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TRITIUM EXCHANGE IN BIOLOGICAL SYSTEMS

W. Siri and J. Evers
University of California, Berkeley, California
United States of America

Abstract — Résumé — Аннотация — Resumen

Tritium exchange in biological studies. Whenever tritium-labelled water is employed as a test solute or tracer in biological systems, an appreciable exchange between tritium and labile hydrogen atoms occurs that frequently affects the nature and interpretation of experimental results. The studies reported here are concerned with the magnitude of the effect that tritium exchange introduces into measurements of total body water and water metabolism in animals and humans. Direct measurements of exchange were made in rats, guinea pigs, pigeons, and rabbits. Tritium-labelled water was administered intravenously or by mouth, and tritium space and turnover determined from the concentration of tritium in blood. The animals were then desiccated to constant weight *in vacuo*. The specific activity of water collected periodically during desiccation increased by 50% as a result of isotope effects. Water from combustion of dried rabbit tissues contained about 2% of the tritium originally given to the animal. Adipose tissue alone contained little or no exchange tritium. The dried tissues of the other animals were rehydrated with inactive water and the appearance of tritium in the water observed. The specific activity of the water increased in exponential fashion, i.e., 1-exp. (kt), with about 90% of exchange occurring with a half-time of 1 h, and the remaining 10% with a half-time of 10 h. The total tritium extracted accounted for 1.5 to 3.5% of the dose given to the animal, which agrees with the difference between the tritium space and total body water determined by desiccation.

An indirect estimate of exchange in humans was derived from concurrent measurements of tritium and antipyrene spaces. The average difference of about 2% in water volume agrees with the direct estimates of exchanges in animals.

It is evident that tritium space should be reduced by about 2% to identify it with total body water. The magnitude and relatively slow rate of exchange may also influence the interpretation of metabolic studies with tritium.

Echanges de tritium dans les systèmes biologiques. Lorsqu'on utilise de l'eau tritiée comme soluté ou comme indicateur dans les systèmes biologiques, il se produit entre le tritium et les atomes labiles de l'hydrogène un échange appréciable qui influe souvent sur la nature et l'interprétation des résultats de l'expérience. Les études présentées ici concernent l'ampleur des effets de ces échanges sur la mesure de la masse totale de l'eau de l'organisme et la détermination du métabolisme de l'eau chez l'homme et les animaux. Des mesures de ces échanges ont été pratiquées directement sur des rats, des cobayes, des pigeons et des lapins. Après avoir administré de l'eau tritiée par voie intraveineuse ou buccale, on a déterminé l'espace et le renouvellement du tritium d'après la concentration de ce dernier dans le sang. Les animaux ont été ensuite desséchés sous vide jusqu'à stabilisation du poids. L'activité spécifique de l'eau prélevée périodiquement au cours de la dessiccation s'est accrue de 50% par suite d'effets isotopiques. L'eau provenant de la combustion de tissus de lapin desséchés contenait environ 2% de la dose originale de tritium administrée. Seuls les tissus adipeux ont révélé peu ou pas du tout d'échange de tritium. Les tissus desséchés des autres animaux ont été rehydratés avec de l'eau inactive et l'on a décelé du tritium dans cette eau. On a constaté que l'activité spécifique de l'eau augmentait selon une loi exponentielle ($1-e^{kt}$) et que 90% de l'échange se produisait avec une période d'une heure et les 10% restants avec une période de dix heures. Le total du tritium extrait représentait 1,5 à 3,5% de la dose administrée à l'animal, ce qui concorde avec la différence, déterminée par dessiccation, entre l'espace tritium et la masse totale de l'eau de l'organisme.

Une estimation indirecte des échanges chez l'homme a été établie d'après les mesures des espaces

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tritium et antipyrine. La différence moyenne d'environ 2% relevée dans le volume d'eau concorde avec les estimations directes des échanges chez les animaux.

Il est évident qu'il faut diminuer de 2% environ l'espace tritium pour l'identifier avec la masse totale de l'eau de l'organisme. L'ampleur et le rythme relativement lent des échanges peuvent également influer sur l'interprétation des études de métabolisme effectuées à l'aide du tritium.

