THE FATE OF UTILIZED MOLECULAR OXYGEN AND THE SOURCE OF THE OXYGEN OF RESPIRATORY CARBON DIOXIDE, STUDIED WITH THE AID OF HEAVY OXYGEN*

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The present investigation was undertaken in an attempt to elucidate the fate of utilized molecular oxygen and to identify the source of the oxygen in respiratory carbon dioxide in the intact animal. As will be shown later, it is believed that erroneous conclusions were drawn from the only previous study with O^{18} as a tracer in connection with this problem.

Current concepts of the mode of oxidation in the animal body hold that the fate of molecular oxygen is to combine with hydrogen from substrates to form water.[1] Therefore, an animal breathing labeled oxygen should produce labeled water. The isotopic water thus formed would be added to the pool of already existing body water, increasing the isotopic concentration of the latter progressively with time.

The question of the source of oxygen in respiratory carbon dioxide may be conveniently discussed in terms of the conventional equation for the complete oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (1)$$

Clearly, at least one-half of the oxygen in the carbon dioxide thus arising must come from some source other than the glucose itself. According to present concepts, carbon dioxide is released by decarboxylation and the extra oxygen in the carbon dioxide is previously introduced, not from molecular oxygen, but from body water. Hence glucose oxidation in the presence of labeled molecular oxygen and normal body water should form carbon dioxide entirely free of label; but glucose oxidation in the presence of normal molecular oxygen and isotopic water should yield

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[1] No distinction is made here between the particular hydrogens removed from the substrate by dehydrogenation and those hydrogens which actually combine with oxygen to yield water, although in general they may be expected to be different. The former are presumed to add to the body water as hydrogen ions and the latter to be removed from the body water likewise as hydrogen ions.

carbon dioxide, the isotope concentration of which is at least 50 per cent that of the body water. Actually, glucose is thought to be metabolized to pyruvate by the glycolytic scheme of reactions, and pyruvate thereupon oxidized to carbon dioxide by the tricarboxylic acid cycle. In this case, it may well be that, before each of the predicted six decarboxylations per glucose molecule occurs, the oxygen of that released carbon dioxide will have been previously in more or less complete isotopic exchange equilibrium with, or derived from, body water. As a result, the isotopic concentration of the carbon dioxide would be raised toward equality with the isotope concentration of the body water. Similar considerations with differences in details would be expected to apply to the complete oxidation of fats and proteins.

Thus far the discussion has been of the source of the oxygen of respiratory carbon dioxide at the instant of its formation by internal respiration, presumably by decarboxylation. However, between the moment of its intracellular origin and its release from the animal in expired air, the carbon dioxide is provided with an opportunity to exchange oxygen with body water during each of the processes involved in transport and external respiration; *i.e.*, traversal of intracellular and interstitial fluid, carriage by the blood, entrance into the alveoli, and removal by ventilation.

At a pH less than 8, the predominant reaction is the following (1).

$$CO_2 + H_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H_2CO_3 \qquad (2)$$

If either the carbon dioxide or the water initially contains all the labeled oxygen, by this reaction the isotope becomes distributed among all three reactants until the atom per cent O^{18} is the same in each, except as the process is influenced by fractionation effects. For the present purposes, the exchange equilibrium may be represented as follows:

$$CO^{16}O^{18} + H_2O^{16} \rightleftharpoons CO^{16}O^{16} + H_2O^{18} \qquad (3)$$

Employing a value of 0.10 sec.$^{-1}$ for the velocity constant, $k_1(H_2O)$, of Reaction 2, uncatalyzed, at 38° and pH 7.4, one can calculate from the appropriate equation of Mills and Urey (2) that 99 per cent of isotope equilibrium is reached in 138 seconds. However, the amounts of carbonic anhydrase in blood appear to be sufficient for exchange equilibrium to be achieved in a fraction of a second (3). Thus, even if the carbon dioxide arising intracellularly were not already in complete isotopic oxygen exchange equilibrium with water, such equilibrium might be expected to be attained subsequently, before exit from the body.

Consequently, both by current concepts of internal respiration and by the carbonic anhydrase-catalyzed exchange during transport and

external respiration, it would be anticipated (1) that the oxygen of respiratory carbon dioxide would approach equilibrium with body water, and (2) that utilized respiratory oxygen would soon find its way into body water.

The experiments to be reported appear to demonstrate that such is indeed the case. In the only previously published study of this question, a brief note by Day and Sheel (4), the inference was drawn that respiratory oxygen does enter directly into carbon oxidation and is exhaled as carbon dioxide, a conclusion at variance with predictions from current concepts of intermediary metabolism. It was therefore considered of importance to reinvestigate the question.

