235. *Some Reactions with Heavy Water.*

By A. H. Hughes, J. Yudkin, I. Kemp, and E. K. Rideal.

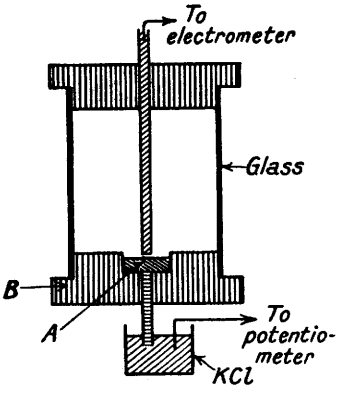
General Introduction.

Although in a number of reactions involving deuterium and its lighter isotope, the reaction velocity with the former is smaller on account of the difference in zero-point energies, yet information is scanty concerning reactions involving heavy water. A number of experiments have been reported indicating that heavy water is toxic to living organisms (see summary by Lewis, *Science*, 1934, **79**, 151); but since the rates of biological processes are in general highly susceptible to small changes in hydrogen-ion concentration and to

the oligodynamic action of small traces of impurities, it was considered advisable to examine a living system in a carefully buffered solution. It will be noted that the experimental data obtained for yeast are in general agreement with the conclusions of Pascu (*J. Amer. Chem. Soc.*, 1934, **56**, 245), *viz.*, that heavy water does indeed possess toxic properties. An attempt has been made to examine separately a few of the simpler reactions which play a part in the living cell, in the hope of limiting the field of enquiry as to the cause of the toxicity of heavy water. Although heavy differs from light water both in fluidity and in surface tension, the differences are not so great as to lead us to anticipate any marked difference in the solvation of the bio-colloids. Again, in reactions in solutions of heavy water it is not at all evident that the rate of any reaction would be governed by the speed of a stage in the reaction which is dependent on the difference in the zero-point energies of H_3O^+ and D_3O^+ or OH' and OD'. It was thus important to examine processes of hydrolysis, and for this purpose both a chemical hydrolysis, *viz.*, that of palmityl chloride by water, as well as an enzyme hydrolysis, triolein by lipase, have been investigated.

Although on inorganic catalysts the reactions involving hydrogenation proceed at different speeds with the two isotopes, it was of interest, as far as the development of a microtechnique rendered it possible, to examine the relative rates in systems involving biological oxidation and reduction. In these cases especially, the effects of an environment of heavy water and the effects of heavy water if participating in the reaction itself have to be distinguished. If the process involves the direct oxidation of part of a complex molecule according to the simple oxidative mechanism postulated by Warburg, no effect other than that of an environment is to be anticipated, but if the oxidation–reduction process proceeds through the mobilisation of hydrogen, as suggested by Wieland, the possibilities of interchange of the hydrogen with that in the water, or of part of the hydrogen being derived from the organic molecule undergoing dehydrogenation and part from the water itself, have to be considered, whilst the reactivities of the two isotopic mobilised hydrogens may differ in a manner analogous to that of free molecular hydrogen and of hydrogen chemiadsorbed at metallic catalytic substrates.

FIG. 1.



I. *The Behaviour of Unimolecular Films on Heavy Water* (with A. H. HUGHES).

It has been shown (Hughes and Rideal, *Proc. Roy. Soc.*, 1933, *A*, **140**, 254) that chemical reactions taking place in unimolecular films can be studied by the method of surface potentials. It is here shown that with suitable modifications the method is capable of dealing with a film spread on a quantity of liquid as small as 0·1 c.c. Two reactions have been studied, the hydrolysis of palmityl chloride by water and the enzymic hydrolysis of triolein,* the latter being presumably a complex reaction some stage of which might be expected to be affected by substitution of heavy hydrogen for hydrogen.

Apparatus.—Fig. 1 illustrates the micro-surface potentiometer employed for dealing with films on a volume of liquid of the order 0·1 c.c. The liquid is contained in a recess *A*, depth 2 mm., cut in an ebonite disc, *B*. Through the base of the recess projects a fine capillary filled with a stiff agar gel and dipping into a solution of potassium chloride, into which also dips a silver wire connected to a potentiometer. The air electrode *C*, of copper wire, whose tip is coated with polonium, passes through a second ebonite disc held firmly to the lower one by a glass shield (1″ in diameter), and is fixed about 1 mm. above the liquid surface. The copper wire is connected to a Lindemann electrometer. In other respects the apparatus is identical with that described by Schulman and Rideal (*Proc. Roy. Soc.*, 1931, *A*, **130**, 259).

