Pharmacology
Identification of molecular sites.		Transport phenomena.	Medicine: breath tests, metabolism, energy expenditure, etc.
Possibility of multilabeling.		Flow rates.	Studies in environmental agriculture.
		Diffusion rates.	Corrosion studies.
		Fate of several atoms in a molecule.	

Transport phenomena

The basic purpose of tagging atoms or molecules is to follow their fate during natural, or man made, processes. Isotopes are particularly useful for the study of migrations in condensed matter. However in the case of the rate of transport of hydrogen the tracer may be quite different from those of the ordinary isotope. Deuterium, e.g. is known to diffuse through palladium at a rate slower than protium by a factor often taken, from basic considerations, as the square root of two. For other elements kinetic isotope effects, usually smaller than the precision obtained on the value of the rate of transport, can be disregarded.

A specific possibility offered by the use of isotopic tracers is the study of exchanges between different phases or chemical species at equilibrium. Natural transport phenomena are often followed by 'natural tracers' i.e. molecules having an isotopic composition different from the average at the investigation site.

Such tracers are used in environmental and agricultural studies and for authentication of products. They are also useful in biological laboratories.

Direct method of following transport phenomena

It consists in sampling the species under study along the route followed and to analyse the tracer.

Indirect methods

As these are specific, a particular example is given as illustration only. Isotopic exchange has been reported as a means to study the mobility of hydrogen on the surface of catalysts (ref. 121). The method relies on the property of hydrogen to be specifically adsorbed outside the active sites which catalyse the isotopic exchange between protium and deuterium, which follows reaction (12).

$$H_2 + D_2 = 2HD \qquad (12)$$

As this exchange does not take place spontaneously, the rate of exchange is controlled by the rate of arrival of hydrogen on active sites where the rate of the reaction is fast, i.e. by the mobility of hydrogen on the surface that can thus be evaluated.

Transport in solids in the laboratory

a) Migration of oxygen in uranium oxide pellets has been investigated, using a SIMS Castaing-Slodzian imaging machine, by labeling the outside of the pellet with ^{18}O. Diffusion of oxygen toward the center of the pellets, studied as a function of time at several temperatures, furnished apparent diffusion coefficients and activation energies for diffusion (ref. 122).

b) Oxygen mobility in YBaCuO thin films, probably fragile under ion bombardment in SIMS, was studied by ^{18}O using Rutherford back scattering (RBS) for the ^{18}O distribution, and Raman spectroscopy for the localisation of ^{18}O in the YBaCuO structure (ref. 123).

c) Concentration gradients may be measured by specific reactions, especially nuclear ones. Depth profiling experiments of ^{18}O in Ta_2O_5 and SiO_2 layers with sharp ^{18}O-^{16}O interfaces illustrating the high resolutions obtainable, were described (ref. 124), using the narrow resonance in the $^{18}O(p, \alpha)^{15}N$ reaction, near 150 kev.

d) Ion movements in the retina could be measured (in vitro) using, as tracers, stable isotopes of calcium, analysed by SIMS, which gives the concentration of calcium at the same time as the location of the tracer. When radioactive ^{45}Ca is employed its location only is obtained, and its use suggests an accumulation of calcium in dead retina. Stable tracers showed that the total calcium flux was nil, and the apparent flux suggested by the use of ^{45}Ca probably due to exchange.

e) Heavier isotopes, that may not necessarily belong to polyisotope elements, can be used, e.g. ^{19}F, injected as constituent of a freon, served to measure cerebral blood flow in cats (ref. 125).

Transport in nature: Uses of enriched isotopes

a) To investigate transport of methane in the atmosphere over transoceanic distances, Cowan used $^{13}CD_4$, i.e. double labeling. Tagging methane with ^{13}C only does not allow a dilution much above one hundred, because of the natural abundance of ^{13}C and its natural variations. Coupling labeling of the carbon with that of one hydrogen improves detectability by almost 104. This factor is enhanced to the fourth power when the four hydrogens are deuterated (ref. 126).

b) Migration of elements in uranium ores from the Oklo quarry in Gabon has been studied by many methods on naturally isotopically enriched elements produced in situ by natural fission chain reactions, two billion years ago (ref. 127).

Transport in nature: Uses of natural variations of isotopic abundances

a) D, ^{18}O, ^{15}N, ^{13}C and ^{34}S, have all been used to study the mixing of continental and marine waters (ref.128). In a research on estuaries (ref. 129), biogeochemical markers were associated with ^{13}C. D and ^{18}O analysis of deep and shallow groundwaters in granite, in connection with studies on the disposal of radioactive wastes, showed that they have a different origin and history. This was confirmed by radiocarbon (ref. 130), salinity and tritium measurements (ref. 131). Ref. 132 gives methods for the determination of the porosity of hydrogeological systems.

b) Hydrogens belonging to biomolecules can often be followed by NMR, without isotopic labeling. Often also natural ^{13}C is a good enough tracer. Numerous examples are given in references of Part I.

Environmental studies

A comprehensive coverage of isotopic studies of past and current environmental changes in the hydrosphere and the atmosphere is found in (ref. 133).

Carbon isotope ratios may serve to identify pesticides, that are also sometimes followed in the enviroment by labeling the chlorine (ref. 134). Carbon and hydrogen isotope ratios of methane carbon dioxide and water allow one to identify bacterial methane gases. The distribution of carbon isotopes in the metabolic products of basic biological systems is described in (ref. 135). Carbon isotopes are useful in investigations of the greenhouse effect (ref. 136). Interpretations of isotopic abundance variations in the environment use information stored in the isotopic record furnished by trees (ref. 137), essentially on hydrogen and carbon isotopes, and measures of the fractionation of oxygen isotopes by respiring marine organisms (ref. 138).

Use of ^{15}N determinations are important for environmental studies. Applications to the soil-plant system are reviewed (ref. 139). Nitrogen 15 concentration varies according to different types of soil utilization. This qualifies it as a tracer to establish the origin of nitrates in waters, and of waters themselves (ref. 140).

Sulfur in the environment: methods to differentiate between anthropogenic and natural sulfur, basic principles and analytical techniques are treated in (ref. 141). Sulfur 34 can be used to trace pollution in aerosols (ref. 142).

Lead isotope ratios help to identify lead sources (refs. 143 and 152). It is, in principle, possible to determine the relative strengths of sources of atmospheric methane by measuring abundances of ^{13}C because ^{13}C ratios may vary, according to the origin of the methane, from δ values, taken with respect to the PDB standard, of -15, to δ values of -90. Effects of environmental exposure on children can be evaluated by stable isotope techniques (ref. 144).

Caveat : when making studies of the environment one should beware of risks of perturbation by kinetic isotope effects. For instance, as the main sink of methane in the atmosphere is a reaction with OH radicals, kinetic effects may be a cause of significant variations in δ when studying methane sources as, for this reaction, $k_{12}/k_{13} = 1.0054 \pm 0.0009$ (ref. 145).

Agricultural research

The main users of enriched stable isotopes are research workers in agriculture. Nearly three out of four papers reporting work with stable tracers isotopically modified are devoted to ^{15}N work on assimilation of nitrogen by plants, and on the use of fertilizers.

Nitrogen 15 abundance measurements, their use for the study of nitrogen fixation in soils and plants, of nitrification, and of mineralisation and assimilation processes, are studied in (ref. 12c). When plants do not fractionate isotopes, isotopic abundances in the tissue of plants are an average of those in the fluxes from the various sources on which the plants draws. This enables one to quantify the relative importance of those fluxes. Water fluxes in field-grown alfalfa, established using ^{18}O and ^{2}H as tracers, are an example of this method (ref. 146).

Determination of ^{15}N can serve to calculate relative proportions of nitrogen in grains coming from natural mineralization of soils or from fertilizers. Numerous reports on related researches originate from China, India, Indonesia, Syria, Thailand, among many other countries. They are often printed in journals written in the national language; abstracts in English are available through INIS. Carbon 13 abundance in grains can be used as an indicator of the efficiency of intake of water by crops (in kg of grains/m^3 of water) because of the influence of water on the mechanism of carbon intake (ref. 147).

Authentication of products, and objects, Archaeometry

Authentications of the origin of wines are made by NMR site specific isotopic analysis.of deuterium. Authentications of fruit juices (ref. 148) are made by mass spectrometric analysis of deuterium and ^{18}O. Mass spectrometric analysis of ^{13}C discriminate beetroot against cane sugar (ref. 149), or between natural and synthetic vanilin (ref. 150), and allowed the termination of the sale of one for the other. A combination of NMR and mass spectrometry is used when authentication of perfumes or aromas is sought (ref. 151).

Archaeometry resorts to isotopic analysis to identify provenances of ancient metals, glasses, marbles,etc., in order to detect forgeries and to determine contemporary trade patterns. Data bases have been established, and discussed of isotopic ratios of lead of ore deposits (ref. 152), for example, of ores from the western mediterrenean (ref. 153). Isotopic analysis of lead, a usual impurity in silver, can help to trace the origin of silver sources of old coins (ref. 154). A data base of carbon and oxygen isotopic ratios serves for Greek and Roman marble (ref 155).

Studies of reaction mechanisms

Molecular structures and reaction mechanisms in organic chemistry and biology have become the subject of most stable isotope applications since NMR spectroscopy applied to ^{13}C, and more recently to ^{15}N, started to develop.

Mechanisms and Molecular structures determinations

In inorganic chemistry. One among numerous examples cited (ref. 5a) concerns the preparation of urea by heating ammonium cyanate. The Wöhler rearrangement might involve randomisation of the nitrogen. Labeling the ammonium nitrogen with ^{15}N gives products almost exclusively labeled at only one of the two possible positions, showing that randomisation does not occur.

Dissolution of actinide dioxides in acidic media is facilitated when another oxidation state is reached. Electrochemical transformation of actinide dioxides has been investigated. The first step of the oxidative dissolution of actinide oxides MO_2 was shown to consist in a solid state oxidation by carrying out the oxidation of ordinary oxides in water labeled with ^{18}O. Raman spectra of MO_2^{2+} species showed bands corresponding to $M^{16}O^{18}O^{2+}$ and $M^{18}O_2^{2+}$, proving that higher oxides had been formed before dissolution (ref. 156).

Measurements of ^{13}C and ^{18}O concentrations in carbonates, by identifying CO_2 sources (atmospheric or dissolved for instance), give useful information on the carbonation mechanism of concretes (ref. 157).

Structure and function determination of complex biological entities. Examples are given (ref. 158):

a) Carbon 13 NMR shows that, in agreement with accepted biosynthetic pathways, palmitoeic acid, synthetised by the yeast *Saccharomyces cervisae* grown in a medium containing 2-^{13}C acetate, is labeled with ^{13}C on alternate carbon atoms only.

b) Investigation of $^{13}CO_2$ bonding states in aqueous haemoglobin.

c) Examples of NMR applications are found especially in the NMR series books published by Springer, particularly in volume 17, on ^{17}O and ^{29}Si, in volume 22 on isotope effects, mostly on ^{13}C and ^{15}N, in volume 23 on deuterium.

In (ref. 159) possibilities offered by isotope labeling and NMR techniques, to establish protein structures in solution, are outlined. In (ref. 160) ^{17}O NMR serves to study systems in which steric interactions are characterised by rotation of functional groups around single bonds, or in which they are accomodated in part by bond angle deformation. It also serves to study inter and intramolecular hydrogen bonded systems.

The structure of asphaltenes in crude oils has been determined by proton and ^{13}C NMR (ref. 161). In ^{13}C spectra, the intensity of the resonance site provides information about the ^{13}C occupation of the site, whereas the multiplet intensity informs about the ^{13}C occupation of contiguous sites. In ^{14}C studies, where sites are established by degradation techniques, this last information can not be obtained (ref. 162).

Isotopes help to develop faster DNA sequencing methods (ref. 163)

Metabolisms

Studies of metabolism are numerous, many are published in *Isotopenpraxis, Am.J.Clin.Nutr.* etc. A few examples are:

a) ^{13}C labeled glucose, was used in the study of the gluconeogenic pathway (ref. 164). Acetoacetate labeled with ^{13}C was used to assess the mechanism of regulation of ketone body flux in patients suffering from scepticimia (ref. 165). The metabolism of glutamine and its role in human nutrition was studied using ^{15}N (ref. 166).

b) The investigation of differences in metabolisms and kinetics of nitroglycerin when administered by two different routes was carried out by parallel simultaneous study of ordinary nitroglycerin, and nitroglycerin labeled with ^{15}N by both routes (ref. 167).

c) The metabolic mechanism of the chiral inversion of "profen" (2-arylpropionic acid) has been investigated by deuterium labeling on the active site. It was found that deuterium could disappear from the site without inversion taking place, proving that an intermediate step had to be the precursor of either of the enantiomeric forms (ref. 168).

d) For in vivo investigation of human cardiac metabolism, and for neurotransmission, NMR is compared with positron emission tomography (PET) and single photon emission tomography, (SPET) (ref. 169).

Breath tests. Stable isotopes are of special interest for metabolic studies using this kind of test. They consist in monitoring assimilation of foods or drugs by measuring the rate of appearance and amount of ^{13}C in CO_2 of respiratory gases after molecules labeled artificially, or naturally, with ^{13}C have been administered orally, or in some studies intravenously (refs. 170 and 171). Breath tests have the advantage of allowing investigations of assimilation of foods of various kinds without having to modify the patients diets, and of avoiding analysis of samples difficult to collect and treat such as faeces. Examples are studies of carbohydrates and protein assimilation (ref. 172), diagnostics of ulcers of the duodenum (ref. 173), studies of hepatic functions using labeled aminopyrine (ref. 174), the recovery of ^{13}C bicarbonate as respiratory $^{13}CO_2$ in parenterally fed infants (ref. 175). The ^{13}C breath test is considered the "Gold Standard" for the detection of *Helicobacter pylori* and therefore of primary importance in the prevention of gastric cancers (ref. 176).

