of ammonium salts present. These results, however, do not preclude the possibility that other substances may stimulate the system. It is realized that several observations reported in this communication are at variance with those in a previous paper from this laboratory (Foulkes, 1953). Many of these receive an explanation in the fact that ammonia proves active only between certain limits of concentration. The apparent failure to inactivate with nitrous acid was due to the addition of ammonium sulphamate after deamination.

In the test system ammonia is actively removed in amounts equivalent to the increase in citrate disappearance and glutamic acid is formed. This is consistent with the suggestion (Krebs, 1952; Krebs, Gurin & Eggleston, 1952) that the presence of tricarboxylic acids in yeast is related to the supply of intermediates for synthetic processes. In yeast, two pathways for the conversion of isocitric acid into α -oxoglutarate have been recognized (Kornberg & Pricer, 1951) and the DPN-activated reductive amination of the latter to glutamic acid is well established (von Euler, Adler & Eriksen, 1937; Adler, Günther & Everett, 1938).

It is of interest that, under our experimental conditions at least, the relevant enzymes are associated with cell particles (cf. Nossal, 1954).

SUMMARY

1. The oxidation of citrate in particulate yeast preparations is stimulated by boiled yeast extract (citrate oxidation factor). This action has been

shown to be partly due to ammonium salts in whole boiled extract, and entirely due to ammonium salts in fractions of boiled extract described previously (Foulkes, 1953).

2. Increased oxidation is accompanied by the combination of considerable amounts of ammonia, chiefly to form glutamic acid.

We wish to thank Dr L. A. Stocken for supplying us with ATP and DPN and Mr R. C. Codnor of the M.R.C. Antibiotics Research Station, Clevedon, for a large-scale preparation of yeast extract *B*. One of us (M.W.W.) is indebted to the Medical Research Council for the award of a scholarship.

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Differential Fractionation of Hydrogen Isotopes in Liver and Mammary Gland

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(Received 29 April 1954)

The validity of the use of isotopes for tracer purposes depends on the assumption that molecules of normal and abnormal isotopic composition behave qualitatively and quantitatively in the same way. The possibility, however, that they might not do so because of mass differences received much attention after the discovery of deuterium in 1932. Thus Eyring & Sherman (1933) predicted on theoretical grounds that deuterium might react only half as fast as hydrogen in chemical reactions at ordinary temperatures, while Wheeler & Pease (1936) found that

the rate of homogeneous reaction of hydrogen with ethylene at 500° was 2.5 times that of deuterium. More recently, differences in the rates of reaction of carbon isotopes have been observed (see review by Ropp, 1952).

Early work on the effect of isotopes in biological processes was devoted mainly to examination of the gross physiological effects of heavy water on the intact organism (see review by Barbour, 1937). There is much contradictory evidence, but in general it may be concluded that concentrations of heavy

water above about 3 % interfere with the metabolic processes of many organisms, both plant and animal. Variations caused by deuterium in the rates of various enzyme reactions have been found (e.g. Sonderhoff & Thomas, 1936) and recently confirmed by Thorn (1951), who showed that succinic acid containing 77 atoms % D in the methylene groups was oxidized by a succinic oxidase preparation at 40 % of the rate of oxidation of normal succinic acid. Thorn emphasized that 'although dilute solutions of D₂O have no apparent effect on many biological processes, it cannot be assumed that deuterium follows the reactions of hydrogen at the same rate, nor does it follow that an enzyme will interact with a deuterated substrate in a manner identical with its normal substrate'. This possibility was recognized by Schoenheimer & Rittenberg (1936), who pointed out that the results of certain experiments could be interpreted quantitatively only if the rates of reaction of protium- and deuterium-containing molecules were known to be the same.