Procedure.—To prevent leakage of the film from the liquid surface, the upper surface of the

* We are indebted to Dr. J. H. Schulman, who is making a detailed examination of the behaviour of the lipases on surface films, for particulars concerning hydrolysis.

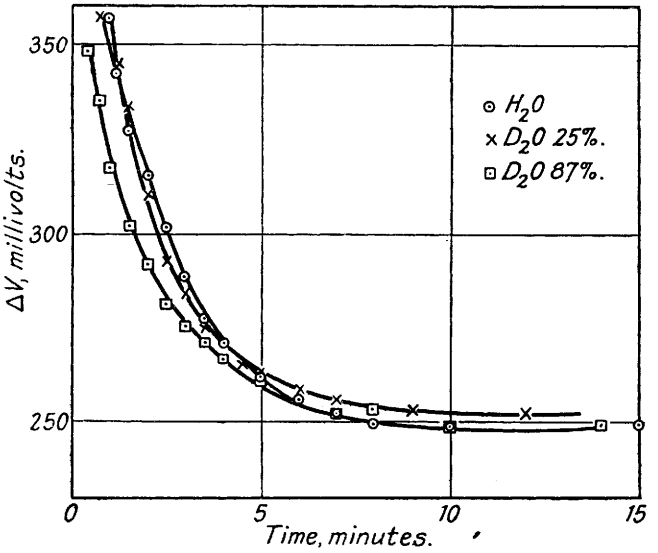
lower ebonite disc was coated with paraffin wax. A major difficulty, the removal of contamination from the aqueous surface, was solved by dusting animal charcoal over the surface to absorb any contamination. A small piece of wax was then melted on to the liquid, and when soft could be peeled off together with the adhering charcoal.

The zero potential of the liquid thus cleaned could be repeated to within $\pm$ 10 mv. A further check of the corrections of this zero could be made with reference to the maximum potential of a film of a known standard substance such as dodecyl alcohol (400 mv.).

The substance to be examined cannot be spread in the usual way by adding drops of a light petroleum solution since the solvent dissolves the surrounding wax. The appropriate volume of the solution was, therefore, allowed to evaporate on the end of a fine glass rod. On dipping the rod in the surface, the substance spreads off to a unimolecular film.

Results.—(i) The hydrolysis of palmityl chloride, which had not hitherto been studied in a surface film, was examined on a larger scale with ordinary water at p_H 7·4 (M/20-phosphate buffer), and found to be highly suitable. The hydrolysis is complete in about 15 minutes at 20°, the final value of the surface potential (ΔV) coinciding with that of palmitic acid under the same conditions.

FIG. 2.



Hydrolysis of palmityl chloride at 19—21°; p_H 7·4.

This reaction was then studied in the micro-trough on 0·1 c.c. of water, with the same result. Two samples of D_2O-rich water (D_2O, 30% and 91·5%) were available. To ensure control of p_H, the 0·1 c.c. of water or heavy water was diluted with 0·005 c.c. of M-phosphate buffer at p_H 7·4. Five runs were done with each filling of the trough, the surface being, of course, cleaned as described between each run. The change of surface potential (ΔV) with time for palmityl chloride is plotted in Fig. 2. No marked differences in the velocities of hydrolysis were observed.

(ii) The hydrolysis of triolein by pancreatin presented a further difficulty in that the triolein film could not be satisfactorily spread on the surface of the enzyme solution. It was, therefore, spread first, and the enzyme, dissolved in buffer solution, was injected under the film through a fine capillary.

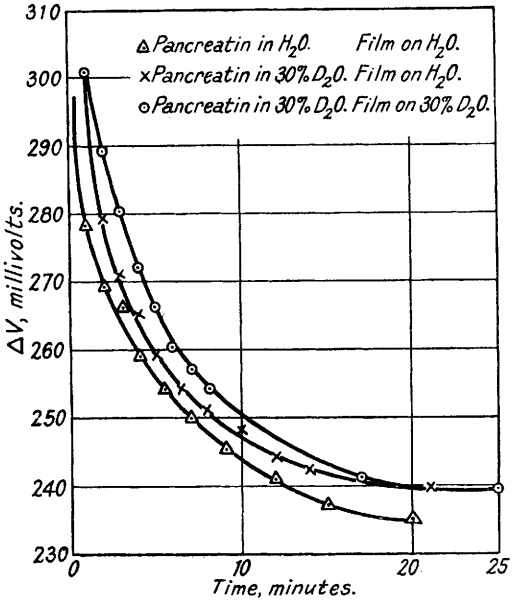
The enzyme solution contained 34 mg. of Merck's pancreatin per c.c. in M-phosphate buffer p_H 7·4; 0·01 c.c. of this solution was injected under a film of triolein spread on the surface of 0·1 c.c. of water in the micro-trough. Four experiments were done with water. The trough was then filled with 0·1 c.c. of 30% D_2O, injection of the pancreatin solution thus reducing the D_2O concentration to 27·3%. To ascertain whether heavy water had any effect on the activity of the enzyme over a prolonged period, a solution of the same concentration of pancreatin was made in 0·1 c.c. of 30% D_2O containing the residue from evaporation of 0·1 c.c. of the M-

