Studies on the Enzymic Oxidation of Succinic Acid Containing Deuterium in the Methylene Groups

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(*Received* 6 *March* 1951)

The assumption on which all metabolic experiments involving tracer isotopes is based is that atoms, groups of atoms, and molecules containing the tracer isotope react in a manner identical with those of normal constitution. However, the theory and practice of the concentration and estimation of stable isotopes shows that discrimination between the isotopes of an element is possible. The question therefore arises as to what extent the living organism provides conditions for discrimination between isotopes. The work to be described here is concerned with differences in behaviour of a normal substrate and the corresponding deuterated substrate in an enzyme reaction.

Differences in behaviour of isotopic compounds are most likely to occur in the case of hydrogen and deuterium compounds because the tracer element is approximately double the mass of the normal element. A normal compound and the corresponding deuterated compound may not be expected to react at the same rate if the difference in mass of hydrogen and deuterium causes a sufficiently marked difference between the zero-point energies (and hence the activation energies for a given reaction) of the two compounds. Indeed, theoretical calculations by Eyring & Sherman (1933) indicated that in chemical reactions at ordinary temperatures, deuterium would be expected to react at least 50% more slowly than hydrogen. These calculations have been supported by results of investigations of relative rates of proton and deuteron transfer (e.g. Wynne-Jones, 1934; Wilson, 1936; Bonhoeffer, 1938). In the biological field, much of the earlier work was devoted to experiments on the effects of heavy water (D_2O) on biological systems. The dramatic demonstration by Lewis (1933) of the failure of tobacco seeds to germinate in pure D_2O was followed by a large volume of work which broadly suggested that low concentrations of D_2O (up to about 10%) had no influence on biological systems (e.g. Dungern, 1936), while higher concentrations in general retarded to varying degrees a number of biological processes, for example, the respiration and alcoholic fermentation of yeast (Pacsu, 1934; Taylor & Harvey, 1934; Hughes, Yudkin, Kemp & Rideal, 1934) and the metabolic rate of mice (Barbour, 1934). Further work was carried out in an attempt to determine whether any alteration of the deuterium/hydrogen ratio occurred in biological systems. This work resolved itself into the careful comparison of the densities of waters of biological origin with the density of a standard natural water. Since the determinations were carried out by density methods, it is not certain, in many cases, to what extent the results might have been affected by variations in the ratio of the oxygen isotopes. The subject of the deuterium/hydrogen ratio constitutes a field on its own (see, for example, Emeleus, James, King, Pearson, Purcell & Briscoe, 1934; Dole, 1936) and cannot be discussed here in detail. It may be concluded from the results, however, that concentration of neither of the isotopes of hydrogen occurs to any great extent in biological systems. On the other hand, the results do not exclude the possibility that small variations in the amount of deuterium may occur in biological systems (say up to about 3% of that normally present), since a variation of this magnitude would be within the limits of error of the several density determinations necessary to control interference from variations in the ratio of the oxygen isotopes (Dole, 1936). In summary it can be said that although on theoretical grounds hydrogen and deuterium may be expected to differ in reactivity, no effect of low concentrations of D_2O on biological systems has been shown satisfactorily, nor has there been a convincing demonstration of biological enrichment of a hydrogen isotope. Such experimental results as these have been taken as the justification for a great deal of tracer work which has been carried out with deuterium.

In discussing the applicability of deuterium as a biological tracer for hydrogen, it is necessary to distinguish between the comparison of the rates at which a compound reacts in media of normal water and D_2O, and the comparison of the rates at which proton and deuteron transfer reactions take place. For example, if a reaction, which involves protons or deuterons, is found to proceed at the same rate in dilute D_2O as in normal water, it is not justifiable to conclude that the deuterium/hydrogen ratio in the reaction products is the same as the deuterium/hydrogen ratio in the medium. And although dilute

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solutions of D_2O 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. The biological work referred to above was concerned with dilute D_2O as a reaction medium; comparatively little appears to have been done in the biochemical field on proton and deuteron transfer.