It is impossible to ascertain whether this is so, however, when the amount of product formed during the course of an experiment is very small compared with the amount pre-existing in the experimental material: e.g. in the intact animal or even in tissue slices. Only in special cases, e.g. when a single cell is allowed to grow into a weighable amount of tissue in a medium of labelled water, is it possible to compare directly the utilization of light and heavy hydrogen. In nature, of course, all combined hydrogen is derived ultimately from water and hence, if fractionation can occur, the abundance of deuterium in combined hydrogen might be different from that normally found in water hydrogen. In spite of much work on the subject (see Kimball, 1949) such differences have never been detected although, as Thorn (1951) points out, variations of up to 3 % in deuterium abundance would be within experimental error. It has therefore been concluded (e.g. Thompson, 1951) that no appreciable fractionation of hydrogen isotopes occurs in biological systems.

The difficulty of detecting fractionation in a biological system during the course of an experiment disappears when two tracer isotopes can be used simultaneously; it is then possible to compare their relative abundances in substrate and product and from the difference, if any, to make at least an approximate estimate of the degree of fractionation of each isotope relative to ordinary hydrogen. Although it might be supposed that any compound, in whose synthesis the hydrogen of water is known to be involved, would show fractionation irrespective of the site of synthesis, it can be shown that this is not necessarily so; only if the rate of synthesis is greater than that of degradation (i.e. there is net

synthesis) will the different rates of reaction of the two isotopes result in an isotopic ratio different from that in the substrate water; but if the rates of synthesis and degradation are equal (i.e. turnover) then the ratio will tend to be the same as that in the substrate water.

The chief assumption that has to be made is that for a product which is synthesized and degraded by a reversible series of reactions, the ratio of the rates of synthesis and degradation of the tritium-containing molecules is the same as the ratio for the deuterium-containing molecules (cf. equation 5).

For such a product we may write the reactions involved in this synthesis and degradation and its removal intact from the site of synthesis as:

Where H* = labelled hydrogen ion; F = immediate unlabelled precursor; H*F = labelled product; k_1 = rate constant for synthesis of labelled product; k_2 = rate constant for degradation of labelled product; k_3 = rate constant for removal of labelled product from site of synthesis; t = time; X = imaginary chemical species formally responsible for removal of labelled product from site of synthesis, reaction (2) being equivalent to the mechanical process of secretion, i.e. when secretion occurs $[\text{X}] > 0$, and when no secretion occurs $[\text{X}] = 0$.

Then the rate of formation of labelled product is given by

$$\frac{d}{dt} [\text{H}^*\text{F}] = k_1 [\text{H}^*] [\text{F}] - k_2 [\text{H}^*\text{F}] - k_3 [\text{H}^*\text{F}] [\text{X}] \\ = k_1 [\text{H}^*] [\text{F}] - [\text{H}^*\text{F}] (k_2 + k_3 [\text{X}]).$$

If the concentration of labelled hydrogen in the substrate water is held constant a steady state is reached: i.e. the isotope incorporation/time curve reaches a plateau. Then $d/dt [\text{H}^*\text{F}] = 0$. Therefore

$$\left. \begin{aligned} k_1 [\text{H}^*] [\text{F}] - [\text{H}^*\text{F}] (k_2 + k_3 [\text{X}]) &= 0, \\ \text{or} \quad [\text{H}^*\text{F}] &= \frac{k_1 [\text{H}^*] [\text{F}]}{k_2 + k_3 [\text{X}]} \end{aligned} \right\} \quad (3)$$

This equation may be applied to either of the two isotopes ²H and ³H and the symbol H may therefore be replaced by D or T. Of the k terms, k_3 has the same value for both isotopes, since the rate of removal of labelled product from the site of synthesis is independent of its isotopic composition. Attaching the appropriate subscripts to k_1 and k_2 we may therefore write equation (3) for the two isotopes:

$$[\text{TF}] = \frac{k_{1\text{T}} [\text{T}] [\text{F}]}{k_{2\text{T}} + k_3 [\text{X}]}, \quad (3a)$$

$$\text{and} \quad [\text{DF}] = \frac{k_{1\text{D}} [\text{D}] [\text{F}]}{k_{2\text{D}} + k_3 [\text{X}]}. \quad (3b)$$

Dividing (3a) by (3b), the isotopic ratio in the product is given by

$$\frac{[\text{TF}]}{[\text{DF}]} = \frac{k_{1\text{T}}}{k_{1\text{D}}} \times \frac{k_{2\text{D}} + k_3 [\text{X}]}{k_{2\text{T}} + k_3 [\text{X}]} \times \frac{[\text{T}]}{[\text{D}]} \quad (4)$$