Обмен трития в биологических системах. При использовании меченной тритием воды в качестве испытательного растворителя или индикатора в биологических системах происходит значительный обмен между тритием и неустойчивыми атомами водорода, что часто отражается на характере и толковании экспериментальных результатов. Сообщаемые в настоящем докладе исследования посвящены определению величины воздействия, производимого обменом трития, на измерение общего количества воды в организме и на метаболизм воды у животных и человека. Непосредственные измерения обмена были произведены на крысах, морских свинках, голубях и кроликах. Меченная тритием вода впрыскивалась в вены или вводилас через рот, и на основании концентрации трития в крови определялось пространственное его распространение и кругооборот. После этого животные высушивались в безвоздушном пространстве до получения постоянного веса. В результате изотопного воздействия удельная активность воды, периодически собиравшейся в течение высушивания, увеличивалась на 50 процентов. Вода, полученная при сжигании высушенных тканей кролика, содержала около 2% первоначально заданного животному трития. Только в жировых тканях вовсе не наблюдалось обмена трития или его было мало. Высушенные ткани других животных снова гидрировались неактивной водой, и определялось появление в воде трития. Удельная активность воды увеличивалась согласно экспоненциальной функции, т. е. 1-эксп. (кt), причем около 90% обмена происходило при полупериоде в один час, а остальные 10% — при полупериоде в 10 часов. Общее количество извлеченного трития составляло от 1,5 до 3,5% заданной животному дозы, что соответствует разнице между пространственным распространением трития в теле и общим количеством воды в теле на основании произведенного высушивания.

На основании одновременных измерений пространств, заполненных тритием и антипирином, были выведены косвенные данные об обмене у человека. Средняя разница в объеме воды приблизительно в 2% согласуется с непосредственным определением обмена на животных.

Представляется очевидным, что занятое тритием пространство должно быть сокращено приблизительно на 2%, чтобы оно совпадало с общим количеством воды в организме. Величина и относительно малая скорость обмена могут также оказывать влияние на толкование производимых с тритием метаболистических исследований.

Intercambio del tritio en los sistemas biológicos. Siempre que se emplea agua tritiada como solución de ensayo o indicador en sistemas biológicos, se produce entre los átomos de tritio y los átomos lábiles de hidrógeno un intercambio apreciable que afecta a menudo a la índole e interpretación de los resultados experimentales. Los estudios descritos en la presente memoria tienen por objeto determinar la magnitud del efecto producido por el intercambio de tritio en las determinaciones del agua del organismo entero y del metabolismo del agua en los animales y en los seres humanos. Se han efectuado mediciones directas del grado de intercambio en ratas, cobayos, palomas y conejos. Se les administró por vía intravenosa o por vía bucal agua marcada con tritio, y se determinó el espacio tritio y la renovación del tritio a partir de la concentración de tritio en la sangre. A continuación los animales se desecaron en el vacío hasta alcanzar un peso constante. Como consecuencia de los efectos isotópicos, aumentó en un 50% la actividad específica del agua recogida periódicamente durante el desecado. El agua procedente de la combustión de los tejidos desecados de conejo contenía un 2% del tritio originalmente administrado al animal. En el tejido adiposo, tomado aisladamente, el intercambio de tritio fue escaso o nulo. Los tejidos desecados de los demás animales fueron rehidratados con agua inactiva, y se observó la aparición de tritio en el agua. La

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actividad específica del agua aumentó en forma exponencial, esto es, con arreglo a la expresión 1-exp. (kt); un 90% aproximadamente, del intercambio se produjo con un período de una hora, mientras que el 10% restante se verificó con un período de 10 horas. El tritio total extraído equivalió a una cantidad que oscilaba entre 1,5 y 3,5% de la dosis administrada al animal, lo cual concuerda con la diferencia entre el espacio tritio y el agua contenida en el organismo entero y determinada por desecado.

Se efectuó una evaluación indirecta del intercambio producido en seres humanos utilizando mediciones combinadas del espacio tritio y del espacio antipireno. La diferencia media de un 2%, aproximadamente, en el volumen del agua concuerda con las determinaciones directas del intercambio en animales.

Es evidente que el espacio tritio debe reducirse en un 2%, a fin de que coincida con el volumen de agua del organismo entero. La magnitud y la velocidad de intercambio relativamente baja pueden influir también en la interpretación de los estudios sobre el metabolismo efectuados mediante el tritio.

Biological tracer studies with tritium, and particularly those involving tritiated water, are potentially subject to at least three effects, other than outright radiation damage, that may influence the precision and at times the interpretation of experimental results.

The first of these effects, which is the principal subject of this report, results from tritium ions behaving like hydrogen ions and, therefore, exchanging with labile hydrogen atoms of solutes in aqueous solution. In tissue, for example, every constituent, with the possible exception of neutral fat, quickly acquires a highly labile tag on addition of tritiated water, either *in-vivo* or *in-vitro*. Conversely, a metabolite labelled in exchangeable hydrogen positions can loose a major portion of its tritium by exchange before it ever engages in metabolic processes.

A second effect is non-exchangeable labelling by metabolic processes and possibly by other mechanisms that are not yet understood. Metabolic incorporation of tritium into cellular constituents in the presence of tritiated water is to be expected in live tissue, but it may not account wholly for the non-exchangeable labelling if the observation reported here on pure albumin solution is valid. Possibly labelling mechanisms related to the WILZBACH process may be involved [1].