Methods

General Procedure—Two types of experiments were carried out: (*a*) that in which the atmosphere was enriched with O_2^{18} (O_2^{18} type)[2] and (*b*) that in which the body water was enriched with H_2O^{18} (H_2O^{18} type). In both types, the animal was placed in a closed metabolism system (see Fig. 1) filled with oxygen, and arranged for collection of dry respiratory carbon dioxide, for the sampling of the atmospheric gas, and for approximate determination of the rate of oxygen consumption. The collection periods, the number of which varied from two to four, were of 2 hours duration unless otherwise indicated. At the termination of the last collection period the animal was decapitated and a portion of body water obtained by vacuum distillation. Ordinarily, one atmospheric gas sample was taken at about the mid-point and one at the end of the experiment.

Experimental Details—Heavy molecular oxygen was concentrated by thermal diffusion. From the heavy molecular oxygen, heavy water was prepared by catalytic combination with normal hydrogen.

Strain A mice maintained on an unrestricted fox chow diet were employed. In the O_2^{18} type of experiment, the isotopic oxygen was added to the atmosphere of the system at *I* (see Fig. 1), after passing through a drying tube and carbon dioxide absorber. In the H_2O^{18} type of experiment body water was enriched by intraperitoneal injection of 1 ml. of H_2O^{18} of 8.47 atom per cent excess O^{18}, 2 to 3 hours before placing the mouse in the metabolism chamber. Preliminary trials had shown that this allowed more than enough time for the injected water to be absorbed.

The isotopic analyses for O^{18} were performed by means of the mass spectrometer. The atmospheric oxygen was analyzed directly from the

[2] The designation O_2^{18} is used to indicate molecular oxygen enriched with regard to O^{18}. It will be realized, however, that in the range of concentrations employed here most of the O^{18} is in $O^{16}O^{18}$ molecules and only a small proportion is in the $O^{18}O^{18}$ form.

sample bulb. In the case of respiratory carbon dioxide, the trap containing the solid sample, still immersed in liquid oxygen, was first evacuated, thus removing the gas phase. The trap was then removed from the refrigerant for introduction of volatilized carbon dioxide into the mass spectrometer. Since no special precautions were taken to mix the solid

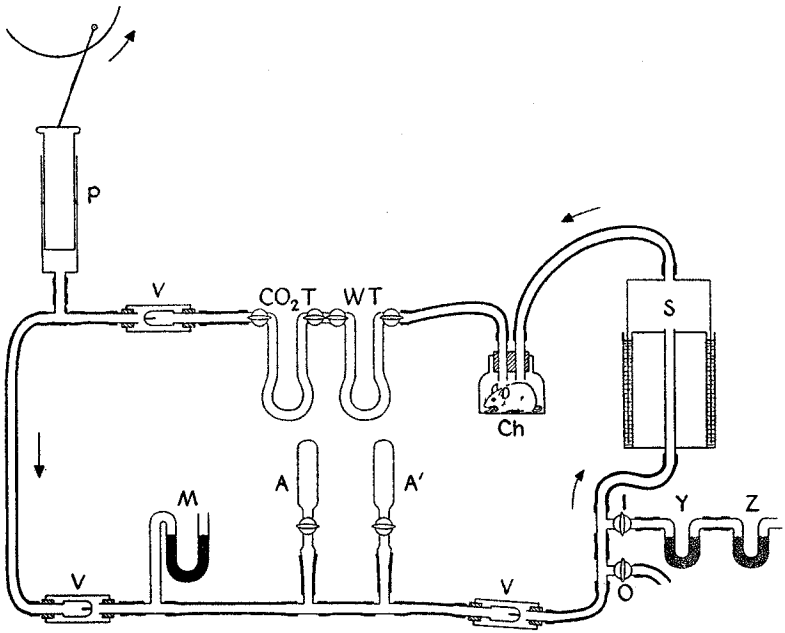

FIG. 1. *Ch*, animal chamber; *WT*, water trap (during the experiment immersed in alcohol-dry ice); CO_2T, carbon dioxide trap (during the experiment immersed in liquid oxygen or air); *V*, valve; *P*, syringe pump (sealed with mineral oil); *M*, mercury manometer; *A* and *A'*, evacuated sampling bulbs for atmospheric gas; *Y*, calcium chloride tube; *Z*, soda lime tube; *O*, *I*, stop-cocks; *S*, spirometer (sealed with mineral oil). Only one water trap and one carbon dioxide trap are shown; others were placed in parallel as necessary. Before each experiment, the system was flushed with oxygen introduced at *I* and removed through *O*, the segment between *I* and *O* being clamped during this procedure.

carbon dioxide, the sample of respiratory carbon dioxide actually analyzed for isotope can be considered only as some portion, but not at all necessarily a representative one, of the collection period in question. Body water was determined by a modification of the procedure of Cohn and Urey (5), in which the isotopic measurement is made on carbon dioxide previously equilibrated with the water sample to be analyzed. Carbonic anhydrase prepared by the method of Meldrum and Roughton (6) was added to the equilibrium chamber to hasten the attainment of isotopic

exchange equilibrium. All values are reported as atom per cent excess O^{18} referred to water as a standard; *i.e.*, the values for O_2^{18} and CO_2^{18} have been corrected for the fractionation which occurs between these molecules and H_2O. A fractionation factor of 1.039 was used for the equilibrium between water and carbon dioxide, a factor of 1.006 for the equilibrium between water and molecular oxygen (7). The over-all error of the isotopic analysis is of the order of 1 to 2 per cent of the determined isotopic concentrations.