As it is to be expected that the isotopic species of product which is formed more slowly will also be degraded more slowly it is now assumed as an approximation that the ratio of the rates of synthesis and degradation of tritium-labelled product is the same as the ratio for deuterium-labelled product: i.e. that

$$\frac{k_{1\text{T}}}{k_{2\text{T}}} = \frac{k_{1\text{D}}}{k_{2\text{D}}} \quad (5)$$

We may now consider the cases of turnover and net synthesis separately.

(i) Under conditions of turnover there is no removal of intact molecules from the site of synthesis and hence, by definition $[\text{X}] = 0$. Then substituting for $[\text{X}]$ and combining equations (4) and (5) we have

$$\frac{[\text{TF}]}{[\text{DF}]} = \frac{[\text{T}]}{[\text{D}]} \quad (6)$$

That is, the isotopic ratio in the product will be the same as that in the substrate water and no fractionation will be observed.

(ii) Under conditions of net synthesis, some product is removed from the site of synthesis, hence, by definition $[\text{X}] > 0$. Then with increasing magnitude of $[\text{X}]$ the term

$$\frac{k_{2\text{D}} + k_3 [\text{X}]}{k_{2\text{T}} + k_3 [\text{X}]}$$

of equation (4) approaches unity and from equation (4)

$$\frac{[\text{TF}]}{[\text{DF}]} \text{ tends to } \frac{k_{1\text{T}}}{k_{1\text{D}}} \frac{[\text{T}]}{[\text{D}]}$$

The isotopic ratio in the product will thus be different from that in the substrate water ($[\text{T}]/[\text{D}]$) by a factor which tends to the value of the ratio of the rate constants of synthesis of tritium- and deuterium-labelled products.

For the purpose of ascertaining whether hydrogen isotopes are fractionated in biochemical systems it is thus necessary to choose a compound which is undergoing net synthesis. The lactating mammary gland is particularly useful for this purpose since it is known from many types of experiment both *in vivo* (Popják, French & Folley, 1951) and *in vitro* (Folley & French, 1949; Balmain, Folley & Glascock, 1951) that rapid net synthesis of fatty acids from small molecules occurs there. In liver fatty acids, on the other hand, it is known that active incorporation of small molecules occurs but concurrently with oxidation and degradation (Stadie, 1945) and it

seems likely that net fatty acid synthesis in the livers of mature animals in nutritional equilibrium is small. Thus if net synthesis and turnover of fatty acids are to be observed separately and simultaneously in the same animal they are most likely to be found in the lactating mammary gland and liver respectively. Furthermore, fatty acids are believed to be synthesized and degraded by a reversible series of reactions (Lardy, 1952; Lynen & Ochoa, 1953) and are therefore of the type of product to which the above simple mathematical argument is applicable.

A preliminary short-time experiment (Duncombe & Glascock, unpublished, quoted by Glascock, 1951) having shown that fractionation does in fact occur in both tissues, the detailed experiments described here were carried out. They confirm that appreciable fractionation of hydrogen isotopes can occur *in vivo* and show that differences in metabolic function between lactating mammary gland and liver can be deduced from quantitative differences in isotopic fractionation.

Part of this work has been published in preliminary form (Glascock & Duncombe, 1952) since when biological fractionation of hydrogen isotopes has been reported by Verly, Rachele, du Vigneaud, Eidinoff & Knoll (1952), Weinberger & Porter (1953), Helvey (1953) and by Eidinoff, Perri, Knoll, Marano & Arnheim (1953). The last workers used a similar method to that described in the present paper, and obtained results on liver fat in quantitative agreement with ours. Thompson & Ballou (1954), however, using a similar method, were able to detect only barely significant isotopic fractionation.

