ISOTOPE EFFECTS IN FULLY DEUTERATED HEXOSES, PROTEINS AND NUCLEIC ACIDS†

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INTRODUCTION

Since the report in 1960 of the first successful culture of algae in 99·7 per cent D_2O, a wide variety of deuterium isotope effects, involving fully deuterated organisms and compounds derived from them by biosynthesis, have been studied at the Argonne National Laboratory. The large-scale cultivation of a variety of green and blue-green algae[2] made possible the growth of fully deuterated heterotrophic organisms[3–5] by making fully deuterated carbon sources and media practicable. It has thus been possible to study not only the effect of deuterium on intact organisms, but to isolate in a purified state individual, fully deuterated compounds of biological importance. Such compounds have proved to be of considerable utility in the study of reaction rates and chemical structure[6–10]. It is the purpose of this communication to (*i*) discuss our most recent work on the cultivation of micro-organisms in D_2O; (*ii*) to present data on the effect of non-exchangeable deuterium on the stability of proteins and nucleic acids; and (*iii*) to describe deuterium isotope effects in the metabolism of fully deuterated glucose and mannose by ascites tumour cells. The isotope effects described here are still in an initial phase of study, but do serve to illustrate the variety of new phenomena now accessible to study.

CULTIVATION OF MICRO-ORGANISMS IN D_2O

Algae

The techniques used in the adaptation and cultivation of algae in D_2O have been described in detail elsewhere[1–3]. Further data have accumulated on the growth rates and temperature optima of the high temperature green algae *Chlorella pyrenoidosa*-71105 and the blue-green *Synechococcus lividus*. *Table 1* summarizes the measured doubling times observed with

Table 1. Growth of algae in D_2O

Species	*Temperature* (°C)	*Doubling time* (h)	
		H_2O§	D_2O§
S. obliquus	27	8	31
C. vulgaris	27	8	29
C. pyrenoidosa-71105	38	2	7·5
S. lividus	53	3	10

§Lowest literature values. See references 1, 3, 5, and this paper.

† Work performed under the auspices of the U.S. Atomic Energy Commission.

light-saturated cultures of these organisms as compared to the better studied *Chlorella vulgaris* and *Scenedesmus*. A deuterium isotope effect of from 3·3 to 3·9 on the exponential growth rate is observed; the deuterium effect to a first approximation thus appears to be independent of the algal species and the temperature ranges studied. There is, however, considerable variability between algal species in the time required for adaptation to heavy water. *S. lividus* grows without lag, at 53° and a light intensity of 400 foot-candles, when subcultured from H_2O. A similar subculture of any of the other three algae of *Table 1* will exhibit a lag period ranging from days to weeks before adaptation occurs.

The optimum temperature for the growth of algae in D_2O is not markedly different from the temperature optimum in H_2O. *Figure 1* shows the exponential growth rate of *C. pyrenoidosa* (71105) in D_2O as a function of

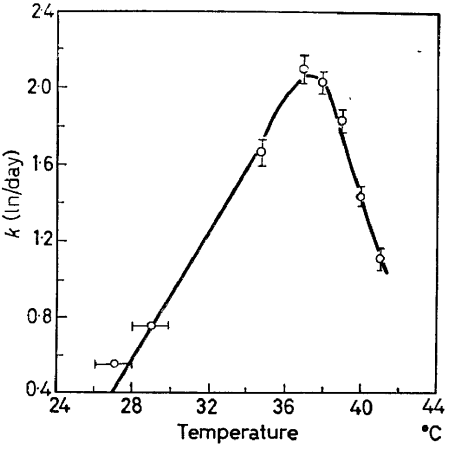

Figure 1. Temperature-dependence of the growth of high temperature *Chlorella pyrenoidosa* in 99·7% D_2O; the specific growth rate is plotted as a function of temperature; the shape of the curve is essentially the same as that for H_2O

temperature. Here the optimum temperature is in the region of 37–38°, while at 42° no growth took place. An entirely comparable temperature dependence has been reported[11] for this alga in H_2O. The identity of the optimum and lethal temperature for growth in H_2O and D_2O is puzzling. If only simple kinetic considerations were involved in the isotope effects, then a quite different kinetic situation should obtain in D_2O. It is thus surprising to find that the rate-limiting steps in growth in D_2O are quite closely related to the H_2O situation.