Studies of nutrition may concern the assimilation and metabolism of major foods that consist essentially of organic molecules, in which case breath tests and analysis of labeled metabolites are used. They may also be focussed on the bioavailability and metabolism of minerals, usually present only as traces in the diet. An example of the first topic is the study of the absorption and metabolic effects of glutamine in humans (ref. 177), reviewed in (ref. 178). The use of stable isotopes in mineral nutrition research is discussed in (ref. 179), the role of trace elements in nutrition of children in (ref. 180). Specific elements are discussed: Fe (ref. 181), Zn, Cu and Fe (ref. 182), the regulation of Zn absorption (ref. 183), intestinal absorption of Cu (ref. 184).

Labeling molecules with two isotopes. Uses of a double label, with two isotopes of the same element, eventually a stable and a radioactive one are demonstrated by the following examples:

a) Measurements of intestinal absorption of calcium are explained in (refs. 185 and 186). One isotope is given orally and, after absorption, appears in the blood. The other isotope is injected intravenously. Both isotopes are later collected in urine, and are the same fraction of their blood content. Thus the ratio of their fractional recovery in urine provides a value of calcium absorption when given orally.

b) In the case of iron (ref. 187), the representativity of the measure of erythrocyte incorporation was assessed as a measure of total iron incorporation. Incorporation of highly enriched ^{54}Fe (stable) in erythrocytes was measured by the increase of its ratio to ^{56}Fe (stable), whereas total body retention of ^{59}Fe was directly established by body counting in a large chamber. The agreement between the two sets of measurements validates the sole use of stable isotopes.

Use of isotopes of two different elements to label molecules enables one, for instance, to compare two metabolisms. The most important example is the use of water labeled with ^{18}O and deuterium to measure energy expenditure in man. The principle of the method is based on the fact that ingested oxygen is eliminated both as CO_2 and water, whereas hydrogen is eliminated as water only. The difference between the two elimination rates is thus a measure of the elimination of CO_2, i.e. of energy expenditure. A thorough discussion of the method, including details of calculation, possibilities of errors, etc., is given in chapter 12 of (ref. 6), and (ref. 188) discusses specially its reliabilty. A major advantage of the method is that the measure can be made on subjects attending to their normal occupations, whereas other methods require either to isolate the subject in a calorimeter chamber, or to modify its diet, or impose other constraints affecting the representativity of results.

As CO_2 in expiratory air is in isotopic equilibrium with body water, elimination of ^{18}O can be followed simultaneously with that of deuterium, on the same fluid, more often urine, but also saliva or sweat. A special double tube mass spectrometer analysing simultaneously ^{18}O in one tube and deuterium in the other saves time and cost for this determination (ref. 189). Measurements were made of energy expenditure of cyclists during the 'Tour de France' (ref. 190).

Measurement of flow rates of fluids in ducts

Though somewhat old, and written for users of radioactive isotopes, (ref. 191) discusses in detail requirements for a valid application of tracers to flow measurements, especially precautions to be taken when mixing a tracer with a flow whose rate is measured. It is shown e.g. that it is not required that a uniform concentration of tracer over the cross section of a duct should be obtained for the method to be valid. It is sufficient that defined surfaces of constant concentration should exist. Evaluations are also made of distances to be respected between points where tracers are injected into fluids, whose flow rates are studied, and places where measurements are made.

Pulsed dose methods

Rectilinear ducts of known, and constant, section area. This conformation is exclusively found in artificial ducts. The transit time of a labeled injection of fluid over a measured distance between two points provides the flow rate. Let D be the flow rate, Δ_t the transit time, x the measured distance and S the area of the section. Then

$$D = S{\cdot}x / \Delta_t \tag{13}$$

The amount of tracer injected, if sufficient for detection after dilution in the main flow, can be arbitrarily chosen but injection has to be short, and the signal, observed at the distance x, sharp enough to enable a sufficiently precise estimation of Δ_t.

As detection of stable isotopes can not be made through walls, this method is nearly never employed when pipes are concerned, though derivations for analysis may be used if they can be made. In open air canals, one might however use heavy water, and in situ detection by an optical method, for small Ds requiring modest amounts of tracer.

Ducts of arbitrary shape. Let c_T be the tracer concentration in the injected volume, c_n the natural tracer concentration, c_s the tracer concentration in the sample and Q a known volume of tracer solution. Q, at concentration c_T, is fed into the duct, the resulting concentration c_s is measured, at a distance sufficient for a steady concentration profile to be established as a function of time. By writing that the amount injected is recovered at the measuring point, one obtains the following equation

$$D = Q(c_T - c_n) / \int (c_s - c_n) t dt \qquad (14)$$

Of course, though the integral is from zero to infinity, it is only necessary to make analysis when signals are above the background line.This method has been used in particular to measure blood circulation rates (ref. 192).

It is possible to simplify the procedure, if perhaps at the cost of a lesser precision, by estimating only the average concentration above background at the measuring point. In order to do this, one can sample the flow during the time Δ_t that the signal passes in front of the measuring point. Writing the equation of conservation of the tracer,and isolating D in the left hand, one obtains:

$$D = Q(c_T - c_s) / \Delta_t(c_s - c_n) \qquad (15)$$

The derivation of (14) and (15) is similar to that of the basic equation of isotope dilution.

Continuous injection method

Instead of a pulsed dose it may be easier to proceed by injecting a continuous flow, q, of tracer solution. At the measuring point the concentration will, after a while, reach a plateau; c_p, and other notations being the same as above, D is given by

$$D = q(c_T - c_n) / (c_p - c_n) \qquad (16)$$

Advantages of the procedure are that the time of making the measurement after reaching a plateau is not strictly imposed, and that problems of choice of the time intervall over which an average concentration must be taken do not arise. A disadvantage is that the amount of tracer required may be larger than when using pulsed doses, depending on the particular configuration under study, and on analytical facilities available.

Flow rates of fluids into a reservoir, simultaneous volume determinations.

An alternative name for flow into a biological reservoir is the rate of appearance. Rates of appearance, R_a, of body fluids into physiological pools are discussed at length (ref. 6). Problems arise when the reservoir is not fed by a single source, through an identifiable duct, in which case one of the preceding techniques could be used.

As this situation is found not only in physiological, but also in environmental and hydrological problems, and perhaps even in industrial ones, we shall use a notation different from the one above, and represent the quantity measured not by R_a but by D_i, inward flux. A tenet of this method is that the volume of the fluid in the reservoir is constant, and that consequently the outgoing flow, D_o, is equal to D_i. When a constant volume assumption cannot be made, a special treatment can be attempted (ref. 6 pp.131-132). Pools of body fluids, or hydrological reservoirs are often interconnected, the case of such systems is treated later.

One can address the problem of establishing the flow rate of one of the constituents of a stream only if the concentration of this constituent remains constant, and an equilibrium situation prevails.

a) Isolated reservoirs, the single pool model

Calculations are made either under equilibrium conditions or rely on the law of variation of the tracer concentration with time. In the general case, the volume of the pool or reservoir is fixed. It can be measured by isotope dilution in many occurences, other possibilities are pointed out below.

The constant injection method. The concentration c_T of the tracer infused into the pool at a constant rate q raises up to a plateau value, which is reached when the outgoing flux carries as much tracer as is injected. This plateau concentration can be measured on any representative sample of the pool. Samples can be taken from any of the outlets of the biological compartments, or of the reservoirs under study, provided that the pools are homogeneous, and that no fractionation of tracer occurs at the outlet, nor, of course, during sampling.

In the following section, we shall assume that c_n is negligible, which is usually the case when deuterium, or a relatively long lived element, with respect to the measuring time, is used as tracer. D_i being the incoming flow of the fluid component under study, c_p its plateau concentration, and D_o its out going flow, conservation of the tracer enables to write, in a steady state, as D_o is equal to D_i

$$D_o \cdot c_p = q \cdot c_T \tag{17}$$

then

$$D_i = q \cdot c_T / c_p \tag{18}$$

The law of variation of concentration in time is easily established, and is

$$c_t = c_p(1-e^{-kt}) \tag{19}$$

where

$$k = D_i/V \tag{20}$$

with V being the volume of the reservoir and $1/k$ is the residence time of the fluid in the reservoir.

Thus when k is small the time required to reach the plateau value is large. A way of circumventing this difficulty, which may be serious in the case of experiments on patients in hospitals, is to 'prime' the pool by a bolus injection that brings directly the concentration of tracer in the pool close to the equilibrium value. However, from (19) and (20), it is seen that it is not mandatory to wait for the plateau value, c_p to be obtained. When calculations of the parameters of the curve can be made during the experiment they provide values for c_p and V.

The pulsed dose, or bolus injection, method. Injection of an amount $Q.c_T$ of tracer into V brings the concentration of V to a value c_o. The concentration decreases after this injection following an exponential law.

$$C_t = c_o e^{-kt} \tag{21}$$

and

$$D_i = Q \cdot c_T / \int c_t dt \tag{22}$$

V is again obtained through $k = D/V$, but can also be calculated from

$$V = Q \cdot c_T / c_o \tag{23}$$

The bolus injection technique was applied to the measurement of cardiac flow rates, an intravenous injection of heavy water was used for the purpose, and simultaneously a measure of total body water could be obtained (ref. 193), and (ref. 116).

When a constant volume cannot be assumed, under favorable circumstances two measurements enable one to solve the problem. For example an equation is given for measuring milk intake in breast-fed babies, using D_2O as tracer for the mothers milk. Experimentally samples of the baby's saliva were taken twice, at a two week interval (ref. 194).

b) the single pool model, non steady state conditions

In biological experiments, it is not always possible to wait for equilibrium conditions, or to use the priming technique. The Steele method, developed to investigate the effect of injected insulin on glucose production, enables to solve the difficulty (ref. 195).

It is assumed that the inward flow of fluid carrying the trace and the continuous injection of tracer mix perfectly and instantaneously with the volume of the pool. Let V be the volume of the pool, c_v its concentration in the fluid, c_p its concentration in tracer, D_i the inward flow rate, c_i its concentration in the fluid (D_i is free of tracer), D_o the outward flow rate with c_o its concentration in the fluid which is equal to c_v and its concentration in tracer to c_p. The latter values are a consequence of the hypothesis of perfect mixing. As before, q is the usual injection flow rate of tracer at concentration c_T.

Variations in the fluid and tracer amounts in V are given by

$$dV \cdot c_v / dt = D_i . c_i - D_o . c_v \tag{24}$$

and

$$dV \cdot c_p / dt = (c_p \cdot dV / dt) + (V \cdot dc_p / dt) = q \cdot c_T - D_o \cdot c_p \tag{25}$$

As

$$dV / dt = D_i - D_o \tag{26}$$

combining (24), (25), and (26) leads to (27) and (28)

$$c_p(D_i - D_o) + V(dc_p / dt) \ = q \cdot c_T - D_o \cdot c_p \tag{27}$$

$$D_i \cdot c_p = q \cdot c_T - V(dc_p / dt) \tag{28}$$

and, assuming a negligible change in volume during dt,

$$D_o \cdot c_v = D_i \cdot c_i - V(dc_v / dt) \tag{29}$$

D_i and D_o are calculated from these relations.

When perfect mixing can only be assumed with a fraction f of the total volume of the pool, $f.V$ in the above relations replaces V. And in practice, instead of calculating exact derivatives measurements at two different times, t_1 and t_2 are taken. Values of c_p and c_v are replaced by the average of their values at t_1 and t_2. Derivatives are replaced by differences between values at t_1 and t_2 divided by $(t_1 - t_2)$.

c) multiple interconnected reservoirs, or pools

In this case, neither the growth nor the decay follows unique exponential laws. The bolus injection technique is the technique mostly used in biological studies. Growth curves are complex, their interpretation requires the establishment of a mathematical model of the system. This task is difficult, models and results, are often uncertain. One should refer to (ref. 6) for a detailed discussion of the problem, and to (ref. 196 and 197) for original mathematical treatments.

Flux of atoms, or molecules between two phases. The first order law.

The treatment applies generally to fluxes between species that can be identified. At equilibrium, it is difficult to measure the rate at which elements exchange between molecular species in the same or different phases; for instance, the rate at which water molecules cross from a condensed phase into the vapor, and vice versa. The so called first order law (30), enables one to calculate the rate R at which atoms are transferred. The relation is a first order with respect to time.

$$\ln(1-F) = -R \cdot t(c_a + c_b) / c_a \cdot c_b \tag{30}$$

Where R is the rate of exchange of atoms or molecules between two species A and B, c_a and c_b are concentrations of A and B in their respective phases, or compounds exchanging atoms, or molecules, are labeled in A, F is the fraction of tracer that has undergone exchange at time t. More precisely, if the number of atoms of the tracer in A is x_o at time 0, x_t at time t, and x_e when equilibrium is reached, then

$$F = (x_o - x_t) / (x_t - x_e) \tag{31}$$

Demonstrations and discussion of equation (30) are found in (refs. 2e and 198).

When experiments aiming at making use of the first order law are carried out, care must be taken that:

a) rates of exchange of tracer and fluid atoms are not affected by a significant kinetic isotope effect, and

b) sampling for analysis introduces no isotopic discrimination.

R, an exchange reaction rate, has the general form

$$R = k\, c_a^{\,m} \cdot c_b^{\,n} \tag{32}$$

m and n can be obtained by varying c_a and c_b, and differentiating $\ln R$ versus $\ln c_a$ or $\ln c_b$.

The activation energy of the reaction is obtained in the usual manner, by plotting the curve $\ln k$ versus $1/T$, where T is the temperature. The curve is a straight line in the case of a regular Arrhenius relation. The method has been applied to study tranfers as different as that of sodium between sea water and fish, or carbon beween atmospheric CO_2 and the oceans. Whilst radioactive isotopes were used for these applications, magnesium ion flux measurements in single barnacle muscle cells were made using stable isotopes as tracers (ref. 199), and the pharmacokinetics of lidocaine absorption could be described by a single first order process (ref. 200).