EXPERIMENTAL

Ten female hooded Norway rats were used in three groups at different times. The first group contained two pregnant rats weighing 344 and 381 g., whose water content, for the purpose of calculating isotope dosage, was assumed to be 75% of the body wt. On the day before the expected birth of their litters they were given by stomach tube sufficient of a mixture of 40 g. heavy water (99.75%) and 1 ml. radio-active water (containing 2 mc of tritium) to raise their body water isotope levels to about 1.5 atoms % excess deuterium and 0.75 $\mu\text{C}/\text{ml}$. tritium. The remainder of the mixture was diluted with normal water so that it contained 2.5 atoms % excess deuterium and was given freely as drinking water. Thus all the water received during the course of the experiment had exactly the same tritium/deuterium ratio. Although the absolute level of isotopes in the blood at any time was of minor importance, the drinking water was made somewhat richer than the calculated value for the blood in an attempt to compensate for the unlabelled water derived from the food and to maintain the blood level approximately constant. That this was not quite achieved is shown by the figures in Table 2, where it will be seen that the isotope levels in the blood samples were about 60% of the expected values.

The two rats, each with its litter adjusted to six in number, were kept in separate cages and given normal food *ad lib*. After 14 days they were both killed by a blow on the head and samples of blood, perinephric fat, mammary gland and liver were collected and kept frozen at -10° till required for analysis. The pups were also killed and kept for isolation of their total carcass fatty acids.

The second group consisted of four rats which had had their litters on the day before the experiment started. The stock-labelled water was made from 80 g. heavy water and 5 mc radioactive water made isotonic with NaCl. Each rat received sufficient of this solution by intraperitoneal injection to adjust the body water isotope level to about the same value as in the first group. The remainder of the water was diluted and the animals were maintained under exactly similar conditions to those in the first group. They were killed on the 14th day after parturition and samples of blood, mammary gland and liver taken and stored at -10° .

The third group consisted of four pregnant rats of body weights 280–350 g. The labelled water used was made from 160 g. heavy water and 20 mc radioactive water. Sufficient of this was given by stomach tube 2 days before the birth of the litters to raise the body-water deuterium level to about 3%, and the remainder diluted for drinking water as before. The isotope levels were increased in this experiment because some of the levels of incorporation were rather low in the earlier experiments. The animals and their litters were kept under similar conditions and killed 11 days later when all the labelled water had been consumed. Samples of blood, perinephric fat, mammary gland and liver were taken as before and stored at -10° .

Preparation of samples

Blood water was obtained by sublimation *in vacuo*, followed by purification for deuterium and tritium analysis as described later.

Total fatty acids were obtained from mammary glands, liver and perinephric fat by refluxing each tissue in 5 times its own weight of alcoholic KOH (4%, w/v) for 24 hr., extracting the unsaponifiable fraction with light petroleum, evaporating organic solvents on the water bath, acidifying with 40% H_2SO_4 and extracting the free fatty acids with light petroleum. The extract was washed until free from mineral acid, dried over anhydrous Na_2SO_4 , and the solvent was evaporated. The free acids were stored in a desiccator containing CaCl_2 and shavings of paraffin wax. Total fatty acids were obtained from some of the pups of the first group by saponifying the whole animal and proceeding as above.

Isotopic assay

Fatty acid samples were combusted on the 150 mg. scale using our technique and deuterium determined by a modification of the falling-drop method (see Glascock, 1954). Tritium was counted as tritiobutane prepared from 10 mg. samples of combustion water as described by Glascock (1951). In the first two experiments both isotopes were determined in each sample by assaying the water resulting from a single combustion; the micropipette on the deuterium apparatus was charged with combustion water, 10 μl . samples were expelled into each of two rigorously dried tubes fitted with standard joints and stoppers and the remainder of the water in the pipette used for dropping. The water in the tubes was sublimed *in vacuo* into two previously prepared tubes of dry butyl magnesium bromide

and duplicate samples of butane prepared. The tritium content of each of these samples was determined by counting at least two measured portions long enough for their activities to be determined with a reliable counting error of not more than 1% (10 000 counts). Drinking water and blood water, diluted if necessary to bring their deuterium contents to a level measurable in the apparatus used were put through the various stages of chemical purification before isotopic assay.