A third factor that must often be considered in tracer studies with tritium is an isotope effect resulting from the three-fold greater mass of the triton relative to that of the proton. Though frequently negligible, an isotope effect may in some instances affect the outcome of a tracer experiment by a factor as great as two or more.

Although hydrogen exchange, non-exchangeable labelling, and isotope effects are useful in their own right as investigative tools, the work reported in this paper is principally concerned with their nuisance value in biological tracer experiments with tritium. It should perhaps also be made clear at the outset that the study of these effects was not a deliberately planned investigation but one that grew somewhat randomly out of a variety of studies on metabolic processes and water kinetics in humans and animals. It is not, therefore, a systematic examination of the problem, but rather an estimate of the probable magnitude of these effects as they may be encountered when tritiated water is used as a test solute for total body water and water kinetics.

The methods employed in this study involved equipment and procedures that are in general use and need not be discussed here beyond noting what they were.

Samples of water from biological fluids and tissues were assayed for tritium with a Tri-Carb liquid scintillation coincidence counter (Packard Instrument Company,

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La Grange, Illinois). The liquid scintillator was that formulated by WERBIN, *et. al* [2], which consists of 0.3 g POPOP, 12 g PPO, and 125 g naphthalene/l p-dioxane. Fifteen ml of scintillator with 0.2 ml water, the usual sample volume, counted with an efficiency of 17%. This volume of scintillator will support as much as 2.5 ml water, although the counting efficiency is then reduced by 50%. Samples were always recounted with an internal standard of tritiated water and appropriate corrections made for quenching. Water samples from urine, blood, and tissues were obtained by vacuum distillation in an apparatus somewhat similar to that described by LINDERSTRØM-LANG [3], which consists of a bent tube with detachable bulbs on both ends, one of which holds the specimen while the other is immersed in a cold bath. Dried tissue samples were first combusted in a conventional Pregl apparatus and tritium then assayed in the water of combustion. In general, errors in counting, pipetting, weighing, etc., were maintained well below 1% by suitable precautions.

The influence of hydrogen exchange on measurements of total body water with hydrogen isotopes was known long before tritium became available for this purpose. The early users of deuterium oxide were aware that it gave an overestimate of total water, but a reliable value for the correction was never established, and few investigators were willing to subject their data on total body water to a necessary but ill-defined correction. On both theoretical and empirical grounds, estimates of hydrogen exchange corrections have ranged from ½% to more than 5% of the total body water indicated by the isotope.

For entirely different purposes, the process of hydrogen exchange in pure protein solutions was carefully examined by LINDERSTRØM-LANG and his associates [3] at the Carlsberg Laboratories, and the mechanism of exchange is dealt with at length in other papers to be found in these proceedings. In general, exchangeable hydrogen atoms are those bound to oxygen, nitrogen, and sulphur, while hydrogen bound directly to carbon is considered to be non-exchangeable. Exchange proceeds exponentially with time in the manner of a first order reaction and presumably with a characteristic rate constant for each hydrogen position. Hydrogens in end groups and side chains appear to exchange most rapidly, while those bound to nitrogen in the backbone of peptide chains undergo relatively slow exchange. Exchange half-times for a single protein species are observed to range from seconds to as long as 24 hours, but it is evident that a substantial fraction of the total exchange must occur with half-times in the order of seconds.

A careful study of exchange curves for pure substances is useful in revealing features of molecular structure but serves little purpose for the intact animal other than to show the gross extent of exchange as a function of time after administration of tritium. For the purpose of arriving at a precise correction for exchange as a function of time after administration of tritium-labelled water, such a curve would be desirable but extremely difficult to establish. The best we can hope for at present is a value based on equilibrium conditions, which may overstate the effect in experiments of very short duration.

Our observations were made on the reappearance of tritium on rehydration of dried tissues and whole animals that had been given tritiated water before they were sacrificed and desiccated. The intervals between tritium administration and sacrifice ranged from ½ to 24 h. Blood samples were taken from the live animal to determine tritium space. After desiccation to constant weight, the dried tissue was rehydrated with inactive water and frequent samples taken for tritium assay for a period of 2 d. Variations in this procedure, which will be noted later, were followed for selected tissues of mice and rabbits. Two measures of the overall

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magnitude of exchange were secured; one from a direct comparison of tritium space with water volume by desiccation, and the other from a direct measurement of total tritium exchanged.

The gross features of exchange observed in these experiments can be summarized in a highly simplified formulation, which should be regarded, however, only as a first order estimate in a more detailed analysis. For simplicity, the initial exchange is regarded as a reversible exchange of hydrogen between P units of tissue and W units of tritiated water. In the live animal, W is identified with true total body water. After desiccation of P_1 units of tissue and rehydration with W_1 units of inactive water, exchange proceeds as before if there has been no substantial alteration in molecular structure.

$$\begin{array}{cc} \text{Initial exchange} & \text{Re-exchange} \\ P \rightleftharpoons W & P_1 \rightleftharpoons W_1. \end{array} \tag{1}$$

The quantity E is defined as g of exchangeable hydrogen/g of dry tissue, and H is g of hydrogen/g of water. The quantities C are counts/min/g of whatever their subscripts indicates.