The exponential growth rate of *S. lividus* is both temperature- and light-dependent. In H_2O at 400 foot-candles one observes a broad optimum at 48° while there is a sharp optimum at 52° at a light intensity of 1500 foot-candles[12]. In D_2O a sharp optimum growth temperature of about 52° is observed when *S. lividus* is grown at 400 foot-candles. In this case a doubling time of ten hours is obtained, which can be compared to the three-hour doubling time in H_2O at 52° and 1500 foot-candles. *S. lividus* will grow at

54° but not at 56° in D_2O. In contrast, Dyer and Gafford[12] report growth in H_2O at 60°. In accord with the experience of these authors[12] we find polymethacrylate (Lucite) containers to be compatible with growth in H_2O, but for still unexplained reasons unsatisfactory for continous cultivation in D_2O.

Fully deuterated heterotrophs

Bacteria, yeast, and moulds can now easily be grown in fully deuterated media when the proper nutritional supplements are provided[3–5]. The preparation and some properties of various fractions and extracts from whole D_2O algae cells have already been described[13]. The extract obtained by autoclaving an aqueous suspension of whole algae has been of the greatest use in growing fully deuterated micro-organisms[5]. Such an extract, in conjunction with the usual inorganic salts and deutero-sugars as a carbon source, has supported luxuriant growths in 99·7 per cent D_2O of *Escherichia coli*[3, 5], *Bacillus subtilis*[5], *B. cereus*, *B. tiberius*[5], and *Hemophilus influenzae*[5]; of the mould *Aspergillus niger*[3]; and of the yeasts *Torulopsis utilis*[4], and several varieties of *Saccharomyces cerevisiae*[4]. In these instances serial subculture into increasing levels of heavy water is generally unnecessary, as a small inoculation of cells or spores generally produces satisfactory growth when the deuterated algae extract is employed.

The growth rate in these D_2O media is lower than in H_2O by 50 to 75 per cent. The yeast *S. cerevisiae* (ATCC7752) in D_2O fortified with 0·8 per cent algae extract and 0·5 per cent deutero-sugars has a doubling time at 25° of nine hours. A lag period of one to two days before growth begins is also encountered with this particular strain. The mould *A. niger* is somewhat better grown in D_2O on a mixture containing deutero-sugars, and 0·5 per cent solids from the ionic fraction[13] obtained by hydrolysis of deuterated algal cell walls. Growth in this case is improved by the addition of traces of vitamins (thiamin, nicotinic acid, calcium pantothenate, biotin, pyridoxin and riboflavin), which can be provided in fully deuterated form from fully deuterated yeast.

A number of nutritional studies were performed with *A. niger* in D_2O using non-deuterated metabolites. It was found that a mixture containing 0·1 per cent each of ten "essential" amino-acids, (arginine, histidine, leucine, isoleucine, methionine, tryptophane, lysine, phenylalanine, threonine, and valine), gave excellent growth from spores in a week. We found no combination of fewer than ten of the "essential" amino-acids that would give a yield equivalent to the full ten.

The protozoan *Euglena gracilis* has also been grown in 99·7 per cent D_2O with inorganic salts, deutero-glucose, vitamins, deutero-glutamic acid, and both hot[5] and cold water extracts of whole deutero-*Scenedesmus obliquus* cells. To obtain growth in 99·7 per cent D_2O it was necessary to serially subculture the organisms into increasing levels of heavy water and to supplement the nutrient medium with a cold water extract of *S. obliquus*. Heated algae extract is beneficial in the presence of the cold water extract, but alone it will not support the growth of *Euglena*. This behaviour indicates the presence of a heat-labile growth factor. Fully motile organisms can be

observed in the deutero-*E. gracilis* cultures and rapidly beating flagella are visible. This organism appears to be the most complex one in which all of the hydrogen has been successfully replaced by deuterium.

The higher plants *Lemna gibba*, *L. perpusilla*, and *L. minor* (duckweed) have proved quite refractory to culture in high concentrations of D_2O. Deuterium concentrations above 55–60 per cent are incompatible with growth. The addition to the nutrient medium of a large number of growth factors, singly and in various combinations, has not yet provided a culture medium superior to one containing only inorganic salts, deutero-glucose, and kinetin. Our more recent experiments with undefined plant extracts indicate that very high D_2O levels may not be inherently incompatible with growth in a higher plant. Experiments aimed at obtaining a fully deuterated higher plant are still in progress.