The transfer of 10 μl . drops of water from pipette to tube in the first two experiments, although rapidly done, admitted the remote possibility of dilution with atmospheric water and hence a spuriously low tritium value. In the third experiment, therefore, a separate combustion was carried out for each tritium determination and the water manipulated entirely in the vacuum apparatus and never allowed to come in contact with the atmosphere. The results of this experiment were similar to those of the other two in which dilution of the water by atmospheric moisture was evidently negligible.

It is to be noted that the tritium content of the water samples was far too small to affect its density measurably and thus to interfere with the deuterium determination; it was of the order of 1 $\mu\text{C}/\text{ml}$., which corresponds with only about 4×10^{-8} atom %.

RESULTS AND DISCUSSION

Accuracy of analytical methods

The determination of deuterium by the falling-drop method was found, both in this and in other work, to give closely reproducible results in the range of concentration 0.1–2.0 atom % deuterium. Thus, as illustrated in Table 1, dropping times for a given sample of water agreed closely, the differences being too small to reflect different deuterium values on the calibration curve. Furthermore, standard deuterium oxide dilutions have been assayed at intervals over 3 years yielding results in which the range did not exceed ± 0.01 atom % excess. In this work some duplicate combustions were carried out on the same sample of fatty acids and again the results agreed within this range. From this it appears that the deuterium contents of the experi-

Table 1. Example of deuterium and tritium assay results

Sample: rat 2, pup 2, total fatty acids. For details see Experimental section.

	Tritium (Counts/min./mg. water)	Deuterium (Falling-drop time (sec.))
Preparation 1	73.0 74.0	85.61 85.62
Preparation 2	75.0 75.5	85.72 85.69
Average	74.4 $\pm 6.99\%$	85.66 ± 0.21 atom % excess D $\pm 8.57\%$ of level in drinking water

Ratio: $^3\text{H}/^2\text{H} = 0.80$

mental samples are accurate to within ± 0.01 atom % excess which is in most cases about 3 % of the abundance measured.

The determination of tritium by the method used also yielded closely reproducible results. The specific activities of samples of the same preparation of butane agreed to within 1.5 % of their mean values both with each other and with those of the duplicate preparation. Thus the value finally obtained for the specific activity of a given sample was calculated

from the mean of at least four closely agreeing butane specific activities. The figures for a typical experiment are given in Table 1.

In determining the ratio of the two isotopes the total error was therefore not likely to exceed ± 5 %. This is in accordance with the findings for the ratios in the blood waters which should almost certainly be the same as the ratio in the drinking water, but which actually had a range of about ± 5 % (see Table 2).

Table 2. *Deuterium and tritium levels and isotopic fractionation in tissues of lactating rats receiving drinking water labelled with deuterium and tritium*

Sample	Rat no.*	² H atom % excess (a)	³ H counts/min./mg. water (b)	² H as % of drinking water ² H (c)	³ H as % of drinking water ³ H (d)	Isotope ratio: (d)/(c)
Drinking water	1-2	2.45	1058	100	100	1.00
	3-6	2.39	1051	100	100	1.00
	7-10	4.90	5064	100	100	1.00
Blood water	1	1.30	559	53.1	52.8	0.99
	2	1.27	574	51.8	54.3	1.05
	3	1.355	602	56.7	57.3	1.01
	4	1.385	573	58.0	54.7	0.94
	5	1.28	590	53.5	56.2	1.05
	6	1.27	536	53.2	51.0	0.95
	7	3.36	3351	68.5	66.2	0.97
	8	3.38	3567	68.9	70.4	1.02
	9	3.40	3667	69.3	72.4	1.04
	10	3.38	3494	68.9	69.0	1.00
					Mean	1.00
Mammary gland fatty acids	1	0.120	40	4.90	3.78	0.77
	2	0.250	88	10.20	8.32	0.82
	3	0.220	77	9.21	7.35	0.80
	4	0.250	86	10.47	8.24	0.79
	5	0.345	121	14.45	11.52	0.80
	6	0.303	103	12.68	9.80	0.77
	7	0.495	361	10.10	7.10	0.70
	8	0.730	597	14.90	11.80	0.79
	9	0.555	437	11.30	8.64	0.76
	10	0.620	480	12.60	9.48	0.75
					Mean	0.775
Liver fatty acids	1	0.065	27	2.65	2.55	0.96
	2	0.200	80	8.16	7.56	0.93
	3	0.245	90	10.20	8.57	0.84
	4, 5†	—	—	—	—	—
	6	0.255	93	10.67	8.88	0.83
	7	0.415	413	8.46	8.16	0.96
	8	0.990	884	20.20	17.45	0.86
	9	0.750	578	15.30	11.40	0.75
	10	1.010	884	20.70	17.45	0.84
					Mean	0.87
Pup total fatty acids‡	1	0.415	141	16.90	13.30	0.79
		0.345	119	14.10	11.20	0.79
		0.370	125	15.00	11.80	0.79
	2	0.195	66	7.96	6.24	0.78
		0.210	74	8.57	6.99	0.80
		0.265	86	10.80	8.13	0.75