At equilibrium in the original tritiation, the distribution of tritium between water and cellular material is readily shown to be proportional to the exchangeable hydrogen,

$$\frac{C_p}{C_W} = \frac{E}{H} = 9E. \tag{2}$$

After desiccation and rehydration, precisely the same ratio should be observed if there are no gross alterations in molecular structure. Obviously, this same ratio should also be found in all subsequent rehydrations, irrespective of the quantities of water used at each step. The total exchangeable hydrogen in the whole animal, in selected tissues, or in specific constituents can be obtained in this fashion, although combustion of the dry material for tritium assay and analysis for total hydrogen are required, which for a whole animal is awkward.

A simpler procedure for estimating total exchangeable hydrogen is based on the specific activities of blood or urine in the live animal and the water of rehydration, in which case

$$E = \frac{HWC_{W_1}}{P_1 (C_W - C_{W_1})}. \tag{3}$$

In applying E to the specific problem of correcting tritium space to true body water, the form of the correction depends upon how E is defined and measured. If it is regarded as exchangeable hydrogen/g of whole body dry mass, then the true total body water may be shown to be

$$W = \frac{HvC_0 - EMC_W}{(H - E) C_W} \tag{4}$$

where vC_0 is the dose of tritium administered in counts/min, M is the body weight, and C_w is the activity in blood or urine. This formulation is not altogether satisfactory, however, because little or no exchange occurs in depot fat and bone mineral, and E is, therefore, dependent upon the degree of obesity.

A more rational approach may be made on the basis of exchangeable hydrogen in lean tissue. The ratio of total protein to total water in most vertebrates appears to

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be about 21/72; one can argue for slightly different values, but the differences introduce only second order uncertainties.

The true total body water in a live animal, assuming E has been determined for lean tissue, is then given by

$$W = \frac{HvC_0}{(H + 0.292\,E)\,C_W} \tag{5}$$

which should, in principle, be applicable to all mammals and relatively free of dependence on fatness of the animal. Tritium space, which is the volume estimated directly from the activity in blood, urine, or other fluids, is based on simple dilution; hence,

$$W^* = vC_0/C_w. \tag{6}$$

The exchange error is, therefore, simply 2.6 times the exchangeable hydrogen:

$$\frac{W^* - W}{W} = 2.62\,E. \tag{7}$$

Before proceeding to experimental results on exchange, a brief analysis is needed in explanation of what appears to be non-exchangeable labelling in these and similar tracer studies. If labelling of organic constituents has occurred by metabolic and other undefined processes, that portion of the labelling that involves non-exchangeable hydrogen will persist through repeated desiccation and rehydration. On combustion of dry tissue after it has passed through one or more such stages, the activity C_c in the water of combustion will be the sum of the activity from residual tritium exchange and non-exchangeable labelling, which can be expressed as

$$C_z = (H_pC_c - EC_{w1})/H \tag{8}$$

in which H_p is the total hydrogen per unit of dry tissue.

Whatever the true time dependence of non-exchangeable labelling may be, it appears reasonable to assume that in solutions with low concentration of tritium, and for short times, labelling proceeds approximately at a constant rate; hence,

$$C_z = RtH_pC_w/H. \tag{9}$$

R is a constant, in units of reciprocal time, that can be related to the observed activities in the original tritiated water, rehydration water, and that of combustion:

$$R = (H_pC_c - EC_{w1})/tH_pC_w. \tag{10}$$

Returning now to experimental results on animals, Fig. 1 illustrates the character of exchange observed in albumin and desiccated tissues of the rat and mouse. The extent of exchange is expressed in % of the original dose of tritium, and for albumin it is normalized to the protein-water ratio in lean tissue. The greater fraction of exchange occurs in an extremely short time, but equilibrium is still not attained at 24 h except in albumin. It is not proposed, however, that these curves for re-exchange represent what takes place in the live animal. Death and desiccation uequestionably alter molecular configurations and structure and almost certainly affects many of the rates of exchange. The obvious evidence for this is the fact that

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we have never brought an animal back to ...on rehydration. On the other hand, the indicated values for exchange at equili... ...n appear to be valid and could be verified by comparison of the calculated t... ...m space with total water obtained from desiccation.

Experimental values for non-exchangeable ...elling were obtained only in albumin for 48 h tritiation and in mice at 24 h. It is e... ...ent from these two points, however, that in some tracer studies non-exchangeab... ...abelling cannot be wholly ignored.