Discussion

Data obtained from the experiments described above indicate that the deuterium isotope effect on the growth of a wide variety of organisms first manifests itself by a lengthy, variable, adaptation period, which is often accompanied by increased nutritional fastidiousness. Marked deuterium isotope effects have been noted on mitosis[15–18] and reproduction[19, 20]. The nature of the responses elicited by D_2O suggests that the primary response to isotopic substitution involves control mechanisms rather than the usual reactions of intermediary metabolism.

The *in vitro* investigations to date on the effect of deuterium on the thermal properties of nucleic acids[21, 22] and proteins[23] offer scant clue to the biological effect of deuterium. The thermal properties of nucleic acids are hardly affected and there is no clear biological implication in the fact that for proteins deuteration at exchangeable positions favours the helical form over the random coil. However, recent studies by Henderson[24] indicate that D_2O may strongly effect the repressor action in the induced formation of β-galactosidase in *E. coli*. Enzyme induction and repression could be the critical determinant in the early phases of deuteration. It may well be that the site of a primary effect of deuterium on living cells is in the operation and regulation of genes. If such is the case, D_2O would be expected to severely affect highly differentiated cells.

DEUTERIUM EFFECTS IN BIOPOLYMERS

Deutero-phycocyanin

Protein chemists have become increasingly concerned in recent years with the contribution of hydrophobic bonds to the configurational stability of proteins[25–29]. Theoretical calculations indicate that interactions involving non-polar amino-acid side-chains can make a considerable contribution to the structural stability of a protein. One would then expect the substitution of deuterium for protium into such side-chains to produce measurable thermal effects in the absence of any changes at exchangeable positions in the protein. The decreased thermal stability of deutero-phycocyanin extracted from the blue-green alga *Plectonema calothricoides* has

already been reported[8]. The thermal denaturation of this deutero-phycocyanin begins at 45° in H_2O at pH 7, whereas the protio-phycocyanin extracted from the hydrogen alga shows the first signs of denaturation at 51°. Denaturation is followed by the quenching of fluorescence[8] or decrease in optical density, and the observed difference in thermal stability is experimentally significant. Since the experiments on the two types of proteins are conducted under identical conditions in H_2O, and since the denaturation point appears to be independent of pH over a wide range†, we conclude that the differences in thermal stability are a result of changes in hydrophobic interactions resulting from isotopic substitution. The ionic bonds and hydrogen bonding will be identical in the two proteins dissolved in H_2O, but the interactions of the hydrophobic side-chains with each other and the solvent are different.

We have also examined the thermal stability of ordinary and deutero-phycocyanin isolated from *S. lividus*. This organism is under natural conditions a thermophile and grows at elevated temperature. Its phycocyanin denatures at a higher temperature than that from *P. calothricoides*. The denaturation point has been determined from a plot of change in optical absorbance at 610 mμ per degree temperature rise *versus* the temperature. *Figure 2* shows a typical measurement. The protein solutions were subjected

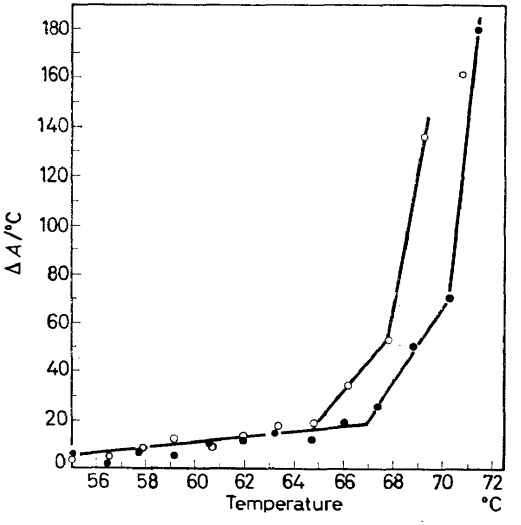

Figure 2. A plot of the decrement of absorbancy of phycocyanin per degree rise in temperature plotted against temperature; reading taken at ten minute intervals in H_2O-phosphate buffer, 0·01 M, pH 6·52; protein isolated from *Synechococcus lividus*

○: Deutero-phycocyanin
●: Protio-phycocyanin

to uniform temperature increments at ten minute intervals. The absorbancy was read immediately before the next incremental temperature increase. Below the point of denaturation the absorbancy reading is stable with time,

† Dr Akihiko Hattori, Argonne National Laboratory, manuscript in preparation.

but above this temperature point, the absorbancy falls continuously with time. The measurement is quite precise and is capable of detecting configurational differences with high sensitivity.