Maternal carcass fatty acids Deuterium and tritium contents of all samples analysed (rats 1, 2, 7, 8, 9, 10) were not significantly greater than 0.

* Rats 1-6 received the labelled drinking water for 14 days before killing; rats 7-10 for 11 days.

† Insufficient material for deuterium analysis.

‡ Samples from three separate pups for each rat.

Isotopic fractionation

Table 2 shows the isotope contents of the drinking water, the blood waters and the fatty acids from the various tissues listed. For each sample the deuterium or tritium levels (columns *a* and *b*) were expressed as percentages (columns *c* and *d*) of the levels in the drinking water for the particular experiment. The drinking water had been assayed at least in duplicate, and, in two experiments, by making two separate and different dilutions. It was, of course, necessary to know the isotope contents of the drinking waters accurately, since all the isotope ratios were based on them.

The tritium/deuterium ratio for each sample was then defined as *d/c* (Table 2, last column). This ratio should be unity if no fractionation had occurred, and less than unity if relatively less tritium than deuterium had been incorporated. It is seen that ratios for the blood-water samples were all approximately unity, within the predicted experimental error, with a mean of 1.00, showing that there is no appreciable fractionation during the passage of the labelled water through the wall of the gut. That such diffusion processes do not involve fractionation has also been shown by Prentice *et al.* (1952) in studies using hydrogen isotopes for the determination of total body water, and by Hurst, Schemm & Vogel (1952), who demonstrated that the kidney does not distinguish between heavy and normal water (see also review by Pinson, 1952).

The ratios for the mammary gland fatty acids are in all cases considerably less than unity, varying from 0.70 to 0.82. A statistical analysis of these figures is given in Table 3 and shows that the difference of their mean from unity is highly significant ($P < 0.001$). This is in accord with the prediction for an organ in which net synthesis is in progress. It is of interest to note that the degree of hydrogen isotope fractionation reported here is of the same order as that found for carbon isotopes by Weigl & Calvin (1949) using an algal culture which discriminated against $^{14}\text{CO}_2$. The relative mass difference between $^{14}\text{CO}_2$ and $^{12}\text{CO}_2$ is about the

same as that between ^3HOH and ^2HOH namely 5%. If mass is the factor chiefly responsible for fractionation both of isotopic water and of carbon dioxide then our findings are consistent with the belief that the reacting entities are ^2HOH and ^3HOH , or more probably the corresponding hydrated ions, rather than free deuterium and tritium ions, otherwise the fractionation would be greater.

It will also be seen (Table 2) that the total fatty acids of the pups of rats 1 and 2 had the same ratio as the fat of the gland they had been suckling. There are two possible explanations for this; either the pups' depot fat (which constitutes the largest part of the total body fat) had been derived from dietary fat, or considerable net synthesis of body fat from smaller molecules had occurred in the pups. The isotopic ratio and absolute levels in the blood water of the latter would almost certainly be the same as in the maternal blood water, any fractionation along the route maternal blood water $\rightarrow$ milk water $\rightarrow$ pup blood water being unlikely. If net synthesis occurred it would thus be taking place in an environment similar to that in the maternal mammary gland. In these experiments there was no means of deciding between the alternatives, although we are of the opinion that it is more likely that the pup carcass fat was derived directly from the milk fat.