In order to assist in clarifying the position it seemed desirable to compare the action of a dehydrogenase on its normal substrate with its action on the deuterium-substituted substrate. The succinic oxidase system, although complicated and not fully understood, appeared to be the most convenient for such a study since the methylene hydrogen atoms of succinate are not immediately exchangeable with the hydrogen ions of the aqueous medium. Using deuterium as a tracer, however, it has been shown that considerable exchange of succinic acid methylene hydrogen atoms with hydrogen ions takes place when a solution of succinate is maintained at 80° for 100 hr. (Wynne-Jones, 1935) or is incubated anaerobically with a crude succinoxidase preparation (Weinmann, Morehouse & Winzler, 1947). It was therefore evidently necessary to use as active as possible a preparation of the succinic oxidase system, in which the rate of reaction is not limited by an insufficient concentration of intermediate carriers, and to obtain information from experiments of short duration.

Erlenmeyer, Schonauer & Süllmann (1936) found that [$\alpha\alpha' D_2$] succinic acid (dideuterosuccinic acid) was oxidized at about 70 % of the rate of oxidation of normal succinic acid in the presence of a crude muscle mince and methylene blue. It was proposed to extend this aspect of their experiments by studying the kinetics of the reaction in greater detail. Since the acid which they used contained two hydrogen and two deuterium atoms in the methylene groups there appeared to be an uncontrollable factor involved as to which two of these four atoms were removed, and therefore it is not possible to compare rates of proton and deuteron transfer using this acid as a deuterated substrate. For this reason the action of the succinic oxidase system on normal succinic acid was compared with its action on [$\alpha\alpha\alpha'\alpha' D_4$] succinic acid (tetradeuterosuccinic acid) in order to compare rates of proton and deuteron transfer. The reactions of the two substrates were further compared with respect to activation energy, and an estimate of the relative affinity of the enzyme for the two substrates was also made.

This work forms part of a thesis presented for the Degree of Ph.D. in the University of London. A preliminary account has been given to the Biochemical Society (Thorn, 1949*a*).

MATERIALS AND METHODS

[$\alpha\alpha\alpha'\alpha' D_4$] *Succinic acid* was prepared by the reduction of potassium acetylenedicarboxylate with sodium amalgam in D_2O, substantially as described by Halford & Anderson (1936). The potassium acetylenedicarboxylate was obtained by the reaction of dibromosuccinic acid with KOH in ethanol (Moureu & Bongrand, 1920) and was not separated from the KBr which was simultaneously produced, since the latter does not interfere with the reduction. For the reduction, 8·0 g. of the salt mixture were partially dissolved in 10 ml. of D_2O (99·77 %), and, while the mixture was cooled in an ice bath and mechanically stirred, an amalgam made from 2·0 g. of sodium and 20 ml. of mercury was added to it over a period of 40 min.

The purification methods of Halford & Anderson were not followed because they were designed to produce an acid containing deuterium also in the carboxyl groups. The D_2O was first recovered from the reaction mixture by vacuum distillation. The resulting mass of crystals was dissolved in 60 ml. of water, and the solution was acidified to congo red, transferred to an extractor with washing to 150 ml., and continuously extracted with ether for 8·5 hr. The crude tetradeuterosuccinic acid, obtained by evaporating the ether extract to dryness, was redissolved in 100 ml. of 0·75 N-H_2SO_4 and treated with 0·75 N-$KMnO_4$ at boiling point. This permanganate treatment, which removes small amounts of incompletely reduced material, is in principle the same as that used in similar circumstances by Weinmann *et al.* (1947), following the method of Goepfert (1940). The purified tetradeuterosuccinic acid was recovered from this solution by continuous ether extraction for 6·5 hr. After evaporation of the ether, the yield of the acid was 70 % (based on the weight of the salt mixture used for reduction). The acid recrystallized from water had m.p. (uncorr.) 181·8–182·6°.

The acid was found to contain 77 atom % D in the methylene groups instead of the theoretical 100 atom %. The reason for the low result was not sought, but deuterium may have been lost by exchange during the purification and recrystallization.

[$\alpha\alpha' D_2$] *Succinic acid* was prepared by reduction of recrystallized fumaric acid by methods similar to those described above. Since the reaction mixture contained no KBr it was treated with acid permanganate immediately after the recovery of D_2O, and was then continuously extracted with ether. The yield was 72 %. The extracted acid, recrystallized from water, had m.p. (uncorr.) 182·7–183·4°.

The acid was found to contain 42 atom % D in the methylene groups instead of the theoretical 50 atom %.