Deutero-phycocyanin from *S. lividus* undergoes denaturation at a temperature about two degrees lower than the protio-protein. *Table 2* lists some of

Table 2. Temperature of onset of thermal denaturation of deutero- and protio-phycocyanin derived from *S. lividus*

In H_2O-*phosphate buffer*			*In* D_2O-*phosphate buffer*		
pH	*Temperature* (°C)		pD	*Temperature* (°C)	
	Protio-	*Deutero-*		*Protio-*	*Deutero-*
6·52	66·6	64·4	7·02	67·6	64·0
6·80	54·2†	49·4†			
6·93	63·8	61·8	7·48	64·0	61·6

† In the presence of 0·5M KCNS and 0·017M Versene.

the results on the denaturation of this protein. While the pH-dependence of the thermal denaturation remains to be determined, the results indicate that once again deuteration at non-exchangeable positions in the protein molecule decreases thermal stability. An increase in the differential effect of deuterium on protein stability in the presence of thiocyanate has also been noted. In a 0·5 M potassium thiocyanate solution (pH 6·80, 0·017 M Versene) denaturation begins at 54·2° for the protio-phycocyanin, and at 49·4°

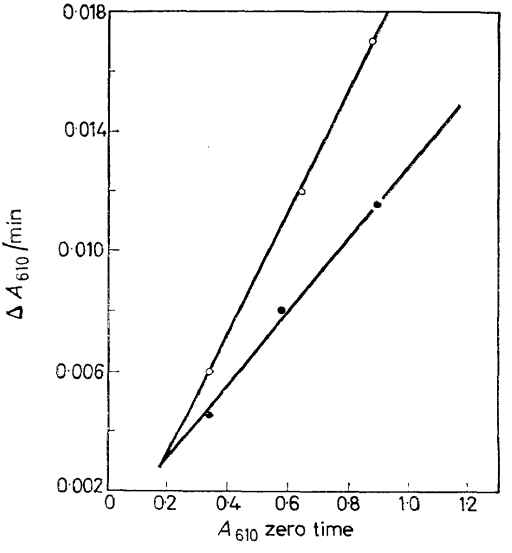

Figure 3. The concentration dependence of the proteolytic hydrolysis of ordinary (●) and deutero (○) phycocyanin; the rate of decrease of absorbancy in the presence of α-chymotrypsin is plotted against protein concentration; enzyme concentration, 0·067 mg/ml; 0·01 M phosphate buffer, H_2O, pH 7·23

for the deutero-phycocyanin. Deuteration thus seems to facilitate loss of configurational integrity in this instance as well.

Differences in side-chain interactions might be expected to modify the susceptibilities of ordinary and deutero-proteins to digestion by proteolytic enzymes. Accordingly, phycocyanin extracted from the blue-green alga *Phormidium luridum*[2] was subjected to treatment with crystalline trypsin and alpha-chymotrypsin. The decrease in absorbancy at 610 mμ with time from the fifth to the tenth minute of digestion was taken as a measure of the rate of lysis. The temperature was maintained at $24{\cdot}5 \pm 0{\cdot}2°$.

Both proteolytic enzymes hydrolyse native deutero-phycocyanin at a faster rate than native protio-phycocyanin (*Figure 3*). It is well-known that denatured proteins undergo proteolysis at a higher rate than the native or intact protein. The more rapid hydrolysis of deutero-phycocyanin is thus consistent with the observation that this class of proteins undergoes thermal denaturation more readily, a consequence presumably of decreased configurational stability. However, the more rapid enzymic hydrolysis may also involve a change in interaction between protein and enzyme arising from the deuterium effect on hydrophobic bonding.

Nucleic acids

It has recently been established[21, 22] that the thermally induced helix-coil transitions in both DNA and RNA are unaffected by a change in solvent from H_2O to D_2O. No significant difference in total hyperchromicity, the midpoint, or the width of the transition[22] are observed when DNA or RNA are heated in D_2O. In experiments with DNA it was also found the substitution of deuterium for hydrogen at non-exchangeable positions did not affect the thermal helix-coil transition[21]. These observations indicate that the differences in the properties of nucleic acids caused by deuterium substitution are not measurable under the conditions of these experiments. However, observations on the effect of electrolytes on the DNA[30] structure convincingly suggest that hydrophobic bonds may play an important part in stabilizing the secondary structure of DNA. It seems reasonable, therefore, to continue to explore nucleic acid systems for deuterium isotope effects.