phosphate buffer. 0·01 C.c. of this solution of pancreatin in D_2O after standing for 12 hours was then injected into 0·1 c.c. of water, and in another experiment into 0·1 c.c. of 30% D_2O.

The change of surface potential (ΔV) of triolein with time in each case is plotted in Fig. 3.

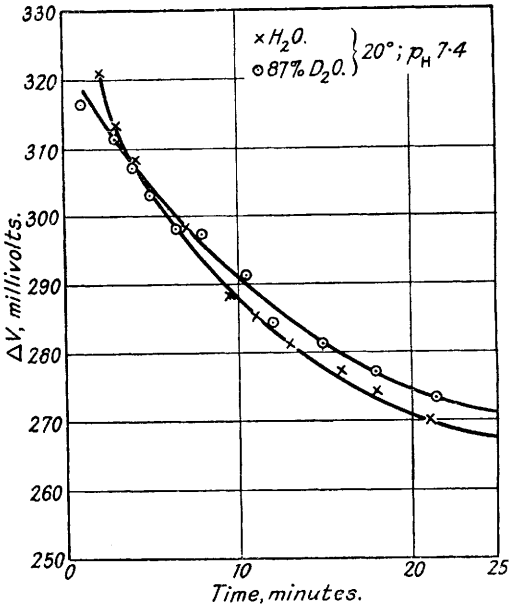
The experiments were repeated with a second sample of Merck's pancreatin with ordinary water, 0·005 c.c. of the buffered enzyme solution being used, making a solution approximately $M/20$ in phosphate; 0·1 c.c. of 91·5% D_2O was then substituted for the water, giving a D_2O concentration of 87% (see Fig. 4).

FIG. 3.



Hydrolysis of triolein by pancreatin at 20°; p_H 7·4.

FIG. 4.



Hydrolysis of triolein by pancreatin.

A relevant correlation lies in the values of the maximum surface potential (ΔV, max.) for various film-forming molecules. These values are determined in the first place by the dipole moment of the polar head group and by the polarity of the surrounding medium. Observed values of ΔV, max. are shown in the accompanying table on water and heavy water mixtures.

Substance.	ΔV, max. (mv.); 20°. H_2O.	28—30% D_2O.	87—91·5% D_2O.
Dodecyl alcohol	400	405	416
Triolein	370	322—363	328—351
Tripalmitin	600—650	611	613
Palmitic acid, p_H 7·4	250—267	251—264	249—267

No differences are manifested, the variations being due to the difficulty of spreading exactly the requisite quantity of substance for a uniform film at its maximum compression, but the similarity of the readings indicates that the induction effects between the polar head group of the film-forming substance and the solution are of the same order for water and for heavy water.

Discussion.—It is at once clear from the observed reaction velocities that an environment of up to 87% D_2O has no marked effect on the rate of hydrolysis of palmityl chloride, or on the presumably more complex enzymic hydrolysis of triolein. Two conclusions may thus be drawn. (1) Any effects due to the higher viscosity of the medium in heavy water are negligible. (2) If the reaction proceeds in stages, then the slow stage is not that involving heavy water or its ions; or alternatively, heavy water and the OD′ ion react at the same speed as water and OH′.

II. *The Effect of Heavy Water on Xanthine Oxidase* (with J. YUDKIN).

In order to study the effect of heavy water on a dehydrogenase system, xanthine oxidase was chosen, partly because both enzyme and substrate are obtainable in a dry form. It was also of interest because water is supposed to be involved in the oxidation, the dehydrogenation proceeding, according to Wieland, *via* a hydrated form of hypoxanthine.