Normal succinic acid was prepared during trials of the methods described, using water in place of D_2O as the medium for sodium amalgam reduction. The acid had m.p. (uncorr.) 182·8–183·6°. (Found: C, 41·2; H, 5·2. Calc. for $C_4H_6O_4$: C, 40·7; H, 5·1 %.) Analysis by Weiler & Strauss, Oxford.

This acid was used for comparison with the deuterated acids in the enzyme experiments.

Deuterium analysis. The deuterated acids were analysed for deuterium by the gradient tube method (Linderstrøm-Lang & Lanz, 1938; Linderstrøm-Lang, Jacobsen & Johansen, 1938; Anfinsen, 1946). The combustion of the samples was carried out substantially as described by Keston, Rittenberg & Schoenheimer (1937) and the samples of combustion water were purified in a distillation apparatus similar to that

described by these workers. The water samples were refluxed in the apparatus for a few minutes over $KMnO_4$ and CaO, and were then distilled *in vacuo* three times.

Succinic oxidase system. The system described by Keilin & Hartree (1940) was used. The preparation of the enzyme system (Keilin & Hartree, 1938) differed from that of the original authors in that the washed mince of pig-heart muscle was broken down with phosphate buffer in a Waring blender, instead of in a mechanical mortar with sand. Cytochrome *c* was prepared according to Keilin & Hartree (1937).

Measurement of oxygen consumption. The rate of oxygen consumption of the system was measured in Warburg manometers at 37–37·5° (unless otherwise stated). The gas phase was air. In the several series of experiments which were carried out the concentrations of the constituents of the mixtures in the flasks varied as follows: phosphate buffer, pH 7·3, 0·04–0·05 M; succinate (the acids were neutralized to pH 7·3 with NaOH before making their solutions up to volume), 0·02–0·05 M; cytochrome *c*, $0{\cdot}76 \times 10^{-5}$–$1{\cdot}4 \times 10^{-5}$ M. The manometers were read at 5 min. intervals for about 30 min., during which period the reaction velocity was sensibly constant. The oxygen consumption was plotted against time, and from the best straight line (judged by eye) the oxygen consumption at 15 min. (unless otherwise stated) was taken as a measure of reaction velocity.

The substrate was usually added from the side arm after equilibration in order to avoid contact of the deuterated substrate with the enzyme before readings were commenced. Preliminary experiments were undertaken in order to determine concentrations of substrate and cytochrome *c* above which the reaction velocity was not increased.

RESULTS

Rates of oxidation of succinic acid, tetradeuterosuccinic acid and dideuterosuccinic acid in the presence of the succinic oxidase system. The rate of oxygen consumption of the succinic oxidase system in the presence of tetradeuterosuccinic acid was expressed as a percentage of that in the presence of succinic acid. The mean result from twelve experiments was 39·0 % (range, 34·1–42·7 %). That is to say, tetradeuterosuccinic acid was oxidized at about 40 % of the rate of oxidation of succinic acid. A typical experiment is illustrated in Fig. 1.

In the same manner, it was found from four experiments that dideuterosuccinic acid was oxidized at about 70 % of the rate of oxidation of succinic acid (mean, 71·2 %; range, 65·5–81·4 %). This value is in agreement with the results of Erlenmeyer *et al.* (1936).

It must be remembered that these results, and all others given in this paper, refer to deuterated acids containing about 20 % less deuterium in the methylene groups than is implied by their names. It is to be expected, therefore, that tetradeuterosuccinic acid and dideuterosuccinic acid would be oxidized by the succinic oxidase system at rates (relative to the rate of oxidation of succinic acid) even less than those recorded here.

Activation energies of the oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system. Two experiments were carried out in which the influence of temperature on the rates of oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system was determined. In the first experiment the rates were determined at 20·2, 28·0 and 37·0°, and in the second at 20·2 and 37·0° only. Duplicate determinations were carried out in all cases. Owing to slight daily variations in the activity of the enzyme each experiment was completed in 1 day. The logarithms of the rates were plotted against the reciprocals of the absolute temperatures as shown in Fig. 2, and by multiplying the negative slopes by $2{\cdot}303R$, the values for the activation energies were obtained, as shown in Table 1.