The interaction of ribonuclease, (Worthington crystalline pancreatic) with ordinary and fully deuterated RNA prepared from *S. cerevisiae* have been examined. In these experiments the rate of development of hyperchromicity at 259 millimicrons was measured as a function of pH and ionic strength in H_2O-acetate and H_2O-*tris* buffers at 0·1 M concentration. Initial rates were linear. In this study the RNA concentration was held constant, $A_{259} = 0{\cdot}65$, and the final enzyme concentration at pH 5·0 was 700 μg/ml. In higher pH ranges, the ribonuclease concentration was reduced by a factor of from 5 to 10.

Figure 4 shows the results of a series of hydrolysis experiments at pH 5·0. At low ionic strength, deutero-RNA interacts with ribonuclease more slowly than protio-RNA by as much as a factor of two. At the optimum ionic strength, which appears to be the same for the two types of RNA, the deutero-RNA reacts faster. The effect of ionic strength upon the reaction

at pH 7·4 has not yet been concluded. However, in 0·1 M *tris*-buffer at pH 7·4, the deutero-RNA again reacts only half as fast as the protio-RNA. A similar result also obtains with RNA prepared from ordinary and deuterated *S. obliquus*.

Other work[31] indicates that the effect of the addition of salt to RNA solutions is to permit a more tightly coiled configuration, because the mutually repelling charged segments of the RNA chain are more effectively

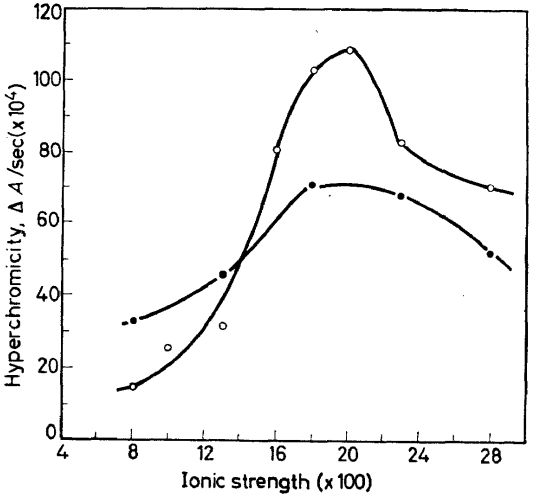

Figure 4. The initial rate of development of hyperchromicity of yeast RNA treated with ribonuclease as a function of ionic strength of the medium; all determinations in H_2O-acetate buffer, pH 5·0, with appropriate amounts of added sodium chloride

●: Protio-yeast RNA
○: Deutero-yeast RNA

shielded from each other. At the optimum ionic strength, then, the RNA molecules are in a configuration most susceptible to interaction with ribonuclease. One can speculate from the data that at low ionic strengths deutero-RNA is less favourably disposed toward enzymic interaction because of weaker hydrophobic bonding. At the optimum ionic strength, the secondary (helical) structure of the deutero-substrate is more rapidly disrupted (greater rate of development of hyperchromicity) because of weaker hydrophobic bonding. It must be emphasized once again that these enzymatic studies were all performed in H_2O and that the observed effects are presumably due to the presence of deuterium at non-exchangeable positions. If these preliminary observations are correct, and if they can be extended to preparations from other sources, it would appear that the factors that determine thermal stability are not identical with those that control the susceptibility of RNA to ribonuclease.

SUGAR METABOLISM

There are at least two general ways of investigating deuterium isotope effects in living organisms. One consists in altering the isotopic composition

of the organism, as by the administration of D_2O-drinking water[32]. The other procedure involves the administration of fully deuterated essential metabolites to an organism of normal isotopic composition. The availability of fully deuterated glucose and mannose makes an experimental implementation of the second approach feasible. To acquire orientation in this new field of investigation, the two ascites tumour cells, Krebs-2 and L-4946 mouse leukaemia were used, and the utilization of the deuterated hexoses was followed in the Warburg apparatus by methods similar to those described by Yushok[33]. In principle, parallel studies were made with ordinary and fully deuterated hexoses at varying temperatures and with varying concentrations of substrate under conditions as identical as possible to secure a valid comparison of the isotope effect.