Generally speaking, applying the first order law may spare users of stable isotopes from going through complicated reasoning, and help to obtain results more easily.

IV. USES OF ISOTOPE EFFECTS AND OF SPECIFIC NUCLEAR PROPERTIES

Definitions

Isotope effect is the name given to any change of physical or chemical property induced by the substitution of one isotope for another, but leaving out differences in nuclear properties. Effects can be either equilibrium or kinetic.

Intermolecular effects is the name given to effects observed when there is a difference of isotopic abundances of an element between different phases, or different chemical species. When isotopic abundances depend on the site within a molecule, such effects are known as intramolecular effects. When different sites of a single molecule may exchange, effects on each site may be different: one speaks of site-specific effects. Site specific effects are necessarily coupled with intramolecular effects. Finally, if in a molecule undergoing a reaction an isotopic substitution is made on a given site, this substitution may induce a secondary isotope effect on neighbouring sites. Such effects are named α if they occur on the first neighbour, β if they do on the second, etc. The magnitude of equilibrium effects is often identical to isotopic separation coefficients for which the usual designation is α.

General considerations

TABLE 6. Uses of isotope effects and of specific nuclear properties of isotopes. The table includes effects that are outside the scope of the report defined Part I.

	Fundamental research	**Applied research**	**Limitations**
Effects in equilibria	Studies of: Molecular structures Reaction mechanisms Bond force constants Condensed phases	In pharmacology: Location of active sites. Estimation of efficiency of chemical processes. Isotope separation.	**Kinetic effects**
	Isotope effects in nature	Measurement of temperature in geological dating.	
		In the water cycle: Water resources, glaciology.	
		Climatology	
		Authentication of natural products.	
		Archaeometry	
Kinetic effects	Structures of activated complexes.	Studies of: Enzyme reactions Solvent effects Tunnel effects Adsorbed species	**Effects in equilibria**
	Reaction mechanisms.		
	Exchange rates of reactions in equilibria		
Specific nuclear properties	Nuclear physics	Production of pure radioactive isotopes. Nuclear medicine. Dating methods. Nuclear industry.	

In the preceding Part, isotope effects, and, principally kinetic isotope effects, were quoted as causes of errors in interpretations of tracer experiments. In this chapter, dealing with equilibrium and kinetic effects, it is shown that they open fields of research specific to the use of stable isotopes, because isotope effects are not usually measurable with radioactive tracers, and, when they are, a useful precision is in most cases difficult to obtain.

The biggest equilibrium and kinetic effects are those of light elements, whose isotopes have large relative mass differences. The largest, at times, by orders of magnitudes, see appendix II, are those of hydrogen isotopes. Isotope effects are larger for the heavy elements than for those of the middle of the classification, because of nuclear volume effects. Nuclear size and shape effects have been interpreted as the origin of specific enhencement of ^{235}U, with respect to other uranium isotopes in chemical exchange reactions (ref. 201). Some effects have been found to be mass independent. For instance, in some meteorite samples, differences of the ^{17}O to ^{16}O and the ^{18}O to ^{16}O ratios from that in normal oxygen were the same, contrary to the expected factor of two expected for the second with regard to the first. Mass independent effects are also produced in the laboratory, especially in the formation of ozone. They are not fully understood (refs. 202 and 203).

Equilibrium Effects

Equilibrium effects are treated separetely from kinetic effects because techniques used, and information obtained are different in both cases,

Measurement of equilibrium effects.

Direct measurements. As precise comparisons of isotopic ratios are best made using the same analytical technique on the same chemical species it is not always easy to measure directly isotope effects. For example, to measure the ^{18}O effect in the CO_2 - H_2O equilibrium it would not be safe to measure the ^{18}O to ^{16}O ratio both on water and CO_2 molecules. Oxygen 18 in CO_2 is measured, in the mass spectrum, by the ratio of peak heights at mass 44 and mass 46. For water, the usual technique is to equilibrate samples with CO_2, and, from the equilibrium constant to calculate the ratio in water. Obviously another method must be used. It consists in reducing water to carbon monoxide, for instance by running the vapor over heated diamond, and to convert the monoxide to dioxide in an electrical discharge. Only CO_2 is thus introduced in the mass spectrometer, either for the analysis of the water, or for that of the CO_2 with which it has been equilibrated (ref. 204).

When studying hydrogen, oxygen or carbon isotope effects of reactions involving organic molecules, isotopic abundances, or ratios, may be measured on the entire molecules. These are burnt, and the water, and carbon dioxide produced are isotopically analysed. This procedure has the drawback of averaging changes over whole molecules. But it is possible, principally by NMR, to study each site during a reaction, and to find those which show at equilibrium changes in isotopic composition. Such changes are obviously larger and can be the source of more specific information.

The Rayleigh technique. The Rayleigh formula, cf. Appendix A, shows that changes of isotopic composition of a species undergoing a transformation, whether chemical or physical accompanied by a volume reduction, are given by a power of this reduction which depends on α , the value of the effect. Mesuring both isotopic changes and volume reductions enables one to calculate associated isotopic effects. Advantages of the method are: a) that all measurements are made on the same species in only one phase, and b) that changes in isotopic compositions larger than those of a single equilibrium step can be obtained. Large volume reductions are induced, facilitate measurements.

The method applies to any system where a species is converted into another *under equilibrium conditions.*

Indirect method. When the establishment of equilibrium is slow, or when, for analysis, separation of exchanging molecules, or of phases in equilibrium, is difficult, or induces an undesirable isotope effect, masking the real one, an alternative route may be found by measuring equilibrium effects in separate exchanges of the two species with a third one from which separation is faster or easier.

Equilibria of oxygen isotopes between rocks can be evaluated by studying their much faster equilibria with water. Equilibrium factors of exchanges between water and alcohols have been studied by making separate measurements, by NMR, of fractionation factors of their exchanges with hydrogen and taking the ratios of results (ref. 205). It may also be possible to avoid separations when NMR is employed.

Uses of NMR.. NMR is especially useful for site-specific effects, and intra molecular equilibria determinations. Although NMR does not usually provide values of isotopic abundances, in the case of deuterium ratios of peak heights, at given sites, provide ratios of abundances and values of isotope effects. NMR can in this way measures inter, and also intra-molecular and site-specific hydrogen isotope effects even when deuterium is at its natural abundance. The analytical technique is discussed in (ref. 206). Other analytical techniques can only identify sites of isotope effects in organic molecules having many similar groups of atoms, or several identical functions (acid, alcoholic, etc.), at the cost of painstaking work.

In natural substances, deuterium abundances differ according to various factors. In plants they follow as an average the local water supply, modified by fractionation factors that can be site specific. Local water concentrations in deuterium are strongly dependant on climate, thus on the geographical situation. Consequently, measuring site specific deuterium concentrations, may be a tool for assigning the origin of wines, perfumes, etc. It can be supplemented by mass spectrometric determinations of ^{13}C whose contents vary according to the assimilation cycle of carbon by plants (ref. 206).

A whole set of primary and secondary thermodynamic hydrogen isotope effects can be directly determined in a "one pot " experiment, without the need for selective deuterium labeling. When working at deuterium natural abundance, molecules are labeled only at one position, instead of possibly several ones when enriched hydrogen is used; thus interferences of signals originating from many labels, that are to be feared with highly enriched molecules, do not occur.

Carbon isotopes site specific ratios, at natural abundances, can also be studied by NMR, though with more limited applications than those allowed by the use of mass spectrometry (ref. 207).

Investigation of equilibrium conditions

The question is how to be certain that equilibrium conditions prevailed during a process involving volume reductions or changes of phase, generally a Rayleigh distillation.

The double label method. When the system under study consists of molecules having either more than one polyisotopic element, or one element with at least three isotopes, the simultaneous study of two isotopes may furnish a proof of equilibrium when αs for both isotopes in the process are known.

a) Volume reductions may be the unknown, for instance, when studying amounts of evaporation from lakes or reservoirs. Volume reductions can be calculated from isotope enrichments if αs are known, because final volumes and concentrations in deuterium and ^{18}O are measurable, and initial isotopic concentrations are known. Initial volumes can be calculated using both isotopes; results only agree if equilibrium prevailed.

b) This method has been used, with D and ^{18}O, to prove that equilibrium was established when cloud water vapor condensed to form hailstones. The temperature of condensation was assumed to explain the deuterium concentration in the condensed phase. It was confirmed by ^{18}O measurements (ref. 208).

In Rayleigh processes, by calculations of tritium enrichments obtained by volume reductions, that are practiced in order to enhance isotope concentrations too low to be measured directly in the initial volume. Assuming that equilibrium conditions prevailed, the Rayleigh equation enables one to calculate initial concentrations by measuring volume reductions and final concentrations. The equilibrium assumption can be validated by considering deuterium whose initial concentration can be measured.Thus for this isotope, the Rayleigh relation contains no unknown and is only verified when equilibrium conditions prevailed.

In exchange reactions, the ratios of isotopic abundances of elements possessing at least three stable isotopes is predicted, at equilibrium, by the rule of the geometric mean. (Appendix A). When they are obtained it is a good presumption that equilibrium has been reached. Oxygen isotope fractionations between minerals and water were studied in this way, using the following technique.

The pseudo equilibrium method. The method was used to measure ^{16}O-^{18}O fractionation factors in equilibrium reactions between rocks and water (ref. 209). The authors started from a system already close to equilibrium in ^{16}O versus ^{18}O, but far from equilibrium in ^{17}O versus ^{18}O. Thus the $^{16}O/^{18}O$ ratio did not change much during the reaction and the influence of inhomogeneities, in solid state minerals, or of other factors of perturbation, was minimal. A sufficient approach to equilibrium was obtained when, according to theory, the $^{17}O/^{16}O$ effect reached a value about one half of that of the $^{18}O/^{16}O$.

Uses of equilibrium effects in the laboratory

The list of problems that measuring isotope fractionations may contribute to solve cannot be exhaustive. Significant examples in various fields are given below, consideration being given only to uses of fractionations produced in the laboratory or occuring in nature other than those relative either to isotope separations or to dating, for which one is referred to the literature. (refs. 7a and 210).

Fundamental research on equilibria, solid state physics. Equilibrium isotope effects are correlated with molecular structures, and help to establish them. In particular, systematic correlations of the isotope chemistry of all hydrogen compounds have been made as a function of molecular structures and of the atomic number of the element bonded to the hydrogen atom.

Condensed phase isotope effects are closely related to the nature of intermolecular forces in liquids and solutions. They have been reviewed (ref. 211). References to vapor pressure studies that led to an understanding of some condensed phase isotope effects and to improved calculations of interactions in the liquid and solid phases, and to analysis of equilibrium effects in terms of bond force constants are found in a special issue of *Zeitschrift für Naturforschung* (ref. 212), dedicated to J. Bigeleisen.

NMR gives evidence of intramolecular effects by detecting unusual effects on the fractionation factors of hydrogen isotopes (ref. 213). In evaporation of ethanol, whereas the hydroxyl hydrogen shows a normal behaviour, i.e. a depletion of the vapour in hydroxyl deuterium, the methyl and methylene groups exhibit a reverse effect: the vapor of those groups is enriched in deuterium (ref. 214).

In solid state physics, there is a shift in the transition temperature of superconductivity in metal doped fullerenes upon isotopic substitution. And there is a difference between fullerenes prepared from a powder of carbon enriched at 50% in ^{13}C, and materials prepared from a 50% mixture of ^{12}C and ^{13}C powders (ref. 215), probably due to differences in binding energies. General Electric prepared diamonds 99% pure in ^{13}C, and also diamonds 99.9% pure in ^{12}C, instead of the natural abundance, which is 98.9% in ^{12}C. In both cases a 50% improvement in heat conductivity over that of natural diamonds was obtained.

Pharmacology Diagnoses. Reactive sites of molecules may be identified as those where isotope effects occur. When pure tracer work is done, molecules must be labeled at positions that are not involved in the mechanism, preferably, with a heavy isotope unlikely to undergo isotope effects. However, to study mechanisms, the label should be located at a site which is implicated in the mechanism (ref. 216). Thus stable isotopes in pharmacology offer the possibility of evaluating mechanisms of drug action, and of drug-drug interactions, of verifying assumptions, with the advantage that the use of stable isotopes is safe (ref. 217).

Another possibility offered by stable isotopes is related to the fact that substitution by stable isotopes, for instance replacing a CH_3 group by a CD_3 group in antipyrin or caffeine, may affect drug metabolic pathways, and change their activity. For example an increase in the hypnotic activity of deuteriated butethal is reported (ref. 218).

Studies of isotope effects in respiratory gases are not to be mistaken for breath tests cited in Part III, the latter using isotopes only as tracers. For example, oxygen isotope fractionation associated with respiration can be used in diagnoses (ref. 219) because it is affected by a history of smoking, by vigorous exercise, and, for patients who suffer anemia, it is related to their level of haemoglobin (ref. 220) and to their blood flow value (ref. 221).

<u>Study of processes in nature</u>

General principles: Isotopic fractionations in nature have the following origins:

a) <u>Evaporation and condensation in the water cycle</u> that affect hydrogen and oxygen isotopes. Fractionation factors, and their temperature dependence, are found in standard texts (ref. 7b and 222). Usually the greater vapor pressures are those of light molecules, but inverse effects, due to intermolecular forces are observed with aromatic molecules (ref. 223).

b) <u>Exchange reactions</u>, of which the oxygen-carbonate exchange is a good example (ref. 7b, 222 and 224). Examples of equilibrium constants and their temperature dependance are given in Appendix B.

c) Production by decay of radioactive parents of some or several stable isotopes. Lead is typical of this case.

d) <u>Differences in the chemistry of the growth of plants</u> and in metabolic paths of animals, affecting mostly the light elements H, C, N and O. The most often quoted differences are between plants following C4 or C3 cycles. Isotopic records stored by trees (ref. 137) and measures of the fractionation of oxygen isotopes by respiring marine organisms (ref. 138) help in interpreting isotopic abundance variations in the environment.

e) <u>Differences of formation of ore deposits</u> by mechanisms, not always identified. Deposits of boron ores, e.g. are known to differ in isotopic composition.