Table 1. *Activation energies of the oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system*

Exp. no.	Activation energy (cal.) Succinic acid	Tetradeuterosuccinic acid
22	11,100	12,400
23	11,400	13,000
Average	**11,250** ± 150*	**12,700** ± 300

* The value of 11,500 cal., previously reported (Thorn, 1949*a*), was incorrect owing to an error in calculation.

The results indicate that there is a difference between the activation energies of the reactions of the two substrates of 1450 ± 450 cal., the slower reaction of the deuterated substrate being associated with the higher activation energy. The value obtained for the activation energy of the reaction of succinic acid is in agreement with that obtained by Hadidian & Hoagland (1939).

The Michaelis constants of the succinic oxidase system with respect to succinic acid and tetradeuterosuccinic acid. The influence of substrate concentration on the rate of oxygen consumption of the succinic oxidase system was determined in the case of succinic acid and tetradeuterosuccinic acid, and from the results the Michaelis constants of the system, with respect to succinic acid and tetradeuterosuccinic acid, were calculated. Owing to the fact that at low substrate concentrations the reaction velocity falls very soon after the reaction has begun, it was necessary to read the manometers at 3 min. or even 2 min. intervals in order to obtain as reliable an estimate as possible of the initial reaction velocity. It was then possible to obtain at least two readings that lay on a straight line, as shown in Fig. 3. The reaction velocity was taken as the oxygen consumption at 6 min., read from the straight line. A number of experiments was performed, out of which three on each substrate, from which reasonable estimates of initial velocities could be obtained,

were used for calculating Michaelis constants. The Michaelis constants were calculated from the data by plotting s/v against s, where s is the substrate concentration and v the initial reaction velocity, according to the method of Lineweaver & Burk (1934). The slopes and intercepts of the lines (one of which is shown in Fig. 4) were calculated by the

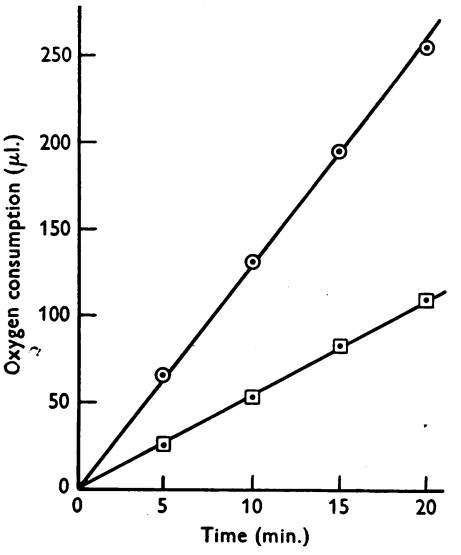

Fig. 1. Oxygen consumption of the succinic oxidase system: ⊙, in the presence of succinate; ⊡, in the presence of tetradeuterosuccinate.

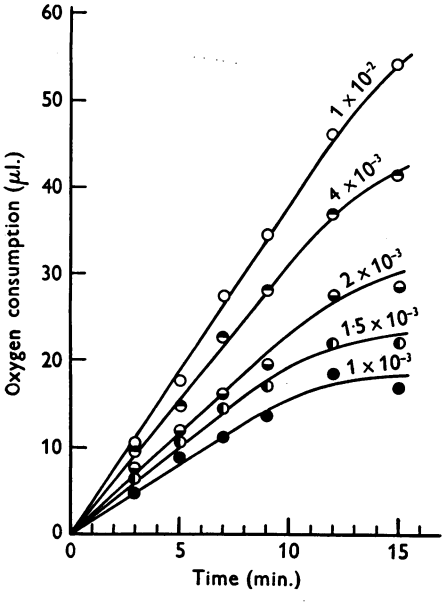

Fig. 3. The effect of succinate concentration (M) on the oxygen consumption of the succinic oxidase system (Exp. 12).