Typically, lactic acid production reached a maximum rate shortly after a

Table 3. Anaerobic glycolysis by Krebs-2 ascites cells

Temperature (°C)	*Mannose*		*Glucose*	
	No. runs	*Mean* H/D *ratio*†	*No. runs*	*Mean* H/D *ratio*†
30·2	1	1·22	2	0·89
35·1	3	0·98	2	1·18
37·0	7	1·25	8	1·03
37·5	9	1·35	10	0·98
39·6	1	1·47	2	0·98
41·1	3	1·32	—	—
42·7	1	1·35	1	1·00
	25		25	

† Mean H/D ratio = mean ratio of maximum rates of glycolysis of glucose or mannose compared with its fully-deuterated form.

brief lag period. *Table 3* gives the ratio of such maximum rates for the deuterated compound relative to ordinary glucose for Krebs-2 ascites cells. In general, although the rates varied from experiment to experiment, the ratios for the deuterated compound *versus* the non-deuterated counterpart were quite consistent, and were independent of initial hexose concentrations. For this tumour cell, no significant differences in rates were found for glucose, but the maximum rate of deutero-mannolysis was consistently 70 to 80 per cent of that of ordinary mannose compound. These results point to a definite isotope effect on anaerobic mannolysis, and are in contrast to its absence in anaerobic glucolysis. The presence of a slight temperature effect is also suggested by the data in *Table 3*, but interpretation is difficult because of the variability of the results.

Similar observations were made with a smaller series of studies at 37° with L4946, an ascitic mouse leukaemia. In this case the isotope effect on mannolysis was somewhat larger than for the Krebs-2 tumour cells, but the relative rates for ordinary and deutero-glucose show a surprising reversal, with the deutero-glucose apparently utilized at a distinctly higher rate than the normal substrate. The data for concentrations ranging from 0·002 to 0·1 M and at several temperatures are summarized for both tumours in *Table 4*.

Table 4. Comparison of anaerobic glycolysis by Krebs-2 and L4946 ascites cells

	L4946		*Krebs-2*	
	No. runs	*Mean* H/D	*No. runs*	*Mean* H/D
Mannose	8	1·56 ± 0·08	25	1·27 ± 0·04
Glucose	4	0·87 ± 0·05	25	1·00 ± 0·02

Table includes runs at all temperatures and concentrations. H/D ratios are of maximum rates of lactic acid production of ordinary hexoses to those of the fully deuterated sugar. Mean values are given with their standard errors.

In both the present work and that of Yushok[33] the rate of utilization of ordinary mannose is less than that of ordinary glucose. This fact implicates either the hexokinase or the phosphomannose–isomerase systems as rate-controlling steps in the degradation of mannose. One suspects, with Yushok, that the slow reaction is the phosphorylation of mannose as the rate of this reaction is only 40 per cent of that for glucose[34], but the isomerase step must still be considered.

If the isomerase step is rate-limiting for the utilization of ordinary mannose, a fairly large isotope effect is expected when deutero-mannose is substituted for ordinary mannose. Rose and O'Connell[35, 36] found a primary deuterium isotope effect of about 1·8 in the enzyme-catalysed interconversion of glucose-6-phosphate-2-D to fructose-6-phosphate-1-D. Deutero-mannose would not be expected to effect the phosphorylation reaction to any significant extent as experiments by Mohan[37] showed no difference in rate in the phosphorylation of ordinary and deutero-glucose by yeast hexokinase and ATP. The large isotope effect in the anaerobic use of deutero-mannose by tumour cells observed in this work indicates that the rate-controlling step is the conversion of deutero-mannose-6-phosphate to fructose-6-phosphate, and that the same step is likely to be rate-controlling in the anaerobic mannolysis of ordinary mannose.

SUMMARY

The results described here are indicative of the range and scope of isotope studies that can now be carried out because of the availability of fully deuterated substances produced by biosynthesis. Based on our experience, it is likely that all but the most fastidious micro-organisms can be grown in fully deuterated media. Ascending the phylogenetic scale, a protozoan has been grown in fully deuterated form. Studies of fully deuterated proteins and nucleic acids indicate that the presence of non-exchangeable deuterium weakens or lessens the degree of hydrophobic bonding in these molecules. Experiments in the utilization of deutero-glucose and deutero-mannose by ascites tumour cells reveal interesting isotope effects, from which conclusions on rate-limiting phenomena in metabolism can be based. The deuterium isotope effect with deutero-mannose is consistent with the hypothesis that the conversion of mannose 6-phosphate to fructose-6-phosphate is the rate-limiting step in the degradation of this sugar to lactate.

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