The isotope ratio in the liver fatty acids (Table 2) is also in every experiment less than unity but at the same time higher than that in the mammary gland fat from the same animal (except in one instance, rat no. 9). Statistical analysis shows that the mean for the livers is significantly lower than unity and is also significantly higher than the mean for the mammary glands (Table 3). This is in agreement with the prediction for an organ in which there is less net synthesis and more turnover of fat than in the mammary gland.

The results reported here show a much greater isotope effect than that reported recently by Thompson & Ballou (1954). These workers found for the various samples they examined a tritium/deuterium ratio never smaller than 0.93. Comment-

Table 3. *Statistical analysis of isotope ratios from Table 2*

- (1) Mammary gland fatty acids:
Mean isotope ratio = 0.7750 ± 0.00930 (7 degrees of freedom)
Significance of difference of mean value from 1.0
 $P < 0.001$
- (2) Liver fatty acids:
Mean isotope ratio = 0.8712 ± 0.02386 (5 degrees of freedom)
Significance of difference of mean value from 1.0
 $0.01 > P > 0.001$
- (3) Difference between liver and mammary gland fatty acids:
Paired 't' test using eight rats with complete results (rats 1-3 and 6-10)
Difference between mean isotope ratios = 0.1012 ± 0.03050 (7 degrees of freedom)
 $P = 0.0136$ (approx.)

ing on a preliminary report of our results (Glascock & Duncombe, 1952) they suggest that the much greater isotope effect observed by us may have been due to the much shorter period over which the isotopes were administered (2 weeks instead of 5), thus allowing 'less opportunity for metabolic equilibration of the isotopes'. If by metabolic equilibration is meant the attainment of a plateau by the isotope incorporation/time curve, then there is good reason to believe that this condition was attained in our experiments. Schoenheimer & Rittenberg (1936) found that the plateau value for the carcass fat of mice was reached in 6 days and, mammary gland and liver being metabolically much more active than adipose tissues, it therefore seems extremely probable that the plateau was reached in our animals. It should be noted that nearly all the samples assayed by Thompson & Ballou consisted of pieces of tissue (carcass, muscle, pelt, brain and liver) which are composed predominantly of protein and were probably in a state of turnover rather than active net synthesis. If this were so, then, on the basis of our prediction, we should expect them to have an isotope ratio approximating to unity. Apart from body water the only other sample assayed was depot fat which showed the lowest isotope ratio (0.93). In our animals the isotope incorporation into depot fat was too small for the ratio to be measured accurately.

The experimental technique of Thompson & Ballou also differed from ours in several important respects. They did not maintain the body-water isotope levels constant by giving the doubly labelled water for drinking but administered it by injection at 3.5 day intervals, causing fluctuations between 1.5 and 4.5 % deuterium. Their methods for determining the isotopes also seem to have been less accurate than ours. For example, their tritium assays were reproducible to within not less than $\pm 5\%$ whereas ours were reproducible to within $\pm 1.5\%$.

It therefore also appears that since it has been shown in the present work that the incorporation of tritium into some compounds may be as much as 30 % less than that of deuterium, the difference between deuterium and protium may be at least as great. Hence calculations of the total amount of water hydrogen incorporated into biological products cannot always validly be made on the basis of isotopic data alone. Either isotope alone may of course be usefully applied to the determination of relative synthetic efficiency, or to the elucidation of metabolic pathways.

Other results

The results obtained in these experiments also throw some light on the contribution of drinking water to blood water and on the relative utilization

of water hydrogen for the synthesis of fatty acids in mammary gland, liver and carcass. For the purpose of this comparison it is necessary to consider only the relative abundance of one isotope—say tritium.

Contribution of drinking water to blood water. Table 2 shows that the isotope level in the samples of blood water varies between about 50 and 70 % of that in the drinking water. Thus from 30 to 50 % of the blood water must have been derived from unlabelled combined hydrogen or free water in the food.