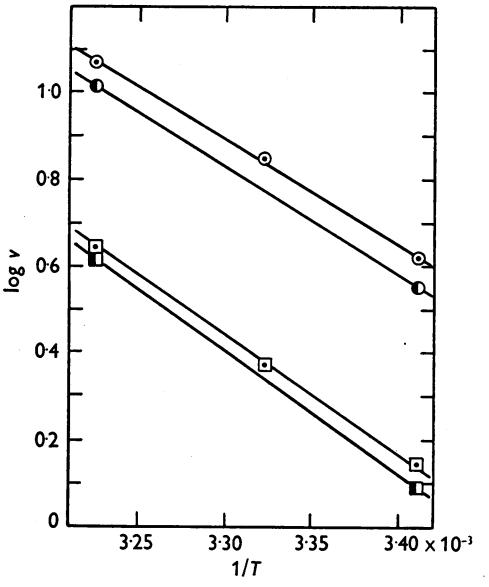

Fig. 2. The effect of temperature on the rate of oxygen consumption of the succinic oxidase system in the presence of succinate and tetradeuterosuccinate. v = rate of oxygen consumption (μl./min.); T = absolute temperature. ⊙, succinate, Exp. 22; ◐, succinate, Exp. 23; ⊡, tetradeuterosuccinate, Exp. 22; ◧, tetradeuterosuccinate, Exp. 23.

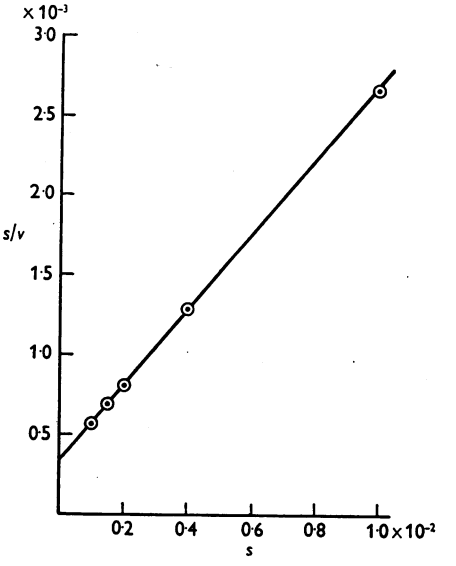

Fig. 4. The effect of succinate concentration on the rate of oxygen consumption of the succinic oxidase system (Exp. 12), plotted according to Lineweaver & Burk (1934). s = molar concentration of succinate; v = initial rate of oxygen consumption (μl./min.).

method of least squares, and from them were obtained the values for the Michaelis constants shown in Table 2.

Table 2. *Michaelis constants of the succinic oxidase system with respect to succinic acid and tetradeuterosuccinic acid*

Succinic acid		Tetradeuterosuccinic acid	
Exp. no.	K_m (M)	Exp. no.	K_m (M)
10	$0{\cdot}86 \times 10^{-3}$	7	$2{\cdot}06 \times 10^{-3}$
11	$1{\cdot}27 \times 10^{-3}$	8	$2{\cdot}63 \times 10^{-3}$
12	$1{\cdot}53 \times 10^{-3}$	9	$1{\cdot}77 \times 10^{-3}$
Average	$1{\cdot}2 \times 10^{-3}$	Average	$2{\cdot}2 \times 10^{-3}$

The average figure for succinic acid is in fair agreement with that of $1{\cdot}0 \times 10^{-3}$ M calculated by Haldane (1930) from the data of Widmark (1922). The results for each acid do not agree closely, which is not surprising in view of the difficulty of making reliable estimates of the initial reaction velocities at low substrate concentrations. Nevertheless, there appears to be a difference between the two sets of figures, indicating a value for the ratio K_m^D/K_m^H of about 1·8, where the symbols refer to the Michaelis constants of tetradeuterosuccinic acid and succinic acid respectively. In order to establish whether or not this difference of Michaelis constants existed, it was considered desirable to use a different experimental approach, rather than to multiply the number of direct Michaelis constant determinations. A method involving mixed substrates was devised, the theoretical basis of which has previously been given (Thorn, 1949*b*). A simpler method, based on the same extension of the Michaelis-Menten theory, has also been described by Whittaker & Adams (1949).