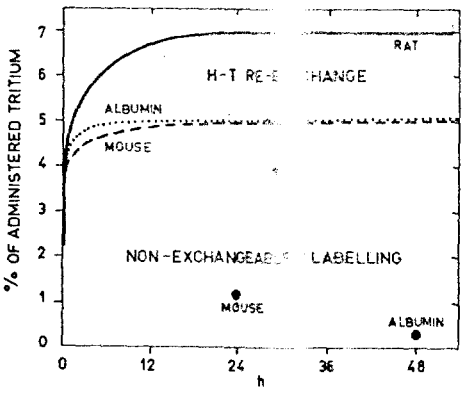

Fig. 1

Character of exchange observed in albumin and d...siccated tissues of the rat and mouse.

In the experiments with mice it accounts for 1...% of the total tritium, and equals 20% of the exchange effect. For albumin, no...-exchangeable labelling was about 20% of exchange labelling.

Table I summarizes our observations on a va...iety of animals and tritium-exposure times. It is immediately evident that something like 2% of the weight of dry tissue is exchangeable hydrogen, which is equivalent to about 30% of the total hydrogen in lean tissue solids. The variations in the values for exchangeable hydrogen are largely accounted for by differences in the fat content of the tissues and animals. It can be seen that little or no exchange occurs in neutral fat. Heart and lung, which obtain little fat, are comparable to albumin, whereas muscle and skin have significantly lower values because of the presence of fat and inclusion of bone in some muscle samples. These tissues were again dried and rehydrated, and within the limits of experimental error gave values for exchangeable hydrogen identical to the first. The rabbit organs were not re-exchanged with inactive water but were combusted directly after drying. The pigeon proves to be a different kind of animal in more ways than simply feather. We have no explanation for their extremely low exchangeable hydrogen.

In general, after 24 h exposure, permanent labelling by metabolic and other processes was fully a fifth as great as that by exchange. This would account for about 1.5% of the initial tritium dose given the animal.

The exchange error in estimates of total body water with tritium are summarized in Table II, which includes for comparison an estimate of the same error based on the volume of water from desiccation and apparent tritium dilution in the live animal. For a variety of reasons, most of them unavoidable at the time, the tritium spaces and hence the corrections based on them are uncertain within several per-

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TABLE I
EXCHANGEABLE HYDROGEN AND NON-EXCHANGEABLE LABELLING

Material	Tritium exposure time h	g % exchangeable hydrogen	Range in g % exchangeable hydrogen	Non exchangeable labelling / Exchangeable labelling
Albumin				
Bovine serum	48	2.0		.022
Mouse (4)	0.5	1.1	1.06–1.11	
Mouse (3)	24	1.0	0.72–1.11	
Mouse (4)	24			
Heart – lung		2.1	1.2–2.9	.22
Muscle		1.9		.06
Skin – subc		1.6		.10
Rat (4)	4	1.5	1.44–1.55	
Guinea pig (2)	4	1.1	0.80–1.55	
Rabbit (3)	4			
Liver		1.4	1.11–1.70	
Kidney		1.2	1.00–1.70	
G.I. Tract		1.0	0.90–1.30	
Muscle		0.9	0.90–1.30	
Plasma solids		1.7	1.66–1.70	
Fat		0.0	–	
Pigeon (2)	6	0.8		

cent. Nevertheless, they tend to corroborate the estimates derived solely from re-exchange, which, on the basis of more detailed analysis of the problem, we believe to be the more reliable values for exchange error.

The fact that the exchange in mice containing tritiated water for only half an hour does not differ greatly from that in mice exposed for 24 h leads us to believe that exchange *in-vivo* occurs more rapidly than is indicated by the exchange curve for the rehydrated tissues of the mouse. The correction for the guinea pig is low because of its gross obesity. This would not explain the low value for the pigeon, however. The values for mice and rats were remarkably uniform among the animals tested, although we have no immediate explanation for substantial differences in value between the mouse and rat.

From this preliminary evidence, it would seem that a correction for hydrogen-tritium exchange depends to some extent on the length of time the animal, or

TABLE II
ERROR IN TOTAL BODY WATER MEASURED WITH TRITIUM

Animal	Tritium exposure time h	Error in total body water by desiccation* %	by re-exchange** %
Mouse (4)	0.5	3.7	4.8
Mouse (3)	24	–	5.2
Rat (4)	4	6.4	7.1
Guinea pig (4)	4	1.6	4.2
Pigeon (2)	6	2.5	3.1

* (Tritium space – T.B.W.) / T.B.W.
** Calculated from re-exchange in desiccated animal.

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TABLE III

ISOTOPE FRACTIONATION IN EXPIRED WATER VAPOUR

	Sex	Age	Wt. Kg	T.B.W. % wt.	Sp. act. expired water / Sp. act. urine or blood
Human	F	31	63	56	0.78
	M	51	70	54	0.86
	F	36	48	66	0.93
	M	28	66	67	0.88
	M	63	88	50	0.96
	M	36	82	49	0.88
Pigeon 1					0.55
Pigeon 2					0.35

human, contained the tritiated water, and perhaps on the animal species and degree of obesity. Although these data suggest tritium exchanges to the extent of about 5% of the administered dose in mammals, it is obvious that a more detailed examination is called for.