The Warburg manometer was used. 0·1 C.c. of *M*/20-phosphate buffer p_H 7·5 was placed into each of two cups and evaporated to dryness. About 10 mg. of the dry enzyme preparation (kindly supplied by Mr. D. E. Green) were weighed into each cup, and about 1 mg. of hypoxanthine into each side tube. 0·3 C.c. of ordinary or heavy water was measured into each cup, and 0·2 c.c. into the side tube. After 10 minutes' shaking in the bath at 37°, the contents of the side tube were mixed with those in the cup, and the oxygen uptake measured.

In 1½ hrs., 10·2 mg. of xanthine oxidase + 1 mg. of hypoxanthine absorbed 22·3 and 22·8 c.mm. of oxygen (at N.T.P.) in water and in 30% D_2O respectively, showing that there is no inhibition of xanthine oxidase in the latter.

III. *The Effect of Heavy Water on the Respiration and Fermentation of Yeast* (with J. YUDKIN).

Small concentrations of heavy water (0·5%) have no effect on the fermentation of yeast (Macht and Davis, *J. Amer. Chem. Soc.*, 1934, **56**, 546), but inhibition is stated to occur in higher concentrations (60% or 100%) (Pascu, *ibid.*, p. 245). It was thought desirable to repeat the experiments, with buffered solutions, and at the same time to measure the effect of the heavy water on respiration.

The measurements were carried out in the Warburg manometer. On the one sample of yeast were measured: (1) the difference between oxygen uptake and carbon dioxide output, (2) the oxygen uptake, (3) the anaërobic carbon dioxide output (*i.e.*, fermentation), (4) the oxygen uptake again.

The Warburg technique permits measurement of these quantities without disturbance of the experimental fluid. The measurement (4) showed whether any serious change had occurred in the sample during the experiment.

The estimations were carried out in ordinary water and in 30% heavy water at 18°. To 0·5 c.c. of the water in each Warburg cup was added 0·05 c.c. of a suspension of baker's yeast in *M*/2-potassium dihydrogen phosphate containing 50% of glucose. In two experiments, 0·05 c.c. contained 25 mg. of glucose, the quantities of yeast (different samples) being 10 and 15 mg. respectively.

Results.—The results, expressed in c.mm. (at N.T.P.) of oxygen or carbon dioxide in 30 minutes at 18° are shown in the table.

	Experiment 1.			Experiment 2.		
	H_2O.	30% D_2O.	% Inhibition.	H_2O.	30% D_2O.	% Inhibition.
O_2 uptake (1)	56	47	16	57	49	14
CO_2 output (aërobic)	62·5	53	15	74·5	59	21
CO_2 output (anaërobic)	116	93·5	19	106·5	94·5	11
O_2 uptake (2)	54	49·5	9	46	41	12

It will be seen that both respiration and fermentation are inhibited to about the same extent, the results for the latter reaction confirming those of Pascu. The smaller oxygen uptakes at the end of the second experiment are attributable to the unavoidably long duration of this experiment; nevertheless, the inhibition is practically unaltered.

We thank Dr. H. A. Krebs for assistance in development of the manometric technique.

IV. *The Effect of Heavy Water on the Cytochrome–indophenol Oxidase System* (with J. YUDKIN).

It has been shown that the respiration of yeast takes place chiefly through the dehydrogenase–cytochrome–indophenol oxidase system (see Keilin, Article "Cytochrome," Ergebnisse der Enzymforschung, Leipzig, Bd. II). To see whether the inhibition of the

respiration of yeast occurs through the dehydrogenases (as does the inhibition by narcotics) or through the indophenol oxidase (as by hydrogen cyanide or carbon monoxide), the effect of heavy water was observed on a cytochrome–indophenol oxidase system. A preparation of sheep's heart muscle, kindly provided by Prof. D. Keilin, was used in the presence of *p*-phenylenediamine. This is capable of being oxidised by oxygen in the presence of cytochrome and indophenol oxidase.

The activity of the system was measured by observing the oxygen uptakes in the Warburg manometer. Into each of two cups were placed 2·5 mg. of *p*-phenylenediamine, 0·5 c.c. of ordinary or heavy water, and 0·05 c.c. of the heart muscle preparation suspended in phosphate buffer p_H 7·4. The oxygen uptake was read every 10 mins. for 1 hour; the rate was found to be constant over this period, and was 38·5 and 40 c.mm. (at N.T.P.) in the hour in water and in 30% D_2O respectively.