The action of the succinic oxidase system on mixtures of succinic acid and tetradeuterosuccinic acid. The rate of oxygen consumption of the system was measured in the presence of a constant total concentration of substrate. In each experiment one flask contained succinic acid alone as substrate, one contained tetradeuterosuccinic acid alone, and the remainder contained mixtures of the two acids in various proportions. Fig. 5 shows the experimental points representing the average of four experiments. The curves in Fig. 5 were calculated by means of the mixed-substrate equation (Thorn, 1949*b*), which was simplified for this purpose as follows:

$$v_t = \frac{VxK'_m + V'x'K_m}{K_m x' + K'_m x + K_m K'_m},$$

where v_t is the total rate of disappearance of the two substrates when both are present in initial concentrations x, x'; V, V' are the 'maximum velocities' for the two substrates tested separately, and K_m, K'_m are the two Michaelis constants. It will be noticed from the figure that the experimental points do not lie on a straight line, and therefore the fact that the enzyme has different Michaelis constants with respect to the two substrates is established. The value of 1·83 for the ratio K_m^D/K_m^H, arrived at from the determined Michaelis constants of the two substrates, is seen to be too great: a value of 1·45 for the ratio fits the experimental points quite well. This implies that the 'affinity' of the enzyme

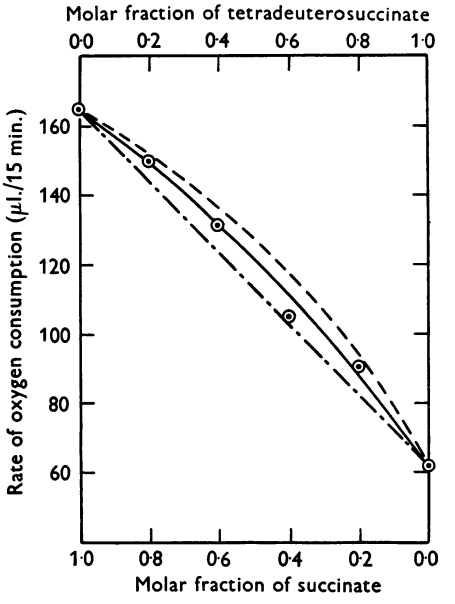

Fig. 5. The effect of the relative proportions of succinate and tetradeuterosuccinate on the rate of oxygen consumption of the succinic oxidase system. The points represent the average of four experiments. The lines are calculated from the mixed-substrate equation; - - - - - -, $K_m^D/K_m^H = 1{\cdot}0$; ——, $K_m^D/K_m^H = 1{\cdot}45$; - - - -, $K_m^D/K_m^H = 1{\cdot}83$.

for succinic acid is 1·45 times its 'affinity' for tetradeuterosuccinic acid. It is considered that this ratio of affinities is more nearly correct than that which is calculated directly from the Michaelis constant determinations.

DISCUSSION

The results reported here may be summarized briefly by the statement that, using the particular enzyme preparation and the methods described, the succinic oxidase system oxidizes normal succinate faster than deuterium-substituted succinate, and appears to have a greater affinity for normal succinate than for deuterium-substituted succinate. The fact that the oxidation of tetradeuterosuccinate by this system is a slower reaction than the oxidation of normal succinate is in itself an interesting result, which might, however, have been anticipated from the results of previous work on base-catalysed proton

and deuteron transfer (Wynne-Jones, 1934; Wilson, 1936; Bonhoeffer, 1938) as well as on theoretical grounds (Eyring & Sherman, 1933). The observed difference between the activation energies of the reactions of tetradeuterosuccinate and normal succinate was found to be of the order of 1000 cal. Wilson (1936) has pointed out that this figure is to be expected on theoretical grounds from a consideration of the zero-point energies of carbon-hydrogen and carbon-deuterium bonds. Further discussion of the results from the point of view of the mechanism of the reaction becomes unprofitable unless it is certain that the rate-limiting step which was being measured was in fact the enzymic dehydrogenation of succinate. It is not, however, certain that this was the case.