The question of an isotope effect is one that can be answered only in the context of the experimental procedure. Obviously, tritium, by virtue of its great mass, will affect equilibrium constants, distribution coefficients, diffusion rates, binding, and even vibrational frequencies. Whether or not alteration of these characteristic constants affects the outcome of a biological tracer experiment depends on the nature of the process investigated.

In the investigation of total body water and water kinetics with tritium, in which a quasi-steady state prevails, it can be said almost with finality that an isotope effect does not occur, or at least is immeasurably small. Numerous investigators have reported no significant differences in the specific activity of tritium in blood, urine, and other biological fluids once mixing was complete. The same conclusion was arrived at by the author after assaying tritium in the blood and urine in some 300 humans and in innumerable animals.

Water involved in metabolism may, however, be another matter. It is also clear that expired water vapour is subject to a large and unmistakable isotope effect. This is strikingly evident in the pigeon, which in the course of extended flight, seems to conserve tritium despite rapid water turnover. In order to estimate the magnitude of this effect, pigeons and human subjects were placed in an open circuit respiratory system in which dry air was inspired, and expired water vapour was collected in cold traps. These tests were conducted some hours or days after administration of tritiated water to obviate interference from mixing. The specific activity of expired water vapour could then be compared with that of blood and urine taken at the same time. The results of these measurements are summarized in Table III.

In the human subjects, the specific activity of expired water vapour relative to that of urine and blood ranged from 0.78 to 0.96. No obvious pattern of dependence emerges from these few subjects, and it can only be concluded that an isotope effect is there and that it is significant.

The pigeon, on the other hand, is equipped with quite a different respiratory apparatus and is able to fractionate HTO and H_2O with respectable efficiency. Two subjects hardly qualify the data for statistical certification, but with a reduction in specific activity of 50% or more, the influence of an isotope effect is unmistakable in these birds.

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DISCUSSION XXXII

P. Springell (Australia): I would like to elaborate a little on an aspect of hydrogen-tritium exchange mentioned briefly in this paper, namely the exchange involving pure protein. In collaboration with Dr. S. J. Leach in the Division of Protein Chemistry, CSIRO, Melbourne, we have recently undertaken an exchange study on ribonuclease in tritiated water.

Information regarding molecular structure may be obtained by the study of exchangeable H-atoms in proteins, and up to now deuterated water has mainly been used for such investigations. However, tritiated water has a number of advantages and we have employed it with some success. The main advantages may be summarized as follows:

(1) In the case of deuterated proteins it is necessary to deuterate as fully as possible in $> 99\%$ D_2O, which alters the conformation and stability of the original protein. On the other hand, the sensitivity of tritium detection methods is such that only tracer amounts of tritium need be employed. We have usually labelled one atom/mole of protein or less, resulting in much less risk of changes in conformation.

(2) In using tritium there are the possibilities of both equilibrium and kinetic isotope effects. The former could lead to a distribution of tritium between protein solute and aqueous solvent which is in favour of the soiute, literature values from such factors in a variety of systems varying between 0.96 and 1.25 (see e. g. A. R. G. LANG and S. G. MASON, *Canad. J. Chem.*, 38 (1960) 373). However, for ribonuclease samples from six different sources, we have found that the number of exchangeable hydrogen atoms, assuming a distribution factor of unity, was the theoretical value of 245 ± 5. In this instance therefore, the equilibrium isotope effect appears to be absent. The use of tritium instead of deuterium, however, does lead to a decrease in the observed *rates* of exchange. This enables us to follow the initial rates of exchange in more detail. This finding also casts some doubt on the interpretation of "slow" and "fast" H-atoms as being, respectively H-bonded or not, within the protein. We now think that these numbers are in part a reflection of the method of analysis and the particular-H-isotope used.

In the course of our work a number of new facts have come to light regarding the importance of the precious history of the protein in determining the ease with which all the exchangeable hydrogens are replaced. When the forward-exchange reaction (ribonuclease + THO) was carried out on commercial samples of crystallized ribonuclease, the incorporation of tritium was slow and incomplete. This is in marked contrast to the results for the back-exchange reaction (N, O-tritiated ribonuclease + H_2O) where exchange was much more rapid.

The marked difference in results obtained between the two procedures is probably a reflection of the differences in pre-treatments of the ribonuclease. In the back-exchange the protein is lyophilized and heated two or three times from concentrated solution before the exchange reaction is commenced and this may cause the less accessible portions of the molecule to be opened up. On the other

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hand, the protein as purchased has presumably had time to refold during several years of storage, so that the sample procedure of dissolution and forward-exchange is insufficient to make all the H-atoms accessible.