Heavy water therefore causes no inhibition of the cytochrome–indophenol oxidase system. Hence, it seems probable that the inhibition in the respiration of yeast takes place by an inhibition of the dehydrogenases, as with narcotics. So far, only one dehydrogenase has been observed, *viz.*, xanthine oxidase (Section II), and here no inhibition was found. But it has been shown that this dehydrogenase is atypical in that it is not affected by narcotics (Sen, *Biochem. J.*, 1931, **25**, 849). The effect of heavy water on other dehydrogenase systems is being studied.

V. *The Swelling of Gelatin in Heavy Water* (with I. Kemp.)

If a sol of gelatin is allowed to set and is desiccated to a xerogel, then on immersion in water it swells relatively rapidly and uniformly to its original form, after which a slow but regular increase in volume takes place. The rate of swelling under ideal conditions is expressed by a unimolecular velocity equation *

$$(dV/dt)_{PT} = K(V_\infty - V) \text{ or } K_{PT} = 1/t \,.\, \log_e V_\infty/(V_\infty - V).$$

Two distinct processes are involved in the swelling of an elastic gel such as a protein, the imbibition of the liquid and the subsequent solvation and separation of the fibrils. Terzaghi (see Freundlich, " Kapillarchemie," p. 592) considered that the rate of imbibition of the liquid into the microcapillaries was the operation governing the process, but unexpectedly high viscosities had to be assumed for the liquids investigated to render this view tenable. It would appear more likely, especially in the light of the investigations of Katz (*Ergeb. Naturwiss.*, 1924, **3**, 316) on the *X*-ray patterns of swelling gels, that under suitable conditions the relatively slow process concerned is the solvation and concomitant separation of and release of strain in the fibrils. Since this process of hydration can be followed by observation of the increase in volume of the gel, it is clearly desirable so to present the xerogel to the liquid that the rate of imbibition does not influence the results. For example, if a very thick layer of the xerogel be placed in water, the outer layers will rapidly imbibe the water and commence to swell before the inner layers have been at all affected. Both the rate of imbibition and the rate of solvation will in these circumstances affect the apparent velocity of swelling and also the initial distribution of the water if but a limited quantity be used (cf., *e.g.*, the experiments of Flusin, *Ann. Chim. Phys.*, 1908, **13**, 480, and of Spence and Kratz, *Kolloid-Z.*, 1914, **15**, 217, where this appears to be the case).

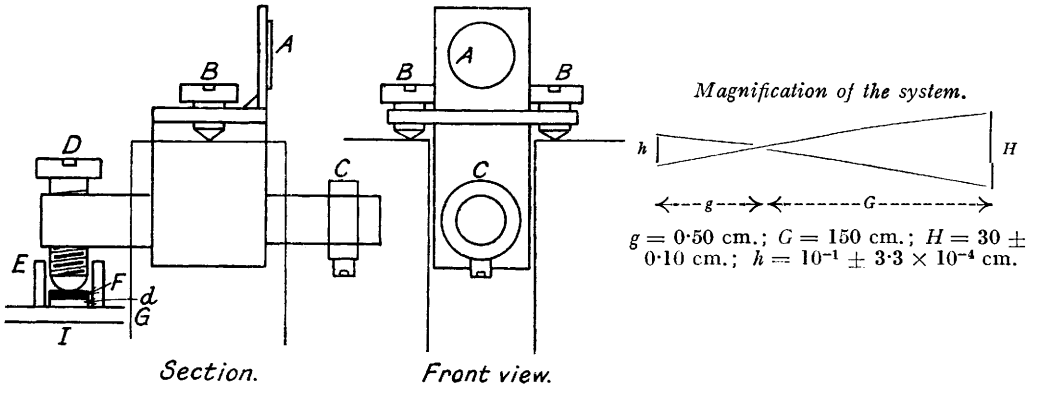
We must thus present the xerogel to the liquid in as finely divided a form as is possible compatible with its structure, or preferably in the form of a thin sheet. Reinke (*Hansteins botan. Abhand.*, 1879, **4**, 1) used laminaria 0·1 mm. thick for the purpose. The usual methods employed consist in measuring either the rate of increase in swelling pressure at constant volume (Reinke) or the rate of increase of weight of the freely swelling gel.

In order to examine the rate of swelling of gelatin in water containing various quantities of deuterium oxide, D_2O, and DHO, a micro-method had to be developed, since the heavy water was only available in small quantities. After a few preliminary experiments, the following simple apparatus was constructed, and it was found that reproducible results could readily

* For the rate of diffusion into a sheet of thickness $2b$, Hill (*Proc. Roy. Soc.*, 1928, **104**, 65) gives $x/x_\infty = 1 - 8(e^{-K\pi^2t/4b^2} + \frac{1}{9}e^{-9K\pi^2t/4b^2} + \ldots)/\pi^2$.

be obtained for examining the rate of swelling under a minute but constant load without disturbing the system.