It has been repeatedly pointed out (e.g. Keilin & Hartree, 1940, 1949; Slater, 1949*a*) that the activity of the succinic oxidase system depends to a large extent on the maintenance of the correct spatial arrangement of the various carriers concerned in the transfer of hydrogen or electrons from succinate to oxygen. The success of a method of preparation of the system can therefore be gauged by the extent to which the organization of the system in the cell is retained. It did not become evident until after this work had been carried out (Slater, 1949*b*) that the more recent preparation of the succinic oxidase system, introduced by Keilin & Hartree (1947), has a much higher activity than the former one (Keilin & Hartree, 1938), which was used in the studies described here. It can therefore be inferred that the older method of preparation interferes with the organization of the system to a greater extent than does the newer method. The importance of these considerations in the present investigation is that interference with the organization of the system may have caused some intermediate reaction to become the rate-limiting step, and the observed differences in reaction rate and activation energy for the normal and deuterated substrates may not represent the actual differences which would have been observed if the removal of hydrogen or deuterium from succinate had been the rate-limiting step. It is interesting to note in this connexion that the activation energy for the oxidation of succinate by the 1947 succinic oxidase preparation has recently been found by Slater (1950) to be 7520 cal., a figure markedly different from that obtained by Hadidian & Hoagland (1939) and in this investigation. It seems possible that the older Keilin & Hartree preparation and the preparation used by Hadidian & Hoagland (that of Stotz & Hastings, 1937) are comparable from the point of view of degree of interference with the organization of the system, and that they therefore give similar values for activation energy, whereas it is certain that the newer Keilin & Hartree preparation represents a more successful attempt to preserve the spatial arrangement of the carrier system transferring hydrogen or electrons from succinate to oxygen (Slater, private communication).

It is therefore necessary to call attention, not to the actual values which were found for the activation energies of the reactions of succinic and tetradeuterosuccinic acid, but to the difference which was found between these activation energies. Similarly, the values which were found for the Michaelis constants cannot be taken to represent the dissociation constants of enzyme-substrate complexes, even within the approximation imposed by the Michaelis-Menten theory, since the kinetics of the reaction appear to be too complicated to allow of such a simple interpretation. Attention should rather be drawn to the difference which appears to exist between the behaviour of succinate and tetradeuterosuccinate when this experimental method is used, and to the fact that such a difference is confirmed when the two substrates are allowed to compete.

A few general conclusions may be drawn about the use of deuterium in biochemical studies. Clearly, experiments in which deuterium is used as a tracer are justified from a qualitative point of view. Quantitative deductions, however, may be in error unless precautions have been taken, since the amount of deuterium or of a deuterated compound which has undergone reaction does not necessarily represent the amount of hydrogen or of a normal compound which would have reacted under the same conditions. The nature of the errors which might be incurred may be outlined.

The majority of tracer experiments with deuterium fall into two classes. In the first class deuterium is used as a label for a carbon chain. That is to say, a compound containing stably-bound deuterium is synthesized and is administered to an animal. It is of interest subsequently to isolate a series of compounds from the animal, and to determine in which compounds deuterium is present at a concentration higher than it is found to be in the body water of the animal. Such compounds can be said to have been derived more or less directly from the compound administered. The method is exemplified by a study of palmitic acid metabolism by Stetten & Schoenheimer (1940). The second class of experiment consists of enriching the body fluids of an animal with a small percentage of D_2O, usually to not more than about 3%, so that all metabolic reactions take place in a medium of labelled hydrogen. The exchangeable hydrogen atoms of metabolic intermediates exchange with the labelled hydrogen of the medium, and the intermediates themselves become labelled with deuterium. In the course of subsequent reactions some of the deuterium introduced in this manner may become no longer exchangeable, and

the compounds so synthesized contain stably-bound deuterium. Deuterium may also be introduced by reduction. Substances isolated from an animal after this type of experiment, which contain stably-bound deuterium, must have been synthesized during the experiment. The work of Stetten and his co-workers on glycogen synthesis under various conditions (e.g. Stetten & Boxer, 1944) may be cited as examples of this type of experiment.

In the first class of experiment, deuterium transfer is not of interest, except in so far as an excess of D_2O appearing in the body water of the animal indicates that a proportion of the administered compound may have been largely metabolized. If, however, the enzymes concerned with the conversion of the compound administered have different affinities for normal and deuterated substrates, the amount of deuterated compound converted will not reflect the amount of normal compound converted under the same conditions. In the case of the mixed-substrate experiments reported here, it can be shown by means of the mixed-substrate equation, and using the ratio $K_m^D/K_m^H = 1{\cdot}45$, that in mixtures of succinate with up to about 20 % tetradeuterosuccinate, the amount of tetradeuterosuccinate undergoing reaction is about 80 % of what it would be if K_m^D was actually equal to K_m^H. It is of interest that Simmonds, Cohn & du Vigneaud (1947) found that the percentage of deuterium in the methyl groups of methionine administered to rats had no influence on the percentage of choline and creatine methyl groups derived from the methionine. It would seem that the enzymes responsible for these transmethylation reactions handle normal and deuterated substrates in the same way.