Applications. Measured fractionations due to mechanisms a) lead to quantitative data in particular of amount of precipitations. Temperature evaluations rest on processes described in b), dating by processes on c). As mentioned earlier we shall not dwell on this last subject. Processes d) and e) provide essentially natural tracers; amounts of fractionations may serve in interpreting their mechanisms.

Studies of the water cycle: General considerations enabling one to use isotope measurements in hydrology, water ressources, climatology:

a) Enrichment of the water of reservoirs occurs due to evaporation. Isolated reservoirs, whose water content is reduced by evaporation, become isotopically heavier; the maximum enrichment, obtained under equilibrium conditions, is given by the Rayleigh formula. Constant volume reservoirs losing a fraction of the input of water by evaporation are most enriched, if evaporation compensates totally the feed, and when equilibrium is reached, by one α. At this maximum the vapor has the same composition as the feed.

b) At a given location, precipitations undergo seasonal isotopic variations due to temperature changes.

c) As clouds loose by precipitation isotopically heavier fractions of their water content they become isotopically lighter along their course.

d) There is a linear relationship, Craig relationship, between ^{18}O and D concentrations of waters or ices, formed under equilibrium conditions in precipitations or condensations, that can be interpreted in terms of atmospheric and climatic processes (ref. 225)

Studies of the water cycle: Cumulative geographical and climatic effects. Polar caps isotope contents

Ice caps of polar regions and of Greenland are archives of past deuterium and oxygen 18 contents in snow falls, because once snow is converted into ice very little isotopic changes occur. Even diffusion of hydrogen atoms induces measurable attenuation of isotopic variations in ice only after tens of thousand years.

Seasonal variations in volume of precipitations cause winter snows to be lighter than summer ones: counting recorded seasonal variations enables one to count numbers of years, and to evaluate yearly accumulation rates.

Average deuterium and oxygen 18 contents of snow in the polar regions are low due to the losses of water affecting, on their way, clouds formed over the oceans; close to the poles, their remaining deuterium content may be only two thirds of the original level. Climatic changes, because they alter ocean temperatures, reflect on these average isotopic contents and can thus be studied through them. Data obtained by isotopic analysis of ice cores cover more than two hundred thousand years (ref. 226). Other analysis, such as that of CO_2 in occluded gas bubbles, give some insight on how parameters influencing the climate are correlated, and thus helps in making predictions, for instance on possible changes due to the greenhouse effect (ref. 227).

Studies of the water cycle: Isotope techniques in water resources. Isotopic contents of waters depend on all previouly mentioned factors: temperatures of evaporation of the water generating clouds, temperature of precipitation, geographical location. It makes isotopic analysis of water a tool in water resource studies.

Evaporation studies and studies of rates of refilling of aquifers are made in many countries. In (ref. 228) a number of examples are given of estimations of losses of ground-water by evaporation in arid regions and of determinations of the origin of groundwater, by ^{18}O and D analysis. The technique can also be applied in alluvial plains or in the Alps. Other investigations use simultaneously chemical and tritium together with stable isotope measurements. Thus tritium and nitrate concentrations serve to estimate the mean residence time in a particular zone of an aquifer, whereas ^{18}O and D concentrations, which are correlated with local climatic fluctuations, showed that the recharge period is in winter, summer rainfall playing no part (ref. 229). Using the laws governing isotopic enrichments of reservoirs, the ratio of water lost by evaporation to total loss of water of a lake with no visible outlet could be calculated from deuterium measurements. The probability of a hidden outlet was evaluated (ref. 230).

Temperature measurements

Temperature variations of isotope effects serve to measure temperatures of sedimentation, crystallisation, etc. The systems must have been quenched after the process took place. Urey, Epstein and Mayeda (ref. 224) derived the first temperature scale based on this principle by analysing the ^{18}O of carbonates of fossil shells, namely belemnites. They verified that when shells were formed equilibrium of oxygen isotopes was established, layer by layer, by exchange with sea water. After formation no measurable exchange occurs over geological times. As the volume of the oceans remains approximately constant, its ^{18}O concentration does not vary. One needs only to measure it and the isotope ratio of carbonates to build a temperature scale. The precision of ^{18}O determinations, 0.1δ enabled one to estimate temperatures to better than $0.5^{0}C$. Further work is quoted in the literature (ref. 7b and 222).

Temperatures of formation of rocks can also be measured by studying the isotopic composition of oxygen. Equilibria between rocks and water can be studied by the pseudo-equilibrium method and equilibria between rocks by the indirect method (cf above).

The temperature of formation of every layer of ice of hailstones was measured in order to find out at what altitude they were formed, as temperature and altitude are univocally linked in the hailstone cloud (ref. 231). Temperatures were calculated from deuterium measurements, the basic underlying assumption being that condensation in the cloud is a Rayleigh process, depleting the uncondensed vapour in deuterium. As explained earlier it was necessary to check that equilibrium was established. It was done, and confirmation of equilibrium obtained, by the double label method, measuring enrichments in deuterium and ^{18}O of the ice layers.

Temperatures may also be derived from equilibria between fluids when separation does not alter their isotope compositions. In natural gas from a well, at Lacq, in France, 30% H_2S gas, with too little water to alter its deuterium content by exchange, was present. Assuming that the measured deuterium concentration of H_2S was due to equilibration with water, supposedly at the deuterium concentration of local streams, at the bottom of the well it was then possible to assign to the bottom a temperature that was later confirmed by direct thermometry (ref. 232).

Kinetic Isotope Effects

Kinetic isotope effects, defined in the same way as equilibrium effects, are tools for investigating reaction mechanisms. Early work by Bigeleisen (ref. 233), and co-workers (ref. 234) paved the way to their use.

Measurement of kinetic isotope effects

To measure kinetic isotope effects three possibilities are offered: the first is to perform separate kinetic experiments on reactants of different isotopic compositions and follow rates in each case. This is the direct, or non competitive, method. The second method is to carry out the reaction on a mixture of labeled and unlabeled compounds and to follow the change in isotopic composition of the reactants: it is the indirect, or competitive method. The third method is the equilibrium perturbation technique. Those methods have been developed in special detail for the study of enzyme reactions; this field is discussed at length in (ref. 13b). All methods are of limited use when reaction rates are too close to diffusion controlled rates.

Direct or non Competitive Methods

The basic characteristic of direct methods is to give access to the overall velocity, *v*, the rate of the first irreversible step *V*, and to *V/K*. (see Appendix B). To establish rates, no isotopic analysis is required. Reactions are followed by conventional methods of measuring the apparition of reaction products, or the diminution of reactant amounts.

As precision on rate measurements is commonly a few percent, and as two series of determinations must be made, except when employing the quasi-racemate technique described below, direct methods are nearly always used with hydrogen isotope labeled compounds that can be subject to very large effects (Appendix B). However experiments with reactants labeled with ^{18}O and ^{15}N have also been made (ref. 225).

Requirements of the method are:

a) to prepare reactants labeled to the highest degree of enrichment obtainable on the reactive site, in order to observe the greatest possible difference in rates.

b) to use, when practicable, a continuous assay technique, such as ultraviolet or visible absorption, or, when studying enzyme reactions, enzymatic methods of measurements.

c) to ensure that rates to be compared are measured at identical concentrations of reactants, and eventually of catalysts, enzymes being a particular case of the latter, and at the same temperature, when rates are determined at one temperature only.

d) to take into account in the interpretation of results that, when several positions may exchange in a reactant molecule and only one is initially labeled, results may concern both intra and intermolecular effects.

e) to take into account the difficulty that may arise in the case of reactions for which the rate of reaction of the protonated (lighter) compound, which is always faster than that of the deuteriated one, becomes too fast to be measurable at high temperatures.

Direct or non Competitive Methods: The quasi-racemate method

This method was first proposed independently by several authors (ref. 236). It consists in comparing directly the reaction rates of an unlabeled compound to its fully labeled enantiomeric form. A fifty percent mixture of each produces an inactive solution (this technique is necessarily employed in the liquid state) designated as the pseudo-racemic. The reaction is started in a polarimetric cell. If the unlabeled compound reacts at a different rate than its enantiomeric labeled form, an optical activity manifests itself. This activity reaches a maximum when the difference in concentration of the two compounds is maximum, and necesarily falls back to zero when both are completely converted to the final products.

With this method rates of reactions are compared under strictly identical conditions and small rate differences of very fast reactions can be measured: the ^{18}O kinetic isotope effect for the hydrolysis of p-nitrophenyl [1-^{18}O]- β-glucopyranoside in 2.0 M HCl was measured by the quasi-racemate technique (ref. 237).

Use of radioactive isotopes with direct methods

In general, for reasons given above, radioactive isotopes are not used with direct methods with the exception of tritium which has been employed, for instance, to study an exchange reaction (ref. 238). NMR was chosen as analytical technique thus avoiding separating labeled and unlabeled reactants.

Indirect or competitive methods

Indirect methods consist in following the ratio of abundances of two isotopes during the reaction. They enable one to calculate isotope effects on V/K, rather than on absolute rates. Reasons for resorting to competitive methods with stable isotopes are the following:

a) For direct measurements, fully isotopically labeled reactants that may be expensive or not available are required. By contrast, taking advantage of the precision and accuracy of measurements of isotopic ratios competitive experiments can be carried out at low enrichments, and even at natural abundances in the case of ^{13}C or ^{15}N. No synthesis of labeled molecule is then necessary.

b) Several causes of error are eliminated by the fact that reactions of both isotopes take place at the same time, in the same vessel. No correction for differences of temperature, or of catalyst or enzyme amount, has to be made.

c) The method is applicable even when a reagent is formed in situ and the rate of formation of the reagent is the slow step;

d) Tritium and ^{14}C are commonly used in competitive experiments, either independently or in conjunction with deuterium or ^{13}C, as small levels of activity only are required with that technique.

Indirect or competitive methods: Isotopic analysis made at the reacting site

The ratio k_1/k_2 of kinetic rates of molecules labeled wih isotopes 1 and 2 is given by the limit at zero conversion of the ratio R/R_p, giving rise to (33)

$$k_1/k_2 = R_o/R_p \qquad (33)$$

where R_o is the initial isotopic ratio of concentrations of isotope 2 to isotope 1, and R_p is their ratio in the product (ref. 239).

When small molecules like CO_2 are studied, the global isotopic effect is identical to, or a large fraction of, that at the reaction site. But organic molecules contain so many carbons and hydrogen atoms that effects are diluted to a non-measurable point in global isotopic compositions. NMR may provide site specific determinations in some cases, otherwise the sometime difficult procedure is to isolate atoms at the reacting site, and to analyse them after insertion in a small molecule, often CO_2 or H_2O.

Indirect or competitive methods: The remote label-double label technique

The technique, introduced by O'Leary and Marlier with stable isotopes (ref. 240) in order to achieve measurements when analysis on the active site is not possible, was already practiced with radioactive ones. The method, fully discussed in (ref. 13a), rests on the principle of independance of isotope effects, (rule of the geometric mean, Appendix B), that has the following consequences:

a) If one labels at the same time sites active in the reaction, where isotope effects should occur, and inactive sites, isotopic changes observed at the latter will be due to differences in reaction rates induced by isotopic substitutions at active sites.

b) Isotopes of two different elements, one labeling an active site and the other a site sufficiently remote from it to be free of isotope effect, can be utilized to measure isotopic effects, as shown by the following example.

Let, in a given reaction, carbon at the active site be C_a, and nitrogen at a remote position N_r. Consider the four isotopic molecules characterized by the nature of the isotopes at the active and remote sites and their four possible reaction rates: k_{24} reaction rate of the molecule in ${}^{12}C_a$-${}^{14}N_r$, k_{25} reaction rate of the molecule in ${}^{12}C_a$-${}^{15}N_r$, k_{34} reaction rate of the molecule in ${}^{13}C_a$-${}^{14}N_r$ and k_{35} reaction rate of the molecule in ${}^{13}C_a$-${}^{15}N_r$.

If there is no isotope effect on nitrogen, $k_{24} = k_{25}$ and $k_{34} = k_{35}$ and the carbon isotope effects, k_{24}/k_{34}, or k_{25} / k_{35}, are identical. As $k_{34} = k_{35}$ it follows that $k_{24} / k_{34} = k_{24} / k_{35}$. The latter ratio can be measured on nitrogen isotopes at the remote site.

The measurement requires that a doubly light and a doubly heavy molecule are available. The absence of isotope effect at the remote site must be checked. This can be done on a compound at natural abundance. The choice of the isotope at the reactive site is imposed by the reaction investigated, the choice of the isotope at the remote site is made according to the easiest way of performing the analysis.

Examples: Remote label isotope effects on atoms heavier than hydrogen (ref. 241). In the alkaline hydrolysis of methylformate both the methoxyl oxygen isotope effect, $k_{16} / k_{18} = 1.009$ in water at 25°C, and the oxygen exchange data, $k_{hydrolysis}/k_{exchange} = 18.3$ in water at 25°C, are consistent with a rate determining formation of a tetrahedral intermediate. The isotope effects for the carbonyl carbon, carbonyl oxygen and nucleophile oxygen during the alkaline hydrolysis are interpreted within the same model.

In methyl benzoate the remote label method allowed measurement of the isotope effects in water at 25°C: $k_{12}/k_{13} = 1.04$ on the carbonyl carbon, $k_{16}/k_{18} = 1.005$ on the carbonyl oxygen, and $k_{16}/k_{18} = 1.006$ on the methyl oxygen. .

Indirect or competitive methods: The pseudo natural abundance technique

This method is similar to the pseudo equilibrium method cited earlier. Experimental errors are minimized when the mixture of isotopically heavy and light molecules, at the remote site, reproduces the natural abundances of the isotopes.