On the practical side I would like to show two slides, one (Fig. 1) of the appa-

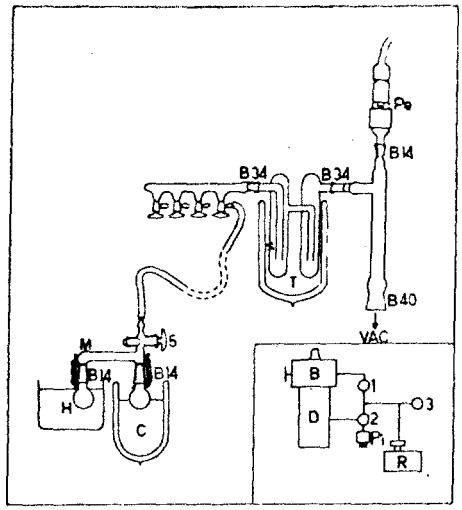

Fig. 1

ratus we use for distilling tritiated water off protein solutions. We have found that by keeping the bath H at -20 °C (bath C is at -70 °C) further exchange is minimized during back-exchange as compared to lyophilizations at room temperature. This is illustrated in Fig. 2 where the initial number of hydrogens exchanging is lowered by 15 H atoms.

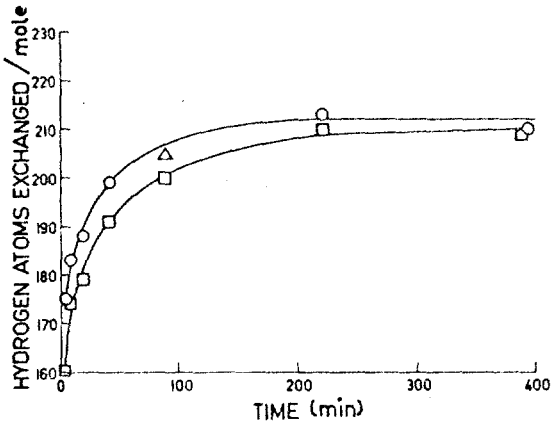

Fig. 2

For estimation we have generated $T_2 + H_2$ by the method of ISBELL and MOYER [*J. Res. Natl. Bur. Standards*, 63 A (1959) 177] with slight modification (Fig. 3). This

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gave us a calibration curve for two ionization chambers which showed a linear relationship between ionization current and radioactivity between 20 and 500 μc THO, thus showing absence of a measurable isotope effect during gas generation. Similarly I should mention that during THO distillation no measurable isotope fractionation effects were noticed.

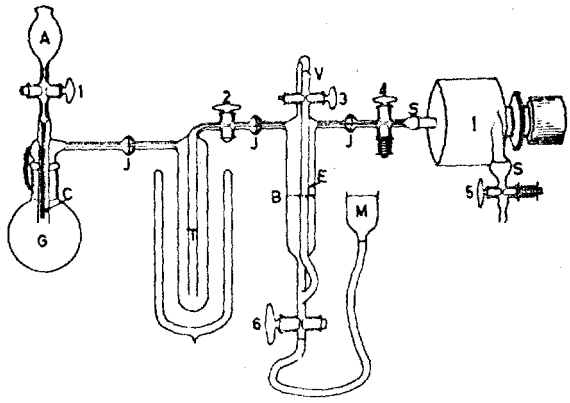

Fig. 3

It is hoped to give a more detailed account of our work at the International Congress of Biochemistry in Moscow in August 1961 and to publish the results in full in the *Australian Journal of Chemistry*.

With regard to the possibility of tritium exchange in case of C-H bonds, we have found no evidence of this in our work using model compounds or ribonuclease. Dr. Wilzbach, answering a question of mine on this subject, also regarded such a possibility as somewhat remote. I wonder whether the residual tritium activity in albumin observed by the authors might stem from strongly adsorbed water. Could Mr. Siri elaborate on how he dried his protein and what was the specific activity of the THO he used for the exchange reactions?

W. Siri (United States of America): The animal tissues and protein samples that we dried were first lyophilized. After nearly complete drying, the temperature was raised to 40 °C in the vessel, so that drying continued to completion at 40 °C. As regards C-H bonds, we have no information on the basis of the work we have done, as to whether or not they are exchangeable. Our conclusions on non-exchangeability in the present case are based on other work.

K. Wilzbach (United States of America): I believe that the levels of radiation in Mr. Siri's experiment are too low to produce any significant amounts of radiation-induced labelling. Therefore, what he calls Wilzbach labelling is very probably either metabolic labelling or relatively slow chemical exchange at activated positions. I think that the designation radiation labelling for these phenomena is a misnomer.

W. Siri: I appreciate Dr. Wilzbach's modesty in refusing to accept credit for this. Perhaps I did not indicate strongly enough that we were not absolutely certain that it was Wilzbach labelling. Our main reason for so describing it was the lack of a better term but we stand corrected if Dr. Wilzbach feels that the term does not apply.