Total body water was determined on five mice of the type employed by drying to constant weight at 110°. The mean value obtained was 62.6 per cent of the body weight, with a range of 61.0 to 65.7 per cent. From the rate of oxygen consumption, a predicted value for the isotope concentration in body water at the end of the O_2^{18} type of experiment was calculated as follows:

$$\text{Predicted } (O^{18})_{bw} = (O^{18})_a \times \frac{\frac{R \times 60 \times t \times 2}{22.4}}{\frac{0.626 \times \text{weight} \times 1000}{18} + \frac{R \times 60 \times t \times 2}{22.4}}$$

in which $(O^{18})_{bw}$ = the atom per cent excess O^{18} in the body water; $(O^{18})_a$ = the atom per cent excess O^{18} in the atmosphere; R = the rate of oxygen consumption in ml. per minute (corrected to standard pressure and temperature); t = the time of the experiment in hours; weight = the body weight of the mouse in gm.

In the H_2O^{18} type of experiments, a predicted value for the body water isotope level was computed from the dilution of the administered water by the preexisting body water, with a correction for formation of normal water by oxygen consumption.

In preliminary experiments it was found that mice exposed to O_2^{18} atmospheres under the conditions employed survived without obvious ill effects.

RESULTS AND DISCUSSION

The essential results, which are listed in Table I, are interpreted to indicate (1) that the oxygen of respiratory carbon dioxide is in exchange equilibrium with body water, and (2) that utilized molecular oxygen is converted to body water.

The following features of the results support the conclusion that the oxygen of respiratory CO_2 is in equilibrium with body water. (*a*) In one H_2O^{18} experiment (Table I, Experiment C) the isotope concentration in the respiratory carbon dioxide (0.51 to 0.52) is in each collection period practically identical with that observed for the body water (0.50). (*b*)

A value for the body water concentration in Experiment D is not at hand. It will be noted that in Experiment C the observed value for the body water agrees with that calculated from the dilution of the administered water. It would seem permissible to employ the corresponding calculated value for the O^{18} concentration for Experiment D. The value thus computed agrees well with that of O^{18} in the respiratory carbon dioxide collection periods. (*c*) In the O_2^{18} type of experiments (A and B, Table I), the O^{18}

TABLE I

Essential Experimental Results

The O^{18} values are in atom per cent excess. Mice were used in Experiments A to E; a rat in Experiment F.

Experiment	Type	Body weight	O^{18} in atmosphere	O^{18} in respiratory CO_2				O^{18} in body water		Remarks
				Period 1	Period 2	Period 3	Period 4	Observed	Predicted	
		gm.								
A	O_2^{18}	27.3	11.0 –10.9	0.08	0.15	0.38		0.43	0.43*	
B	"	29.0	5.00 – 4.97	0.00	0.02	0.13	0.21	0.21	0.20*	Collection Period 1, ½ hr. before introduction of isotopic oxygen
C	H_2O^{18}	24.6	(0.00)†	0.51	0.52	0.51		0.50	0.51‡	1 ml. H_2O with 8.47 atom % excess O^{18} intraperitoneally
D	"	23.5	0.00	0.52	0.51				0.53‡	" "
E	Control		0.00 – 0.01	0.02	0.02			0.63§		Control; see text
F	O_2^{18}	66	3.98 – 3.94	0.01	0.04	0.06		0.06	0.06*	Collection periods 1 hr.

* Prediction from oxygen consumption; see methods; R = 1.2 ml. per minute for Experiment A; 1.3 ml. per minute for Experiment B.

† Assumed value; no analysis.

‡ Prediction from dilution of injected H_2O^{18} by body water.

§ Water introduced into respiration chamber.

concentration in the last respiratory carbon dioxide sample may be compared with that of the body water. In Experiment A, the former is 0.38, the latter 0.43. In Experiment B, the value for the final carbon dioxide period is identical with that of the body water. It will be recalled that this comparison involves carbon dioxide collected for the 2 hour period preceding the time of the body water sample and that, moreover, the carbon dioxide analyzed was not necessarily a representative portion of the total collected sample. For these reasons, further detailed consideration of the comparison is not undertaken, except to point out that the isotope

concentrations in the final respiratory carbon dioxide and the body water are of the same order of magnitude.