Let a and b be the numbers of isotopically pure heavy and light reacting molecules, and the kinetic effect $1+\varepsilon$. The corresponding numbers are a' and b' in the product, thus a'/b', normally equal to a/b times the isotope effect is $a.(1+\varepsilon)/b$. Suppose that the natural product contains mostly the light isotope, (which is a good approximation for elements H, C, N, O), and that a small amount of natural molecule, $b'\varepsilon'$ contaminates the product, the measured ratio in the sample becomes a''/b'', with $a''= a'$, $b''= b'(1+\varepsilon')$. Thus

$$a''/b'' = a'/b'(1+\varepsilon') = a\cdot(1+\varepsilon)\,/\,b\cdot(1+\varepsilon') \sim (a/b)\cdot(1+\varepsilon-\varepsilon') \qquad (34)$$

Let a kinetic effect be 1.08, with a 2%, contamination. Then $\varepsilon = .08$ and $\varepsilon' = .02$. The result is 1.06, which is a 25% error on the effect.

If now one starts with a pseudo natural mixture of pure isotopes with $a/b = n$, then a'/b' should be equal to $n.(1+\varepsilon)$. Contamination adds $b'\varepsilon'$ to b', but also $nb'\varepsilon'$ to a'. And the measured ratio, a''/b'', becomes

$$a''/b'' = [b'n(1+\varepsilon) + nb'\varepsilon']/(b' + b'\varepsilon') = n\cdot(1+\varepsilon+\varepsilon')/(1+\varepsilon') \sim n\cdot(1+\varepsilon-\varepsilon\varepsilon') \qquad (35)$$

With the same isotope effect and contamination as before, the measured ratio becomes 1.0784, which is negligibly different from 1.08. The reason of the difference between the two experiments is that in the first one contamination is almost exclusively on one of the labeled molecules, as in the second it affects both molecules in the same proportion.

Use of radioactive isotopes. Tritium and ^{14}C had been used with double label techniques even before stable isotopes, but as pseudo natural mixtures of radioactive isotopes they are not appropriate, it is not possible to compensate for contamination errors as with stable ones.

Use of radioactive isotopes: The flooding dose technique. This technique was designed to overcome the difficulty of identifying the true precursor enrichment for the calculation of protein synthesis when employing the constant tracer infusion method (ref. 6). It consists in injecting a large amount of unlabeled amino acid together with the tracer, thus flooding the precursor pool and minimizing differences in isotopic enrichment of the free amino acid in plasma and tissue compartments (ref. 242). The rate of synthesis of the specific protein was determined by dividing the rate of incorporation of the tracer by the average enrichment of the precursor.

Use of radioactive isotopes: Use of Multiple isotope effects in enzyme catalysed reactions. This technique consists in using deuterium substitution to selectively slow down the rate of one step in a reaction and observing the change on a second isotope effect. The subject is treated at length (in chapter 9 of ref. 13b). When the two isotope effects are on the same step, the second one is either enhanced, because the step has become slower and more rate limiting, or remains unchanged if the step was already rate limiting. It is quoted that the first use of the method was with formate dehydrogenase, where the ^{13}C isotope effect was the same with deuteriated and normal formate, showing that hydride transfer from formate was rate limiting.The use of the multiple isotope method is only straightforward when the second isotope effect is not a tritium effect.

Use of radioactive isotopes: The equilibrium perturbation technique for enzymatic reactions. The method consists in mixing the labeled reactant (substrate) with the unlabeled product of the reaction to obtain calculated equilibrium concentrations. If there is an isotope effect, when adding the enzyme the unlabeled lighter product reacts faster, usually, than the labeled substrate. A transitory change in concentration is induced before the system comes back to equilibrium; it can be followed by optical methods or by isotopic analysis. The former has enabled to measure effects as small as 1.003 (ref. 13b page 314).

Uses of kinetic effects

Overview of uses of kinetic effects. Interpretation of isotope effects enables one to identify which atoms are involved in the rate determining steps. Rates of equilibration of isotope exchange reactions give information on factors influencing those rates such as the activity, and state of catalysts surfaces, may be obtained. Studies of kinetic effects have grown into investigations of factors influencing the magnitude of these effects and of the structure of transition states (ref. 243), in studies of reaction models. This is achieved by making comparisons between calculated and measured values of isotope effects. Such comparisons can be made with better accuracy and precision than those between calculated and measured absolute rates. They show how a comparison of calculated and observed isotope effects enables to choose a transition state structure. Two isotope effects are investigated simultaneously in this example, one on sulfur, the other on carbon isotopes, illustrating the usefulness of investigating several different isotope effects in the same molecule. Another example (ref.13b p.278) is the study of the thermal decomposition of azo compounds followed on hydrogen, nitrogen, and carbon isotopes.

Kinetic isotope effects are used to investigate details of chemical mechanisms of enzyme actions (ref. 244 and, mostly, ref. 13b). Biochemical reactions are predominantly studied by measurement of hydrogen isotope effects, because hydrogen is present at nearly all molecular reacting sites, and hydrogen isotope effects are larger by orders of magnitude than those of other elements, nitrogen being a special case. However, among other considerations, the readiness with which hydrogen atoms exchange between molecules introduces difficulties of interpretation and necessitates to investigate also carbon, nitrogen, and oxygen isotope effects, and even effects on heavier atoms.

Calculations of reaction rates, outlined in Appendix A, involve classically a transition state in equilibrium with the reacting species. The basic text giving fundamental relations, and results depending on the structure of this state (conformation, bond lengths, bond angles, etc.) is (ref. 245). In (ref. 13b and 246) more detailed and up-to-date compilation of theories and results are found.

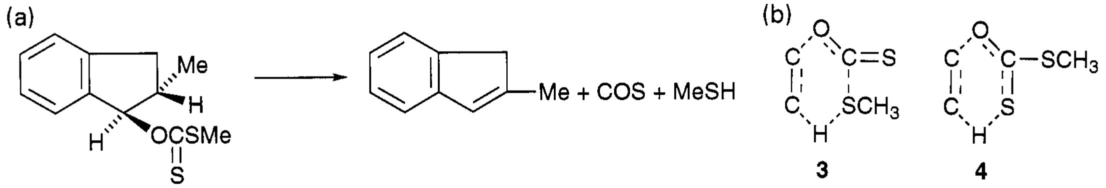

Fig. 1 (a) The Chugaev reaction. (b) Possible transition states.

Figures 1a and 1b, and table 7 are reproduced from Reaction rates of Isotopic molecules Melanders L., Saunders W. by permission of John Wiley & Sons Inc.

Special problems: Rates of isotope exchange reactions independently of kinetic isotope effects

Measurements of equilibration rates in a mixture of $^{16}O_2$ and $^{18}O_2$ can be used to study the sintering of catalysts (ref. 247). This exchange combined with the exchange of oxygen isotopes with CO and with solid oxides of metals of catalysts can serve to study the mobility and reactivity of oxygen species on their surfaces (ref. 248).

Special problems: Solvent isotope effects

Solvent isotope effects are observed mostly when enzymatic rate measurements are made in H_2O and D_2O, or in mixtures of both in various proportions. A difference between solvent effects and ordinary enzymatic reaction rate effects is that in the latter only a few sites of the substrate are labeled and effects are site specific. When D is substituted for H in the solvent, as enzymes have hundreds of exchangeable hydrogen sites, the effect is global. It is necessary also to ascertain whether effects observed are true isotope effects, or the result of differences in physico-chemical properties of ordinary and heavy water, such as neutral pH, viscosity, etc. And it must be verified that no isotope effect affects the exchange reaction with the solvent, and masks or modifies effects on rates of enzymatic reactions.

A discussion of solvent effects (ref. 13b, pages 73 to 126) covers the various situations mentioned above. Curves plotting reaction rates of enzymatic reactions, versus atom fractions of the solvent in deuterium, are interpreted in detail; their shapes vary, among other factors, according to the number of proton transition states involved in the reactions, and, also according to wether the reaction shows a direct or reverse isotope effect.

The mechanism of the reaction of Cr^{2+} with $(NH_3)_5Co(OH)_2{}^{3+}$ has been investigated by the effect, on the reaction rate, of substituting D_2O for H_2O. The decrease in rate, by a factor of 3.8, has been compared with the decrease in rate observed on the reaction of known mechanism of Cr^{2+} with the same molecule at oxidation degree two only. Results show that the transfer of hydrogen from reducing agent to oxidizing agent was not necessarily implied in the mechanism, and that the O-H bond in the bridging group was stretched in the activated complex (ref. 249).

In biochemistry, a study of the human immunodeficiency linked to Virus-1 Protease is cited (ref. 244).

Special problems: Solvolytic reactions

Two mechanisms can be rate determining in such reactions, either a dissociative one, SN1, or an attachement, SN2. A simple example (ref. 250) is schematized by (36).

$$\begin{array}{llllllll}
RX & \xrightarrow{k_1} & R+X & \xrightarrow{k_2} & R^+//X^- & \xrightarrow{k_3} & R^++X^- & \\
 & & & \downarrow k_4 & & \downarrow k_5 & \quad \downarrow k_6 & \quad \downarrow k_7 \\
(36) & & & & & & & \\
ROS & & ROS & & ROS & & ROS &
\end{array}$$

It was expected that isotope effects would be different whether the rate determining step of the solvolysis of RX to give ROS would be step 1, 2 or 3 rather than step 4, 5, 6 or 7. As this was not verified, a sophisticated treatment of the data was recognized necessary, in order to use isotopic results to conclude to mechanisms.

Special problems: Tunnel effects

Tunnel effects can be large (ref. 250 and Appendix B). An example of an experimental study of tunnel effects is shown by an investigation of apparent deviations from the Swain-Schaad relation between kinetic deuterium and tritium effects (ref. 257).

Specific Nuclear Properties

When properties of their nuclei, other than magnetic resonance, are utilized, stable isotopes are either used directly as targets which are studied when undergoing nuclear reactions, or they are irradiated to prepare radioactive isotopes.

Direct uses

In nuclear physics such uses are numerous, and too diverse, for examples, to be typical (ref. 252). They usually require small, often miligrams, quantities of isotopes. Electromagnetic separators, the so called Calutrons, located either in the US or in Russia, are sources of isotopes for those kinds of experiments. Enrichments above 99% are required when low backgrounds are important, 95% often suffice when structures of nuclei and nuclear reactions are investigated, and 50% when the purpose is to make ion sources.

^{126}Xe, ^{76}Ge, were employed in large quantities (thousands of liters, kilograms) for the investigation of double b decay, fabrication of GeLi radiation detectors. They were produced by centrifugation (ref. 253).

NMR imaging in nuclear physics using rare gases has seen an interesting development by the use of 70% enriched ^{129}Xe the nuclear spins of which were polarized by laser optical pumping; polarized ^{129}Xe can be stored frozen for hundreds of hours. Images of lungs, heart, air vessels of mice have been obtained (ref. 254). In neutron diffusion experiments elements depleted in isotopes having large capture cross sections, such as ^{157}Gd, must be used.

Analysis by photon and by neutron activation, were mentioned in Part II for the determination of ^{48}Ca at low levels in biological samples (ref. 255), or the use of enriched ^{86}Sr and ^{50}Cr for isotope dilution analysis.

In nuclear medicine, neutron capture therapy is developed utilizing isotopes that can belong to molecules which selectively target tumor cells; when the capture cross section is large, if the accompanying radiation emitted is absorbed by it, the tumor will be destroyed leaving normal tissue intact. Boron 10 is the preferred isotope for this application because of its large cross section for thermal neutrons, (3838 barns), the high linear energy transfer of alpha and ^{7}Li particles emitted, and the number of biochemically usable compounds into which it can be inserted (ref. 256). Gadolinium 157 is being studied though it emits γ rays which are less effective in destroying tumors because, in addition to a much larger cross section, (255000 barns), it has compounds that can be developed as contrast agents for NMR imaging (ref. 257).

Uses after irradiation

These are in fact uses of radionuclides requiring enriched isotopes for their production, in order to minimize secondary reactions producing undesirable nuclides.

Uses in nuclear medicine. In medicine, radionuclides of interest are normally the short lived ones. Irradiations of the stable precursors are carried out either in cyclotrons or reactors. Several precursors may have the same daughter radionuclide, and conversely a stable isotope subject to irradiations of different kinds, e.g. deutons instead of protons, gives rise to different daughter nuclides. A summary of production and uses for nuclear medicine is available. (ref. 258).

We select a few examples. Reactor irradiations of enriched ^{235}U produce ^{99}Mo, which decays into ^{99m}Tc, the most widely used radioisotope in medicine. with applications to cancerology, endocrinology, etc. One also can collect ^{133}Xe, useful for lung explorations. Iron 59 is produced by neutron capture by ^{58}Fe, etc. Cyclotron irradiations by protons or deutons produce thallium 201, used in cardiology, from kilograms of ^{203}Tl; ^{123}I, with a half life of 13 hours and a 136 keV gamma emission is widely used for explorations of the heart, brain and central nervous system receptors. It is also useful in scintigraphy of the thyroid; it is obtained either by reactions on enriched ^{124}Te, or enriched ^{124}Xe. Fluorine 18 one of the best nuclides for positron emission tomography, (PET), is prepared by reactions on ^{18}O. Other stable parent isotopes for PET are listed in (ref. 259). Separations of the starting isotopes are difficult because isotopes such as ^{124}Xe, ^{18}O, and, to a lesser degree, ^{124}Te have low abundances; and enrichments of Xe and O isotopes can not be obtained by calutrons. Single photon emission tomography (SPET) uses γ emitting atoms, like ^{201}Tl, that have the advantage of having a longer half-life than ^{11}C or^{18}O. SPET, PETand magnetic resonance spectroscopy have been compared (ref. 169).