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J. Varshavsky (Union of Soviet Socialist Republics): We know from the work of the school of Linderstrøm and Lang that the rate of hydrogen exchange varies in the different O-H and N-H bonds of proteins. More specifically, it is known that the hydrogen atoms of N-H bonds participating in the formation of hydrogen bonds have great difficulty in entering into an exchange and behave to a large extent in a manner similar to the hydrogens of the C-H bonds. The same picture is found in nucleic acids and other high-molecular compounds of living organisms. I would be interested in knowing whether the authors of the paper have considered the places of possible tritium introduction into the bonds in the light of the sharp differences in the rates of exchange for the various hydrogen atoms and whether, generally speaking, they attempted to go beyond "gross" investigation to the possibility of interpreting their results in molecular terms.

W. Siri: Let me say first that we were concerned not with the kinetics of the reaction but rather with its gross effects, insofar as they involve the biologist and the whole organism. With regard to exchangeable hydrogen, the only way we could differentiate between it and what we — perhaps naively — chose to call Wilzbach labelling was this: after 3—4 desiccations and rehydrations, there still remained a residual radioactivity far greater, i. e. by orders of magnitude, than we could account for by any of the known exchange processes. If this is still exchange, we have no explanation for it. We are not kineticists or molecular chemists, so we must leave the question to the experts. I can only say that in the experiments we have reported on, there was a residual — and very substantial — radioactivity in the dried tissues of the animals after repeated desiccations or lyophilizations and rehydrations with inactive water. I find it surprising that exchange could occur in the initial tritiation in hydrogen positions and then remain so firmly bound throughout subsequent desiccations and rehydrations. We would welcome any information on this point.

P. R. Schloerb (United States of America): Approximately 0.5% of administered isotope water is excreted in the water each hour. This figure is quite uniform and in a 3—4 hour equilibrium period approaches the magnitude of the correction factor described by Mr. Siri. If this excretion factor is omitted, the two errors would therefore tend to cancel each other. Does the speaker include urine water in the bladder as a part of "total body water"? Should the variable amounts of water in the gastro-intestinal tract, although readily exchangeable, be considered as tissue water?

W. Siri: You are quite right and this is one of the reasons why estimates of total body water based on desiccation (i. e. simple measurement of the amount of the water removed from the animal on drying and correction for exchange), show differences and are not always as reliable as they might appear to be. Estimates of this type are confused by such factors as urinary excretion, loss in weight, high rates of metabolism in small laboratory animals and a variety of other things, including mixing. To obtain what could be considered a fully reliable estimate of total body water, it would be necessary to do a complete water balance, collecting every bit of water which is lost by evaporation from the lungs, via the urine and in other ways. This involves a more elaborate experimental procedure than we were able to apply in these experiments, which were concerned with metabolic problems of body water rather than with the question of the total body water. However, I agree that the question of a precise definition of the total body water and the method by which it is measured is still open. The water contained in the bladder probably cannot be regarded as a true part of the body

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water of the animal but that in the gastrointestinal tract must unquestionably be so regarded, because it is exchangeable, i. e. the turnover rate in the gut is relatively fast. This is not true of the bladder, for example, in the human.

J. Hasan (Finland): Was there any difference between the desiccation times for mouse and rat tissue? What was the average time required for desiccation of the samples which you used in your studies?

W. Siri: We have not been able to observe any significant difference in desiccation time as between mouse and rat tissue. We have followed the weight changes of these tissues very carefully. You will recall that the initial part of the desiccation was done by lyophilization. The second part was done in vacuum at 40 °C. We normally continued the desiccation for at least 24 h. There were certainly no measurable changes in the weight of these tissues after 12 h. In the case of the whole animal, however, the order of magnitude of desiccation time differs considerably and we continued desiccation of the rat for as long as 2 weeks. This was 4—5 d after the weight had reached a constant value.

J. Hasan: I asked my question because I was wondering whether an isotope effect might not be occurring during the desiccation, so that the concentration of tritium in the sample water was increasing with time and simultaneously exchanging with the tissues (which Dr. Springell has shown to be possible even in the case of a frozen sample). This might explain the difference found by Mr. Siri between the exchange rates of mouse and rat tissues. From the metabolic rates, a difference in the opposite direction might be expected.

W. Siri: Unquestionably there is such an effect. I did not show our data on the change of activity in the animal or in the tissues, but I can assure you that the last portion of water that comes off in the desiccation has about a 20—30% higher specific activity than the water that comes off initially. However, I think that this fact has relatively little influence on the experiments I have described, because the tissues were in fact freeze-dried. It is unlikely under these circumstances, i. e. the presence of a solid state, that exchange would take place, even though it took a number of hours to dry the tissues.

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