The following features of the results support the conclusion that utilized molecular oxygen is converted to body water. (*a*) The O_2^{18} concentrations in the respiratory carbon dioxide in the O_2^{18} type of experiment (Table I, Experiments A and B) increased with time. Since the isotopic concentration of the carbon dioxide is found to reflect that of the body water, this observation is in qualitative agreement with the continued formation of isotopic water from the utilized oxygen. With approximately uniform oxygen consumption throughout the experiment, one might have anticipated a relatively uniform increase in the O^{18} concentration in the body water, and thus, in the respiratory carbon dioxide, from collection period to collection period. The failure to observe such a constant rise in Experiments A and B is attributed to the circumstance that the value for a given carbon dioxide sample is not necessarily representative of the collection period in question. (*b*) In the O_2^{18} type of experiments (A and B) there is good agreement between the observed body water values at the end of the experiment and those predicted from the rate of oxygen utilization. The significance of this finding is that the major portion of the utilized inspired oxygen is in fact accounted for in the body water.

Experiment E was carried out to estimate the degree to which the complete, or practically complete, isotopic equilibrium between body water and respiratory carbon dioxide may have occurred between the time the carbon dioxide left the animal body and its collection in the trap. Enriched water was added to the usual animal chamber, which was immersed in a water bath maintained at about 30°. Another chamber containing the mouse was placed between the spirometer and the chamber containing heavy water with a calcium chloride drying tube between the two chambers. In this manner, dry normal respiratory carbon dioxide from the mouse was provided with what may be taken as at least as good an opportunity to exchange with isotopic water before being trapped as occurred in the body water type of experiments. It was found that the O^{18} concentration of the respiratory carbon dioxide under these conditions was only 3 per cent of that of the isotopic water. Therefore, the complete equilibrium observed when the animal body water was enriched is attributed almost entirely to processes occurring within the animal body itself.

The O^{18} concentrations in the body water have a bearing on the size of the pool of oxygen in the body compounds other than water which is exchangeable with body water during the time of these experiments. Apparently this pool of oxygen was not great enough to be detected in the form of a discrepancy between observed body water isotopic concen-

trations and values computed either (*a*) in the O_2^{18} type of experiment by the oxygen consumption method or (*b*) in the H_2O^{18} type of experiment from the extent of the anticipated dilution of injected water. Any exchangeable oxygen would have operated to reduce the observed value below the calculated one, an effect which was not actually demonstrable.

Day and Sheel (4), using rats and an artificial atmosphere containing oxygen equivalent in isotope composition to water of 300 parts per million excess density (0.24 atom per cent excess O^{18}), found that the expired carbon dioxide contained oxygen isotopes in proportions corresponding to about 40 parts per million excess density (0.032 atom per cent excess O^{18}); *i.e.*, 13 per cent of the concentration of the inspired oxygen. As previously noted, these investigators interpret their findings as evidence that respiratory oxygen enters directly into carbon oxidation and is exhaled as carbon dioxide.

The results of the present experiments are in apparent disagreement with those of Day and Sheel in that the former provide no evidence for direct combination of carbon with molecular oxygen to yield respiratory carbon dioxide; all the O^{18} in respiratory carbon dioxide can be explained by equilibration with body water. To investigate whether a species difference between the rat and mouse may account for the discrepancy between the two sets of data, an experiment was carried out in which a rat was placed in an atmosphere enriched with heavy oxygen. The findings (Table I, Experiment F) exhibit relationships similar to those for the corresponding mouse experiments. The isotopic concentration in the carbon dioxide increased with time, but during the 3rd hour its value was still only about 1.5 per cent that of the utilized oxygen. Moreover, this value was identical with that of the body water at the end of the experiment and with the value predicted from the rate of oxygen consumption and the amount of body water. Since the duration of the experiment of Day and Sheel is not given, conceivably these investigators exposed the rat to the heavy oxygen atmosphere for much longer periods than those employed in the present studies. In this case, enough oxygen may have been converted to body water to enrich it to the extent indicated by the respiratory carbon dioxide values reported in their paper.

Although we have not in these experiments assessed the degree to which the isotopic exchange equilibrium between respiratory carbon dioxide and body water is established between the time of intracellular origin of the carbon dioxide and its exit from the animal, there is reason to believe it to be complete. Without such an evaluation no inferences are permissible from the present results concerning the isotopic composition of the respiratory carbon dioxide at the moment of formation in internal respiration, by decarboxylation or otherwise.

SUMMARY

With the aid of heavy oxygen, it has been found in the mouse and rat that (1) the oxygen in respiratory carbon dioxide is in isotopic equilibrium with the oxygen of body water, and that (2) at least a large majority, and perhaps all, of utilized molecular oxygen is soon converted to body water.

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