Isotope generators are of common usage in nuclear medicine. To obtain pure radioisotope the parent must have a high specific activity. For instance ^{188}Re, that has a variety of therapeutic applications, can be 'milked' from ^{188}W prepared by neutron irradiation of 96.07 % ^{186}W, and deposited as oxide on a support. by a process described in (ref. 260).

Other uses in nuclear physics. ^{50}Cr was used in kilogram quantities to produce ^{51}Cr for calibration of neutrino counting equipment for solar neutrino research (ref. 253).

CONCLUSIONS

It may be surprising that, in view of the many areas where decisive contributions have been made by employing stable isotope techniques, these are still principally tools in the hands of specialists. And it is not patent that the fact that stable isotope work is a key that opens doors that remain close to radioactive tracer applications is well recognised.

When writing this report it became apparent that the work of users could be hampered by causes that were mentioned in several papers.

Isotopes, especially those of metallic elements, were not always available. It happened that when delivered neither the quantity nor the enrichment were conform to specifications. But even isotopes that are the most in demand, D, ^{18}O, ^{15}N, ^{13}C, have been difficult to procure at times. Costs are high and analytical methods are expensive and slow. Other nuclides like ^{33}S or ^{43}Ca could be good tracers because of high sensitivities vis a vis NMR detection. Calcium 43, would be particularly useful for detection in vivo and in situ, for which other calcium isotopes are not suitable. But both, having low abundances and two more abundant stable neighbours, are difficult to enrich economically. It should not, however, be forgotten that the cost of separated isotopes is, most of the time, only a fraction of that of synthetized labeled molecules.

Though stable isotopes, per se, are innocuous, their medical usage is slowed down by a somewhat unexpected problem. Their natural abundances are in many cases subject to detectable variations. To prevent such variations from distinguishing labeled from unlabeled molecules, the level of labeling must be significantly above natural abundances. Doses of drugs administered to patients may thus become much larger than those required with radioactive ones, and testing for innocuousness, which requires a large number of tests, may become prohibitively expensive, not to mention the possibility that the necessary level of drug might not be aceptable in some cases.

In spite of the above mentioned difficulties the number of papers reporting uses of stable isotopes is steadily growing. Meetings of the International Isotope Society, which gather stable and radioisotope users are well attended, and so are the biennial Gordon Conferences on stable isotopes that tackle more theoretical problems. The International Agency for Atomic Energy, IAEA, organizes symposia and publishes proceedings of the meetings, and those of panels of experts that cover work on isotope in the life sciences, in hydrology, in climatology, water ressources, environment, etc. Some of IAEA's reports are quoted in Part I. They show, that in all these fields developing countries, countries of the east and middle east, make ample use of isotope techniques in agricultural research and water ressources in particular.

In addition, many regional meetings take place: the German Arbeitsgemeinschaft Stabile Isotope holds an annual conference. In France, in 1993 and 1994, three symposia were held respectively on identification of alcohols and perfumes by isotopic NMR techniques, on production and applications at large, on structural determination of proteines by applying NMR and isotope methods. Isotopenpraxis publishes a large quantity of stable isotope work in the fields of environment and health research, while Journal of Labelled Compounds and Radiopharmaceutics, is the journal of the International Isotope Society.

In many countries, efforts by persons or institutions convinced of the potential benefit of a wider use of stable isotopes have been made. In the United States, P. Klein and his wife have worked, for many years, to develop biological uses of stable isotopes. In the eastern countries stable isotope institutes were established to develop production and applications. Thoses at Cluj, in Romania, and in Leipzig in the former DDR, were particularly famous.

But nowadays stable isotope institutes in former eastern countries have been closed. Separation facilities of isotopes by calutrons have almost ceased to operate. A table of remaining producers is given (in ref. 261), a review of the US potential is found (in ref. 2c), production and uses in France are summarized (in ref. 262).

If difficulties listed before are not smoothed out, the benefits of using stable isotopes will not be reaped as they should. This means, for example, that they would not easily play the role, in quality insurance (ref. 263), in traceability of chemical measurements (ref. 264), in medicine, in pharmacology, that their specificity assigns to them.

Especially frequent is the request for improvements of analytical techniques. For some applications they may come from the adoption of new methods, and new instrumentation. Progresses must be made by universities and research centers, because atomic energy commissions are no longer as active as they were, and users do not seem to offer yet a large enough market to induce industry to make a major effort. Fortunately, references in this report show indications of interesting activities going on in several laboratories.

Perhaps cooperative programs could be set up to speed up the obtention of results. Perhaps also methodology of isotopic applications and analysis could be taught in some universities at the post graduate level.

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APPENDIX A. ISOTOPE EFFECTS BASIC RULES AND CALCULATIONS

Equilibrium Effects

Order of isotope exchange reactions

Isotope exchange reactions are first order with regard to the isotope concentration in the labeled molecules, as shown when deriving the first order law.

Fractionation factors due to chemical reactions

Fractionation factors are linked, but not identical, to equilibrium constants of reactions, because the statistical repartition of isotopes between molecules, that may affect equilibrium constants, does not induce isotope effects, as shown later on an example. They can be expressed as the ratio of partition functions of the products and of the reactants

The theory of equilibrium isotope effects has been extensively developed over the past forty years, in particular by Bigeleisen (ref. 265). To calculate the ratio of partition functions it is assumed that the Born-Oppenheimer approximation is valid and that translation, electronic, vibrational and rotational partition functions can be separated. Only the two latter are important for isotope effects.

Calculations based on Bigeleisen's papers derive those functions from the vibrational and rotational spectra of the molecules. It is noteworthy that isotopic shifts in spectra can be obtained by calculation for simple molecules. Urey calculated useful values of partition functions, and equilibria (ref. 266), one can find also summaries. of his data (refs. 2e and 13b).

The rule of the geometric mean

This rule is a consequence of the former assumptions, that has been expressed first by Bigeleisen. Multiple isotopic substitutions are independent or, formulated in another way, the overall fractionation factor for isotopic substitutions, by the same isotope, on several sites of a molecule, is the product of the fractionation factors on each individual site. The name rule of the geometric mean is given when two fractionation factors are equal, because each one is the geometric mean, i.e. the square root, of the total fractionation.

For instance in the case of water and hydrogen the fractionation factor for the reaction

$$H_2O + D_2 = D_2O + H_2 \qquad (A1)$$

is the product of the fractionation factor of

$$H_2O + HD = HDO + H_2 \qquad (A2)$$

and of

$$HDO + D_2 = D_2O + HD \qquad (A3)$$

Reaction (A1) is never fully observeded because the fast equilibration reaction (A4) takes place.

$$H_2O + D_2O = 2HDO \qquad (A4)$$

Equilibrium constants of chemical reactions and fractionation factors

At equilibrium, in the absence of isotope effect, a statistical repartition of isotopes between molecules having different numbers of exchangeable atoms is established and causes the concentration of labeled molecules to be in the ratio of these numbers. Equilibrium constants differ from unity, but this introduces no isotopic separation as shown by considering the following equilibrium reaction .

$$HDO + NH_3 = H_2O + NH_2D \qquad (A5)$$

Let brackets represent molecular concentrations. The ratio of deuterium to protium in water will be

$$[HDO]/\{[HDO]+2[H_20]\} \qquad (A6)$$

and, in ammonia.

$$[NH_2D]/\{[NH_2D]+3[NH_3]\} \quad (A7)$$

At very low deuterium contents [HDO] and $[NH_2D]$ may be neglected with respect to $[H_2O]$ and $[NH_3]$. As there is no isotope effect abundances of deuterium in water and ammonia are equal, at equilibrium, thus

$$[HDO]/2[H_2O] = [NH_2D]/3[NH_3] \quad (A8)$$

as the equilibrium constant K of reaction (A5) is

$$K = [NH_2D].[H_2O]/[NH_3].[HDO] \quad (A9)$$

the absence of isotopic effect represented by (A8) implies that $K = 3/2$

Equations such as (A9) are often written as in (A10),

$$K = \{[NH_2D]/[NH_3]\}/\{[HDO]/[H_2O]\} \quad (A10)$$

a form that expresses K as a ratio of directly measurable ratios.

Effect of temperature

In intermolecular effects whether of equilibrium reactions, or of changes in phase, fractionation factors depend mainly on temperature. Starting from the expression of the free energy

$$\Delta G = \Delta H - T\Delta S \quad (A11)$$

as ΔG changes sign with temperature, it follows that, for isotope effects in chemical reactions, or for vapor pressure effects, there is theoretically a temperature at which, the effects change direction. This temperature, however, may have no physical meaning as it may be outside the stability range of the molecules, or above their critical point.

Effect of pressure

Pressure has generally little or no effect.

The Rayleigh distillation formula

Consider ratios R of isotopic abundances. The equilibrium factor a between vapor and liquid is the ratio of abundance ratios, R_l in the liquid and R_v in the vapor. It is greater than one when Rs are chosen to be the ratios of abundances of the less volatile isotopic species to the more volatile one. Most of the time lighter isotopic compounds are more volatile, however there are many exceptions: benzene, toluene, etc. (ref. 233). It seems to be due to intermolecular deuterium bonds in the condensed phase being weaker than the normal hydrogen bond. Then, when distillation reduces an initial volume of liquid V_o to a final one V_f, the final ratio in the liquid, R_{fl}, is given with regard to the initial one, R_{ol} by (A12).

$$R_{fl} = R_{ol}\,(V_o/V_f)^{(\alpha - 1)/\alpha} \quad (A12)$$

From this formula it can be seen that a reduction in volume by a factor of e, basis of neperian logarithms, i.e. 2.718, induces an enrichment in the liquid of $(\alpha - 1)/\alpha$, which is in most cases practically equal to $\alpha - 1$, because a is close to unity. A reduction by e^2 (~7.38) doubles this enrichment, etc. *The formula applies to every physical or chemical process that produces a reduction of the reacting volume in a thermodynamically reversible way.* Discussions of conditions of application are found in the literature, (ref. 2e and ref. 3).

Kinetic isotope effects

Definitions and basic relations

Effects are primary when a bond to the isotopic atom is formed, or broken, during the reaction. For instance an effect is primary when an hydrogen atom migrates from a carbon atom to another one during a reaction. It is secondary if the hydrogen remains attached to the same atom. Primary effects may be orders of magnitude larger than secondary, or other effects, with the exception of tunnel effects on hydrogen.

Schematically the basis of the theory of rate processes is summarized as follows: reactants R, form with substrates, S, transition state compounds, RS, in equilibrium with them. RS in turn forms products P.

$$R+S = RS \rightarrow P \tag{A13}$$

The transition state compound, also termed activated complex, dissociates into products P according to a first order law. V_p rate of formation of P is given by

$$V_p = \nu\cdot RS] \tag{A14}$$

where ν is the decomposition frequency of RS, and

$$[RS] = K\cdot[R]\cdot[S] \tag{A15}$$

where K is the equilibrium constant of (A13). As, according to theory

$$V_p = k\cdot[R]\cdot[S] \tag{A16}$$

where k is the absolute rate constant, combining (A14) and (A16) leads to

$$k = \nu\cdot[RS]/[R]\cdot[S] = \nu\cdot K \tag{A17}$$

According to the absolute reaction rate theory

$$K = (k_B T/h\nu).K^{\#} \tag{A18}$$

where k_B is Boltzmann's constant, h Planck's constant, T the absolute temperature, and $K^{\#}$ is the equilibrium constant calculated from the partition functions of the transition state, the ***decomposition mode excluded***. Combining (A17) and (A18) gives

$$k = (k_B T/h).K^{\#} \tag{A19}$$

From (A19) the ratio of absolute rates for two isotopically differing species is obtained by the ratios of $K^{\#}$'s

$$k'/k = K'^{\#} / K^{\#} \tag{A20}$$

This ratio can be calculated from the ratio of partition functions in the same way as ordinary ratios of equilibrium constants for isotopically differing reactions, the decomposition mode excluded. Calculations are carried out with a number of different sets of force constant changes with isotopic substitution, and with different assumptions concerning the geometry of the transition state. Chosen models are plausible when there is an agreement between calculated and observed values of isotope effects.

Specific isotope effects on reaction rates

Effects on two isotopes of the same element

We concentrate on the case of hydrogen and the three isotopes: protium, H, deuterium, D, and tritium,T. A relation between the ratios of absolute rate constants has been calculated in this case, it is the Swain-Schaad relationship (ref. 267):

$$(k_H/k_D)^{1.442} = (k_H/k_T) \qquad \text{(A21)}$$

an alternative form of (A21) is

$$(k_H/k_T) = (k_D/k_T)^{3.263} \qquad \text{(A22)}$$

another way of writing this relation is identical to that chosen for equilibrium isotope effects, i.e.

$$r = \ln(k_1/k_3)/\ln(k_1/k_2) = [1-(m_1/m_3)^{1/2}]/[1-(m_1/m_2)^{1/2}] \qquad \text{(A23)}$$

where subscripts 1, 2, 3, designate respectively H, D, and T, and ms are for masses.

Tunnel effects

Tunnel effects are encountered in reactions in which a species reacts without going over the potential barrier. They are mostly effects on reaction rates of molecules where hydrogen is nearly pure protium, because tunnelling for protium is much bigger than for deuterium or tritium. Therefore tunnelling isotope effects on reaction rates can be important.

Experimental evidence of tunneling is given by deviations from the Swain-Schaab rule, and also by deviations from the law of the geometric mean, leading, in the case of hydrogen isotopes to

$$K_{\mathrm{HH}}/K_{\mathrm{HD}} > K_{\mathrm{DH}}/K_{\mathrm{DD}} \qquad \text{(A24)}$$

Tunnelling produces deviations from the linear Arrhenius logarithmic plot of rates versus the reverse of absolute temperatures that result in apparently abnormal isotope effects. For example k_{H}, because of tunnelling, will be larger, at low temperatures, than predicted by the conventional relation (A25)

$$k = A\exp(-E/RT) \qquad \text{(A25)}$$

a linear extrapolation to $1/T = 0$ will be smaller. When comparing k_{H} and k_{D}, as this effect is negligible with deuterium, A_{H} may thus appear smaller than A_{D}, in contradiction with reality..