On combustion a typical sample of diet was found to yield 65 % of its weight of water. The average daily consumption of this diet was 45 g. corresponding to 29 g. water when fully oxidized. The average daily intake of labelled water during the experiment was 37 g. Thus even supposing the diet to have been fully oxidized the contribution made by it to body water could not have been more than 44 %. Although complete oxidation of the diet is unlikely there appears to be fair agreement between the results derived from analytical and isotope data.

Relative utilization of water hydrogen by the various tissues for fatty acid synthesis. The isotope level in mammary gland fatty acids lies between 5 and 15 % of that in the drinking water, whereas in the liver it lies between 3 and 20 %. It is to be noted that a high or low isotope level in the mammary gland is generally paralleled by a high or low level in the liver of the same animal, and the wide range of values probably reflects different metabolic activities.

The isotope contents of the pup total fatty acids from rats 1 and 2 and the respective mammary gland samples show some interesting differences. The isotope levels are approximately the same in pup total acids, maternal liver acids and mammary gland acids for rat 2; on the other hand the isotope level in pup fatty acids of rat 1 is very much greater than that of the fatty acids of the mammary gland from which they had been feeding. This may be because the true milk fat is much richer in isotope than the gland tissue fat, and the glands of rat 1 had been milked dry by the pups shortly before killing. This would have the effect of leaving predominantly tissue fat, of relatively low isotope content, in the glands, the isotopically richer milk fat entering the pups. If this is so, however, it is in contrast with the finding (Cowie *et al.* 1951) for the isolated cow udder. After perfusion for 2 hr. with blood containing [*carboxy*- ^{14}C]acetate, the udder fatty acids had a specific activity 7–10 times higher than that of the milk fatty acids.

Our finding that virtually no water hydrogen is incorporated into the maternal depot fatty acids (Table 2) is most unexpected. Schoenheimer & Rittenberg (1936) administered heavy water to mice and found that after 6 days the deuterium level in

the total body fatty acids (which must consist largely of depot fatty acids) had reached a plateau at 13–14 % of that of the body water. In the present experiments we might therefore expect the depot fat to have a similar isotope content. The difference between our results and those of Schoenheimer & Rittenberg is at present inexplicable, and further work on this point is in progress.

SUMMARY

1. A theory is presented in which it is predicted that biological fractionation of hydrogen isotopes during the reversible incorporation of water hydrogen into any metabolite will be observed only under conditions of net synthesis and not under conditions of turnover.

2. There is reason to believe that in the fatty acid metabolism of mammary gland and liver net synthesis and turnover are, respectively, the predominant processes. The incorporation of hydrogen from water doubly labelled with tritium and deuterium into the fatty acids of these and other organs has therefore been studied.

3. Rats were maintained on a regimen of doubly labelled water for 11–14 days, and the isotopic abundances determined in blood water and in the fatty acids extracted from mammary gland, liver, perinephric and pup carcass fat.

4. Defining the tritium/deuterium ratio in the drinking water as unity it was found to be unity in the blood water, but significantly less than unity in all other samples assayed, with the exception of perinephric fat, in which isotopic incorporation was too small for the isotopic ratio to be calculated accurately.

5. The mean values of the ratios for fatty acids of maternal mammary gland and liver were 0.775 and 0.87 respectively, which are significantly different from each other.

6. These findings are in accord both with the belief that more net synthesis of fatty acids occurs in mammary gland than in liver, and with the theory that under such conditions differential fractionation of hydrogen isotopes will be observed.

7. The isotopic abundances in pup carcass fatty acids varied considerably, but the isotopic ratio was approximately the same as that of the fatty acids of the gland from which they had been feeding. Alternative reasons for this are discussed.

8. The results are also discussed in relation to the contribution of drinking water to blood water, and the relative utilization by various tissues of water hydrogen for fatty acid synthesis.

We wish to record our thanks to Dr S. J. Folley, F.R.S., for his interest in the work and for making available the experimental animals; to Dr M. Gordon for his advice on chemical kinetics; to the Statistics Section of the Institute for statistical analyses; to Dr A. T. Cowie for administering the labelled water and to Mr B. W. E. Peaple for tritium determinations.

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