The case of experiments of the second class is more complex since, in addition to the possibility of different affinities of enzymes for normal and deuterated substrates, there arises the question of hydrogen and deuterium transfer reactions. In practice, however, it seems likely that the former possibility would be an unimportant source of error. In terms of the experiments reported here, an enrichment of 3 % D_2O in the body water of the animal would mean that the succinic system would be likely to encounter only occasional molecules of a deuterated succinic acid, and even then the acid would probably only contain one atom of deuterium. The ratio of the affinities of the system for monodeuterosuccinic acid and normal succinic acid must be very near to unity, and preferential oxidation of the normal substrate would be unlikely to occur. It remains to consider the question of proton and deuteron transfer reactions. There can be no doubt that deuteron transfer reactions are slower than the corresponding proton transfer reactions. Reviewing the question, Bonhoeffer (1938) found that in cases of base-catalysis (in which the rate-determining stage is the ionization of protons or deuterons from carbon atoms) proton transfer was in all cases from three to ten times faster than deuteron transfer. It is of further interest that Halford, Anderson, Bates & Swisher (1935) studied the base-catalysed reaction,

$$CH_3.CO.CH_3 + HOD \rightleftharpoons CH_2D.CO.CH_3 + HOH,$$

and found that the equilibrium constant was 2·1. In view of the different rates of proton and deuteron transfer, and this information about the equilibrium distribution of deuterium between water and acetone, it seems that the chance exists for unequal distribution of deuterium between body water and metabolites to occur in animals. The point may be raised, and indeed has been raised in this context (Schoenheimer & Rittenberg, 1936), that if unequal distribution of deuterium takes place between body water and metabolites, then enrichment of a hydrogen isotope should take place under normal circumstances. There is, however, no satisfactory evidence that such enrichment does take place in biological systems (see introductory discussion).

These paradoxical lines of evidence must presumably be harmonized by a consideration of the deuterium/hydrogen ratio in the experiments from which the evidence is drawn. Under natural conditions, the proportion of deuterium is so small that unequal distribution of deuterium between body water and metabolites would seem to be negligible, and within the limits of error of the methods of determination. If the proportion of deuterium is increased, there should be reached a stage when unequal distribution of deuterium between body water and metabolites exceeds experimental error, and above this critical level it would be no longer possible to draw quantitative conclusions unless a correction factor could be applied.

The experiments reported here were not designed to study equilibria of this nature, and discussion of this aspect of the problem cannot usefully be prolonged. Schoenheimer (1946) has stated that 'The living organism does not discriminate between isotopes of the same element, stable or radioactive, and treats all alike'. Sufficient evidence, however, seems to be available to make it necessary in the case of hydrogen and deuterium to qualify such a viewpoint with some reference to the proportion of the heavy isotope below which the statement is true within experimental error. Furthermore, as was pointed out in the introductory discussion, the fact that the metabolic rate of an animal is not influenced by a given concentration of deuterium in the body water does not seem to justify the assumption that unequal distribution of the isotope between body water and metabolites does not take place to a significant extent at that concentration of deuterium.

SUMMARY

1. Succinic acids were prepared, containing 77 atom % and 42 atom % of deuterium in the methylene groups.

2. Succinic acid containing 77 atom % D in the methylene groups was oxidized by a succinic oxidase preparation at about 40 % of the rate of oxidation of normal succinic acid under the same conditions. Succinic acid containing 42 atom % D in the methylene groups was oxidized at about 70 % of the rate of oxidation of normal succinic acid.

3. The activation energy of the reaction involving succinic acid containing 77 atom % D in the methylene groups was found to be 1450 ± 450 cal. higher than the activation energy of the reaction involving normal succinic acid.

4. The affinity of the succinic oxidase preparation for normal succinic acid was found to be about 1·45 times its affinity for succinic acid containing 77 atom % D in the methylene groups.

5. The results are discussed, particularly in relation to the use of deuterium as a tracer in biological experiments.

The subject for this investigation was suggested by Prof. F. G. Young, F.R.S., to whom I wish to express my thanks for interest and advice. I also wish to thank the Medical Research Council for a Personal Grant.

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