Kinetic effects in exchange reactions

When there is an isotope effect in an exchange reaction the first order law becomes

$$\ln(1-F) = -Rt(\alpha/c_a + \beta/c_b) \qquad \text{(A26)}$$

instead of equation 28 of Part III. In (A26) above, α is the kinetic isotope effect in the forward direction, and β the kinetic isotope effect in the reverse one, thus α/β is the isotope exchange equilibrium constant. By performing successively experiments with c_a # c_b and $c_a \ll c_b$, α/c_a is accessible. If one then repeats the experiment with another isotope of the same element, as will mostly be done with hydrogen isotopes, and rarely with those of carbon or oxygen, ratios of αs and βs for both isotopes can be obtained, providing the influence of masses on kinetic isotope effects.

Secondary isotope effects

Secondary isotope effects are often more directly tied to the structure of the transition state than primary ones, because the isotopic atom does not belong to the reactive bond, and therefore complications linked with the influence of the reaction coordinate are absent. A relation between a kinetic effect and the corresponding value of the equilibrium is derived when the free energy of the transition state can be written as in (A27), making abstraction, as usual, of the free energy connected with the decomposition mode.

$$dG_T = x dG_P + (1-x) dG_R \tag{A27}$$

Subscripts T, P, R refer respectively to transition states, products, reactants. The transition state has a structure close to that of the reactant when x is small, and it ressembles that of the product when x is close to one. The basic relation of the absolute rate theory, coupled with (A27), leads to the following relation between kinetic and equilibrium effects

$$x = \ln(k_1/k_2)/\ \ln(K_1/K_2) \tag{A28}$$

or, relating x to the maximum kinetic isotope effect, obtained for $x = 0.5$

$$\ln(k_1/k_2)/\ln(k_{1max}/k_{2max}) = 4x(1-x) \tag{A29}$$

Equations (A28) and (A29) apply principally in the case of secondary isotope effects (ref. 268). They enable to evaluate x and qualitatively obtain information about the transition state. Note that (A29) has two solutions in x, equally acceptable in the absence of other information, and that the above relations apply to heavier than hydrogen isotope effects.

Enzymatic reactions

The first steps of enzyme-catalysed reactions are always the formation of complexes with the substrates. Though usually several steps take place, we shall discuss only the simplest one step process, schematically represented by (A30), as it already enables to write fundamental equations of enzymatic reactions,

$$\mathrm{E + S \rightarrow ES \rightarrow E + P} \tag{A30}$$

E represents the enzyme, S the substrate, ES the enzyme complexed with the substrate, P the products.The steady state condition is expressed by (A31)

$$d[\mathrm{ES}]/dt = 0 = k_1\{[\mathrm{E}]-[\mathrm{ES}]\}\cdot[\mathrm{S}] - (k_{-1} + k_2)[\mathrm{ES}] \tag{A31}$$

writing the rate ν

$$\nu = d[\mathrm{P}]/dt = k_2[\mathrm{ES}] \tag{A32}$$

solving [ES] from (A30), and remembering that [E] << [S],

$$K = (k_{-1} + k_2)/k_1 \tag{A33}$$

and, taking into account that, when the enzyme is totally complexed, the maximum reaction velocity is V, given by (A34)

$$V = k_2[\mathrm{ES}] = k_2[\mathrm{E}] \tag{A34}$$

one gets the relation known as the Michaelis-Menten expression

$$\nu = V[\mathrm{S}]/(K + [\mathrm{S}]) \qquad \text{(A35)}$$

V/K is given by (A36)

$$V/K = k_1 k_2[\mathrm{E}]/(k_{-1} + k_2) \qquad \text{(A36)}$$

(A36) relates V/K to the enzyme concentration. It keeps the same form even when more than one step is to be taken into consideration. Isotope effects on V/K are the only measurable quantity by competitive isotopic methods, i.e. methods, described in Part IV, where isotopic reactions are carried out simultaneously on the mixture of isotopic species.

Let us append inverted commas, ('), to isotopic quantities, it can be shown (ref. 269), that

$$\nu/\nu' = k_2[\mathrm{ES}]/k'_2[\mathrm{ES'}] = \{k_1 k_2[\mathrm{S}]/(k_{-1} + k_2)\}/\{k'_1 k'_2[\mathrm{S'}]/(k'_{-1} + k'_2)\} \qquad \text{(A37)}$$

ν and ν' are the reaction rates of the normal and isotopic reaction. Let us write, for both k and k'

$$k = \nu/[\mathrm{S}] \qquad \text{(A38)}$$

Then

$$k/k' = V/K \,/\, V'/K' \qquad \text{(A39)}$$

Isotope effects on V and K must be measured by separate, direct, rate measurements.

APPENDIX B. NUMERICAL VALUES OF ISOTOPIC EFFECTS

When planning to use isotopic tracers either in the laboratoty or in nature, in order to estimate the care to be taken to avoid undesirable thermodynamic or kinetic effects, an estimation of the magnitude of possible isotopic fractionations when handling samples is necessary.

This appendix gives examples of isotope effects stemming from various causes. Special attention should be paid to the largest effects, those of hydrogen isotopes, which are the most likely to lead to mistakes.

Table 7 together with table 8 shows how partition functions enable to calculate hydrogen isotopic exchange reactions and help to evaluate reactions that may not be experimentally easy to study.

TABLE 7. Ratios of Partition Functions for Hydrogen and Water Molecules. Reproduced, with table 8 by permission of the Royal Society of Chemistry from Urey H C. *J. Chem. Soc.* p. 569 (1947).

	Temperature K				
Molecule pair	273.1	298.1	400	500	600
HD/H_2	7.6282	6.7366	4.7672	3.8941	3.4030
D_2/HD	2.4017	2.0704	1.3715	1.0762	0.9154
$(D_2/H_2)^{1/2}$	4.2803	3.7346	2.5570	2.0482	1.7650
HT/H_2	12.496	10.537	6.5542	4.9642	4.1258
T_2/HT	5.1548	4.1182	2.2041	1.5298	1.2003
$(T_2/H_2)^{1/2}$	8.0260	6.5873	3.8008	2.7558	2.2254
DT/D_2	3.6482	3.4426	2.9246	2.6652	2.5034
T_2/DT	0.9617	0.9020	0.7545	0.6799	0.6346
$(T_2/D_2)^{1/2}$	1.8731	1.7622	1.4855	1.3452	1.2605
HDO/H_2O	32.7400	24.9460	11.7240	7.5602	5.6614
D_2O/HDO	8.3185	6.3065	2.9282	1.8791	1.4044
$(D_2O/H_2O)^{1/2}$	16.503	12.543	5.8592	3.7689	2.8196
HTO/H_2O	95.507	65.266	22.681	12.281	8.2027
T_2O/HTO	28.575	19.085	6.2523	3.2871	2.1593
$(T_2O/H_2O)^{1/2}$	52.241	35.293	11.908	6.3535	4.1603
DTO/D_2O	5.9628	5.3294	3.9068	3.2674	2.9088
T_2O/DTO	1.6805	1.4857	1.0573	0.8696	0.7658
$T_2O/D_2O)^{1/2}$	3.1656	2.8138	2.0324	1.6857	1.4925

Each third entry is obtained by multiplying figures of the two preceding lines and taking the square root of the product.To calculate table 7 rotational corrections were not made for any of the values for water.

TABLE 8. Equilibrium Constants for Hydrogen and Water Exchange. Same ref. as table 7, p.568.

	Temperature K					
Equilibrium	0	273.1	298.1	400	500	600
$[HD]^2/[H_2][D_2]$	0	3.18	3.25	3.48	3.62	3.72
$[HDO]^{1/2}/[H_2O][D_2O]$	0	3.94	3.96	4.00	4.02	4.03
$[HDO][H_2]/[H_2O][HD]$	∞	4.29	3.70	2.46	1.94	1.66
$[D_2O][HD]/[HDO\{D_2]]$	∞	3.46	3.05	2.14	1.75	1.53
$[HT]^2/[H_2][T_2]$	0	2.42	2.56	2.97	3.24	3.44
$[HTO]^2/[H_2O][T_2O]$	0	3.34	3.42	3.63	3.74	3.80
$[HTO][H_2]/[H_2O][HT]$	∞	7.64	6.19	3.46	2.47	1.99
$[T_2O][HT]/[HTO][T_2]$	∞	5.54	4.63	2.84	2.15	1.80
$[DT]^2/[D_2][T_2]$	0	3.79	3.82	3.88	3.92	3.94
$[DTO]^2/[D_2O][T_2O]$	0	3.55	3.59	3.70	3.76	3.80
$[DTO]\{D_2]/\{D_2O][DT]$	∞	1.63	1.55	1.34	1.23	1.16

In table 8, equilibrium constants are obtained by ratios of ratios of partition functions found in table 7. For instance, the first line is the result of the division of the first line of table 7 by the second one. Though data presented are rather old they are not obsolete.

Tables 9, 10, 11 show constants of isotopic equilibrium reactions involving also other elements. Comparisons between calculated and experimental values are given.

TABLE 9. Equilibrium Constants of Isotopic Reactions. (Reproduced from ref. 11a.) Exchange reactions shown in this table lead to fractionation factors identical with equilibrium constants, because they take place between molecules having the same number of exchangeable atoms.

Reactions	Equilibriuim Constants	Temperature °C
$HD(g) + H_2O(g) = HDO(g) + H_2(g)$	3.70	25
$HT(g) + H_2O(g) = HTO(g) + H_2(g)$	6.26	25
$H_2O(l) + HDS(g) = HDO(l) + H_2S(g)$	2.35	25
$H^{12}CN(g) + {}^{13}CN\text{-}(sln) = H^{13}CN(g) + {}^{12}CN^{-}(sln)$	1.03	25
${}^{13}CO_2(g) + H^{12}CO_3(sln) = {}^{12}CO_2(g) + H^{13}CO_3(sln)$	1.0075	23
${}^{12}CO_3^{-}(sln) + {}^{13}CO_2(g) = {}^{13}CO_3^{-}(sln) + {}^{12}CO_2(g)$	1.017	0
${}^{12}CH_4(g) + {}^{13}CH_4^{-}(l) = {}^{13}CH_4(g) + {}^{12}CH_4^{-}(l)$	1.002	77
${}^{15}NH_3(g) + {}^{14}NH_4^{-}(sln) = {}^{14}NH_3(g) + 15NH_4^{-}(sln)$	1.035	25
$1/3C^{16}O_3^{-}(sln) + H_2{}^{18}O(l) = 1/3C^{18}O_3^{-}(sln) + H_2{}^{16}O(l)$	1.036	0
$1/2C^{16}O_2(g) + H_2{}^{18}O(l) = 1/2C^{18}O_2(g) + H_2{}^{16}O(l)$	1.047	0
${}^{34}SO_2(g) + H^{32}SO_3^{-}.= {}^{32}SO_2(g) + H^{34}SO_3^{-}$	1.019	25
${}^{76}GeS + {}^{70}GeF_4 = {}^{70}GeS + {}^{76}GeF_4$	1.033	0

TABLE 10. Experimental (K_{exp}) and Theoretical (K_{cal}) Equilibrium Constants for Isotope Exchange Reactions.

Reaction	T°K	K_{exp}	K_{cal}
$HD + DI = D_2 + HI$	600	1.21 ± 0.02	1.207
$HT + H_2O = H_2 + HTO$	298	6.26 ± 0.1	6.350
$1/2C^{16}O_2 + H_2{}^{18}O(l) = 1/2C^{18}O_2 + H_2{}^{16}O(l)$:	273	1.046	1.044
${}^{15}NH_3 + {}^{14}NH_4^{+} = {}^{14}NH_3 + {}^{15}NH_4^{+}$	298	1.034	1.035
$H^{12}CN + {}^{13}CN^{-} = H^{13}CN + {}^{12}CN^{-}$	295	1.026	1.030
${}^{12}CO_3^{2-} + {}^{13}CO_2 = {}^{13}CO_3^{2-} + {}^{12}CO_2$	273	1.017	1.016
$1/3C^{16}O_3^{2-} + H_2{}^{18}O = 1/3C^{18}O_3^{2-} + H_2{}^{16}O$	273	1.036	1.033

Reproduced from J. Bigeleisen, *Science* **147** 3657 p. 467 (1965) by permission from the American Association for the Advancement of Science

TABLE 11. Fractionation Factors at 25°C for $^*CH_3I + CH_3X = CH_3I + {}^*CH_3X$. Reproduced from I. Melander and W.J. Saunders, *Reaction Rates of Isotopic Molecules,* p. 238 (1980), by permission of John Wiley & Sons Inc.

X	$^*C = {}^{13}C$	$^*C = {}^{14}C$
F	1.0306	1.0577
Cl	1.0103	1.0194
Br	1.0073	1.0134
I	(1.0000)	(1.0000)

A special case of equilibrium is that between liquids and their vapors. Isotope effccts are tabulated, table 12.

Except in the case of hydrogen isotopes they were all measured at low temperatures, a region where they reach a few percent for oxygen isotopes. It is seen that all separation factors decrease when the temperature rises. and that the lighter species are the more volatile, except in the case of the deuteriated versus normal methane at the boiling point. This is interpreted as a consequence of stronger light hydrogen bonding in the liquid state that more than counter balances the 'normal' trend of the lighter component to be more volatile.

TABLE 12. Separation Factors in Distillation, derived from Vapor Pressure Ratios. After M. Benedict, T.H. Pigford and H. Levi, *Nuclear Chemical Engineering* 2nd ed. .(1981) p. 714. By permission of McGraw-Hill Inc.