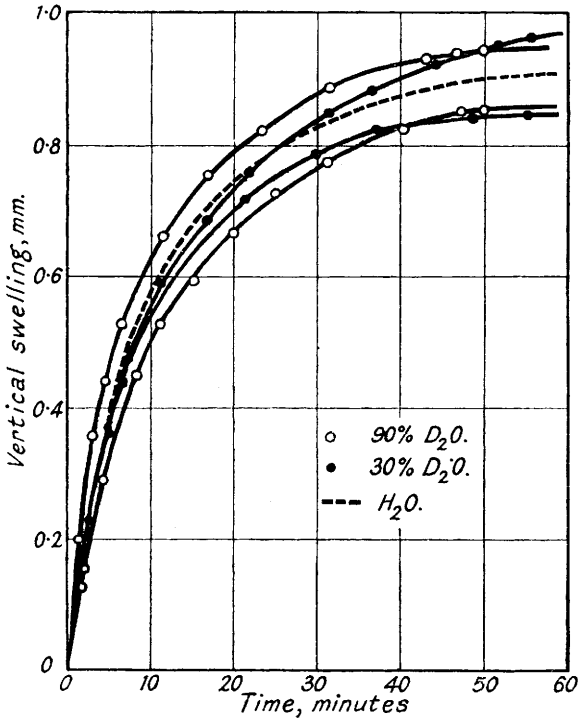
FIG. 5.



The apparatus consists essentially of an optical lever balanced on a pivot *B* by means of the adjustable counterweight *C*. The end of the screw *D* at the other end of the optical lever just touches a thin brass disc *F*, which rests lightly on a disc of gelatin *d* punched out with a steel punch from Eastman Kodak gelatin. The diameter of the disc is 0·23 cm., the thickness 0·20 mm., and the weight 1·0 ± 0·1 mg. (with $\Delta = 1{\cdot}33$, w is computed to be 1·06 mg.). The disc of gelatin is placed in a small cylindrical glass cell *E* cemented on to a polished glass plate *G* which, together with the optical lever, is mounted on a heavy brass bed-plate *I*. The whole apparatus is enclosed in a vessel provided with a glass window for the beam of light reflected from the mirror (*A*) attached to the balanced arm.

The swelling of the gelatin disc in the small cell is effected by the addition of 5·0 c.mm. of water with the aid of an Agla micro-syringe, and measured by timing the rate of traverse of the reflected light spot on a vertical scale. It was found essential to ensure absence of grease of all kinds from the system; gelatin discs which had been touched by hand exhibited a variable behaviour on swelling. Washing the discs in freshly distilled light petroleum was found to be fairly effective in restoring them. In Fig. 6 a few of the curves obtained for the rate of swelling of gelatin are shown.

FIG. 6.



Swelling of gelatin in heavy water.

The velocity of swelling under these conditions was found to follow a unimolecular law and was not appreciably affected by small changes in the hydrogen-ion activity of the water. On the other hand, a few experiments indicated that the degree of extension of the gel on the addition of 5·0 c.mm. of water was at a minimum near the isoelectric point p_H 4·8. As is indicated in the following table, the replacement of water by heavy water up to 90% deuterium content at least has no appreciable effect on the swelling, since the observed diminutions lie within the limits of experimental accuracy.

N/10-*Sodium acetate–acetic acid buffer.*

p_H.	Temp.	Max. swelling, mm.	k, sec.$^{-1}$ × 10^{-3}
3·7	18·1°	0·80	1·9
3·7	18·0	0·97 } 0·89 ± 0·05	1·3 } 1·6 ± 0·2
4·6	16·9	0·77	1·3
4·6	17·9	0·67 } 0·72 ± 0·04	1·3 } 1·3 ± 0
		Distilled water.	
6·5	18·0	0·90 ± 0·02	1·9 ± 0·1
		Heavy water, 30% D *content.*	
6·8	17·4	0·97	1·5
6·8	17·7	0·85 } 0·91 ± 0·04	1·9 } 1·7 ± 0·14
		Heavy water, 90% D *content.*	
—	19·2	0·95	2·5
—	19·1	0·85 } 0·90 ± 0·04	1·7 } 2·1 ± 0·3

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