	Separation factors. Values at two temperatures				
Compounds & separation factors as function of the ratios of vapor pressures of pure isotopic compounds	Separation factors at the triple point	Separation factors at the boiling point	Pressure in Torr at the triple point (1torr = 133.322 Pa)	Temperature at the triple point in °C	Normal boiling point, in °C.
ortho-H_2/HD	3.61	1.81	54.	-259.4	-252.9
$(NH_3/ND_3)^{1/3}$	1.080	1.036	45.6	-77.7	-33.6
$(H_2O/D_2O)^{1/2}$	1.120	1.026	4.6	0.0	100
$(H_2O/T_2O)^{1/2}$	-	1.029	-	-	100
$(H_2S/D_2S)^{1/2}$	-	1.001	-	-	-60.7
CH_4/CH_3D	1.0016	0.9965	87.5	-182.5	-161.9
$^{12}CH_4/^{13}CH_4$	1.0054	-	87.5	-182.5	-161.5
$^{12}CO/^{13}CO$	1.0113	1.0068	111.3	-205.7	-191.3
$(^{14}N_2/^{15}N_2)^{1/2}$	1.006	1.004	96.4	-209.9	-195.8
$^{14}NH_3/^{15}NH_3$	1.0055	1.0025	45.6	-77.7	-33.6
$^{14}NO/^{15}NO$	1.033	1.027	164.4	-163.6	-151.8
$N^{16}O/N^{18}O$	1.046	1.037	164.4	-163.6	-151.8
$H_2{}^{16}O/H_2{}^{18}O$	1.010	1.0046	4.6	0.0	100
$C^{16}O/C^{18}O$	1.008	-	111.3	-205.7	-191.3
$^{16}O_2/^{16}O^{18}O$	-	1.0052	-	-	-183.0
$^{3}He/^{4}He$	70.40 at 1°K	3.08 at 3.3°K			
$20Ne/^{22}Ne$	1.046	1.038	325	-248.6	-245.9
$^{36}Ar/^{40}Ar$	1.006	-	516	-189.4	-185.7
$^{128}Xe/^{136}Xe$	1.000	-	317	-111.8	-109.1

Table 13 gives values of the fractionation factors linked to reaction (B1), at temperatures ranging from the solidification point of ammonia to 40°C where its vapor pressure is about 4000 kPa, or 40 bars.

$$NH_3 + HD = NH_2D + H_2$$
(B1)

As explained in Appendix A, fractionation factors, corresponding with the equilibrium constant of reaction (B1) as written above, are the two thirds of this constant, because the exchange takes place between molecules having a different number of exchangeable atoms; and ammonia being more deuteriated at equilibrium than hydrogen, fractionation factors are bigger than 1. Two sets of fractionations are considered: fractionation in the gas phase and fractionation between liquid ammonia and gaseous hydrogen. The latter is the product of the former factor and the liquid vapor fractionation factor for ammonia which is also found in the table.

TABLE 13. Fractionation Factors of the Hydrogen-Ammonia Isotope Exchange and of the Liquid-Vapor Isotopic Ammonia Equilibrium, as Functions of Temperature, where α_g is the fractionation factor in the gaseous phase, α_v is the liquid-vapor isotopic equilibrium factor and α_l is the liquid ammonia gaseous hydrogen isotopic separation factor

Temperature °C	α_g	α_v	α_l
- 76	7.74	1.072	8.29
- 65	7.30	1.067	7.78
- 60	6.90	1.062	7.32
- 55	6.53	1.058	6.92
- 50	6.21	1.054	6.54
- 45	5.91	1.050	6.21
- 40	5.64	1.047	5.90
- 35	5.39	1.044	5.62
- 30	5.16	1.041	5.37
- 25	4.95	1.039	5.14
- 20	4.75	1.036	4.92
- 15	4.58	1.033	4.73
- 10	4.41	1.031	4.55
- 5	4.26	1.029	4.38
0	4.11	1.027	4.22
+ 5	3.98	1.025	4.08
+ 10	3.85	1.023	3.94
+15	3.74	1.022	3.82
+20	3.63	1.020	3.70
+25	3.52	1.019	3.59
+30	3.43	1.017	3.49
+35	3.34	1.016	3.39
+ 40	3.25	1.015	3.30

(There is a slight discrepancy between older values given on table 12 for α_v at the triple point and boiling point of ammonia and those of this table)

Table from E. Roth *Energie Nucléaire* 1 6 p. 335 (1962)

TABLE 14: Maximum Calculated Isotope Kinetic Effects. From J. Bigeleisen, *Science* **110** p.14 (1949). With permission from the American Association for the Advancement of Science.

Stable Isotope	Tracer isotope	k_S / k_T
^{1}H	^{2}H	18
^{1}H	^{3}H	60
^{6}Li	^{7}Li	1.1
^{9}Be	^{7}Be	1.15
^{9}Be	^{10}Be	1.08
^{10}B	^{11}B	1.3
^{12}C	^{13}C	1.25
^{12}C	^{14}C	1.5
^{14}N	^{15}N	1.14
^{14}N	^{13}N	1.14
^{14}N	^{16}N	1.25
^{16}O	^{18}O	1.19
^{19}F	^{18}F	1.25
^{23}Na	^{22}Na	1.03
^{23}Na	^{24}Na	1.03
^{24}Mg	^{27}Mg	1.08
^{31}P	^{32}P	1.02
^{32}S	^{35}S	1.05
^{35}Cl (natural abundance)	^{36}Cl	1.03
^{37}Cl (natural abundance)	^{38}Cl	1.14
^{40}Ca	^{45}Ca	1.08
^{127}I	^{131}I	1.02

The tracer may be either stable or radioactive. The ratio of rates is written to be greater than 1. Most of the time, *but not always,* the table shows that the lighter isotope, having the faster reaction rate, is the stable one.

Kinetic isotope effects are to be avoided in tracer work. Results of calculations by Bigeleisen in table 14 show maximum values that such effects might have.

Measured kinetic hydrogen isotope effects confirm the larger values predicted by Bigeleisen, and even can be greater, as shown on table 15. The authors of the compilation consider that deuterium kinetic effects (k_H/k_D) larger than 20 can not be explained without a contribution of tunneling, and similarly tritium kinetic effects (k_H/k_T) bigger than 50.

TABLE 15. Large Kinetic Hydrogen Isotope Effects. From a compilation by I. Melanders and W.J. Saunders in *Reaction Rates of Isotopic Molecules* p. 142 (1980). By permission of John Wiley & Sons Inc.

Reaction	k_H/k_D	k_H/k_T	*t* °C
4a,4b-dihydrophenanthene-d_{12} + O_2 / 2,2,4-trimethylpentane	95		-31
Me_2CTNO_2 + 2,4,6-trimethylpyridine / *tert*-BuOH-H_2O		78	32
Me_2CDNO_2 + 2,4,6- trimethylpyridine / *tert*-BuOH-H_2O	24.8		25
$PhCTOHCF_3$ + MnO_4^+ / H_2O-OH		57	25
Ph $CDOHCF_3$ + MnO_4^+ / H_2O-OH	16		25
2,4,6-(*tert*-Bu)$_3C_6H_2OT$ + Me / heptane		54	60
Me 4-NO_2-valerate-4-*d* + 2,3,6-trimethylpyridine / *tert*-BuOH-H_2O	27		30
Me_2CDNO_2 + 2,4,6,-trimethylpyridine / H_2O	19.5		25
p-$NO_2C_6H_4CD_2NO_2$ + quinuclidine / toluene	15.6		25
p-$NO_2C_6H_4CD_2NO_2$ + Bu_3N / toluene	14.0		25

Table 15 illustrates also differences between deuterium and tritium reactions.

Table 16 gives ratios of tunnel effet correction factors for isotopic molecules of hydrogen, therefore the contribution of tunnel effects to global isotopic effects for the reactions that are compared.

TABLE 16. Isotope Effects Calculated from Ratios of Tunnel Effect Correction Factors. Figures in this table are calculated after reference 5c page 71

Reactions compared	*T* K			
	250	500	750	1000
$H + H_2 \rightarrow H_2 + H$ / $D + D_2 \rightarrow D_2 + D$ (These are *Ortho-Para* conversions)	2.903	1.420	1.203	1.124
{ $T + H_2 \rightarrow TH + H$ reactions as below with or { $H + HT \rightarrow H_2 + T$	1.041	1.029	1.015	1.010
{ $H + D_2 \rightarrow HD + D$ reactions as below with or { $D + DH \rightarrow D_2 + H$	2.063	1.268	1.133	1.082
$D + H_2 \rightarrow DH + H$ } { $T + D_2 \rightarrow TD + D$} or compared to or $H + HD \rightarrow H_2 + D$ } { $D + DT \rightarrow D_2 + T$	2.625	1.364	1.175	1.107
{ $H + T_2 \rightarrow HT + T$ same reactions with or { $T + TH \rightarrow T_2 + H$	3.138	1.437	1.207	1.123
{ $D + T_2 \rightarrow DT + T$ same reactions with or { $T + TD \rightarrow T_2 + D$	3.561	1.489	1.229	1.130
for comparison $H + H_2 \rightarrow H_2 + H$ and $H + HD \rightarrow H_2 + H$ *ortho-para* conversion and exchange reaction	1.199	1.067	1.035	1.023

The larger tunnel effects are those of reactions written at the left of the first column. Dividing by correction factors of the other reactions provides the contribution of tunnelling to the ratio of the rates of those isotopic reactions. When several reactions appear one above the other their correction factors are the same and contribute equally to isotope effects. Numbers in the table are greater than one because of the order in which reactions are written.

Tables 17 and 18 enable one to compare inter with intra-molecular isotope effects, and experimental with theoretical results. They are reproduced from tables by Bigeleisen published in *Phys. Chem.* **56** pp. 825-827 (Oct. 1952) by permission of the American Chemical Society.

TABLE 17. Carbonyl Isotopic Intermolecular Effects of ^{13}C in Decarboxylation Reactions.

Reaction	*t* °*C*	Experimental effect	Calculated values
$CH_2(COOH)_2 \rightarrow CO_2 + CH_2COOH$	138	1.037 ± 0.002	
same reaction	137	1.034	1.035
same reaction	149	1.041	
same reaction	173	1.034	
same reaction	196	1.036	1.031
$Cl_3CO_2^- \xrightarrow{OH^-} CHCl_3 + HCO_2^-$	70.4	1.0338 ± 0.0007	1.040

TABLE 18. Intramolecular Isotope Effects for Carbon and Nitrogen Isotopes.

Reaction	Bonds intercompared	*t* °C	k_1/k_2 experimental	k_1/k_2 theoretical
$CH_3(COOH)_2 \rightarrow CO_2 + CH_2COOH$	$^{12}C - ^{12}C$ vs $^{12}C - ^{13}C$	138	1.020 ± .001 1.021 ± .001 1.026 ± .001	1.020
$CH_3(COOH)_2 \rightarrow CO_2 + CH_2COOH$	$^{12}C - ^{12}C$ vs $^{12}C - ^{14}C$	150 153 138	1.12 ± 0.03 1.06 ± 0.02 1.087 - 1.105	1.038
$BrCH(COOH)_2 \rightarrow CO_2 + BrCH_2COOH$	$^{12}C - ^{12}C$ vs $^{12}C - ^{14}C$	115	1.41 ± 0.08	1.038
$(COOH)_2 \xrightarrow{H_2SO_4} CO + CO_2 + H_2O$		100	1.033	
$NH_4NO_2 \rightarrow N_2O + 2H_2O$	$^{14}N - ^{16}O$ vs $^{14}N - ^{18}O$	220	1.023 ± . 003	1.026

The next two tables deal with solvent isotope effects. For more detailed data, along with the theory of solvent effects and of their uses in studies of reactions see Cook's book (ref. 13 b).

Low isotope effects in table 19 are interpreted as pointing out to a non linear transition state. Substituent effects for β methyl versus β hydrogen show that the solvent change is producing some change in the extent of proton transfer in the transition state.

Table 20 illustrates the fact that, when studying solvatation changes in a proton transfer reaction by kinetic isotope effects, changes in the isotopic composition of the solvent could alter absolute rates by a large factor without modifying significantly the isotope effect on the substrate.

TABLE 19. Deuterium Isotope Effects in Elimination from Dimethyl-2-phenyl [2,2-$^2H_2$]ethylamine. Experiments are made in Mixtures of Dimethyl Sulfoxide and Cosolvent at 59.8°C. Reproduced from Melanders I., Saunders W. J. *Reaction Rates of Isotopic Molecules* p. 156 (1980), by permision of John Wiley and Sons Inc., New York.

Cosolvent (%)	k_H/k_D	k_{Me}/k_H [a]
None	2.9	0.38
H_2O (10)	2.9	0.54
H_2O (20)	2.7	0.77
H_2O (30)	2.8	0.61
tert-BuOH (20)	3.0	0.58
tert-BuOH (40)	3.1	0.62
tert-BuOH (60)	2.9	-

[a] Ratios of rates for dimethyl-2-phenylpropyl- and dimethyl -2-phenylamine oxides. The value in the line H_2O (30) is based on rates extrapolated from different temperatures.

TABLE 20. Primary Hydrogen Isotope Effects in Elimination ,from 4-(4-nitrphnoxy)-2-butanone of 4-Nitrophenol. Experiments were made in ordinary and deuteriated methanolic metoxide and in ordinary and deuteriated aqueous hydroxide. Numerical data are reproduced from Th. Casamassina, Ph. Huskey *J. Am. Chem. Soc.* **115** p. 14-20 (1993), by permission of the American Chemical Society.

Rate constants in M^{-1} s^{-1}	Solvent MeOH	Solvent MeOD	k_{MeOD}/k_{MeOH}	Solvent H_2O	Solvent D_2O	k_{D2O}/k_{H2O}
k_H	8.53 ±0.23	18.72 ±0.20	2.195	6.397 ±0.078	9.76 ±0.15	1.526 ±0.029
k_D	1.316 ±0.028	2.925 ±0.010	2.228	0.8488 ±0.0077	1.305 ±0.031	1.537 ±0.039
k_H/k_D	6.48 ±0.22	6.40 ±0.24		7.54 ±0.112	7.48 ±0.21	