The present buffers and solvent systems may not necessarily be the best or the most convenient for separating mixtures of DNP amino-acids, and other schemes of separation using buffered columns could almost certainly be devised. The primary object of the present work was not centred in devising the best analytical method, but in eliminating some of the difficulties encountered using unbuffered columns. The application of these methods will be described in a forthcoming paper.

SUMMARY

The use of buffered silica-gel columns in the partition chromatographic separation of 2:4-dinitrophenyl amino-acids is described.

I am indebted to the Director and Council of the Wool Industries Research Association for permission to publish this paper and to Mr G. R. Lee for assistance with the experimental work.

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The Mechanism of the Action of Notatin

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Notatin is an enzyme discovered by Müller (1928) in *Aspergillus niger* and in *Penicillium glaucum*, and named by him glucose oxidase. Müller showed that this enzyme catalyses the oxidation of glucose by molecular oxygen to an acidic product which he identified as gluconic acid. Subsequently it was found by Franke & Lorenz (1937), using a more highly purified preparation, that hydrogen peroxide was formed in this reaction; these authors also suggested that the enzyme was a flavoprotein. A more complete purification was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945), who named the enzyme notatin. Independently, Van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) also purified the same enzyme, designated by these authors penicillin B. The experiments of the American workers suggested that the prosthetic group was the alloxazine-adenine dinucleotide. This was definitely proved by Keilin & Hartree (1946, 1948*a, b*), who examined the physical

and chemical properties of the enzyme in great detail and also determined its specificity range very thoroughly.

The overall reaction catalysed by notatin is generally represented as follows:

This equation does not make clear which is the essential, enzyme-catalysed step in the reaction and, indeed, there has been some discussion as to whether notatin is a dehydrogenase (Franke & Lorenz, 1937; Franke & Deffner, 1939), or an oxidase (Müller, 1936). The latter hypothesis assumes that the enzyme 'activates' molecular oxygen, one of the oxygen atoms combining with the aldehyde form of glucose, whilst the other reacts with water to form hydrogen peroxide. However, the fact that notatin contains alloxazine-adenine dinucleotide suggests that the enzyme is a dehydrogenase. According to this second concept notatin catalyses the transfer of two hydrogen atoms from glucose or from the

hydrated aldehyde form of glucose (Franke & Lorenz, 1937) to molecular oxygen and the latter acts entirely as a hydrogen acceptor.

The present authors (see Bentley, 1948) have found that the oxygen obtained from the decomposition of hydrogen peroxide, by catalase, in water enriched with respect to ^{18}O , contains the normal abundance of ^{18}O . Under these experimental conditions, therefore, there is no exchange of oxygen atoms between hydrogen peroxide and water during the decomposition of the hydrogen peroxide. It thus appeared possible to establish definitely the mechanism of the action of notatin by carrying out the glucose oxidation in water enriched with ^{18}O , and subsequently decomposing the hydrogen peroxide with catalase. If the notatin acts as an oxidase, the oxygen evolved on decomposition of the peroxide should contain half the isotope excess of the water used as a solvent:

If, on the other hand, gaseous oxygen is a hydrogen acceptor in this reaction, the hydrogen peroxide will not contain any excess ^{18}O :

EXPERIMENTAL

Materials

Notatin. This was a highly purified preparation supplied to us by Prof. D. Keilin.

Catalase. Preparations were made from horse liver and horse erythrocytes by the methods of Sumner, Dounce & Frampton (1940) and of Bonnichsen (1947), respectively. The haematin content of the catalase solutions was determined according to Keilin & Hartree (1936). The solutions used were between 10^{-7} and 10^{-8}M .

Glucose. In most of the isotope experiments a commercial sample of glucose hydrate was used which consisted almost entirely of the α -form. Pure anhydrous α -glucose was prepared according to Hudson & Dale (1917); it had $[\alpha]_D^{18} = +101.5^\circ$ (5 min.). β -Glucose was prepared by the pyridine method of Behrens (1907) and recrystallized from aqueous ethanol as described by Hudson & Dale (1917); it had $[\alpha]_D^{18} = +20.8^\circ$ (6 min.).

Isotope experiments in H_2^{18}O

These experiments were carried out in a flask (25 ml. capacity) attached through pressure tubing to a 10 ml. gas burette. The flask could be shaken mechanically.

Glucose monohydrate was dissolved in H_2^{18}O and the notatin added in a length of melting-point tubing, so that initially it did not dissolve. The apparatus was swept out with normal O_2 and then closed with the burette full of O_2 . The flask was agitated so that the notatin dissolved and then shaken mechanically at room temperature, the O_2 uptake being followed in the burette. When there was no further change, the contents of the flask were transferred to one leg of the gas sample tube described by Sprinson & Rittenberg (1948).^{*} The other leg contained a few drops of

a catalase solution. The solutions were frozen in an ethanol- CO_2 bath and the tube evacuated to 10^{-3} mm. The tube was then closed by turning the cap, and the solutions allowed to thaw and degas. The contents were again frozen and the evacuation was completed. After thawing, the solutions were mixed to decompose the H_2O_2 and the liberated O_2 admitted to the sample system of the mass spectrometer in the usual way. Water samples were analysed by equilibration with CO_2 in the presence of carbonic anhydrase (Cohn & Urey, 1938; Bentley, 1948).

Possible exchange of oxygen in the decomposition of H_2O_2 , by catalase. In a large number of control experiments H_2O_2 at various concentrations was decomposed, *in vacuo*, by catalase in the presence of H_2^{18}O (1.39 atom % excess ^{18}O). The evolved O_2 was analysed in the mass spectrometer, but was never found to contain a significant excess of ^{18}O . In view of the relatively low degree of enrichment of ^{18}O in the water used, it cannot be concluded that no exchange occurs in the catalytic decomposition of H_2O_2 . But it follows that if such an exchange occurs, it must be relatively small and would not affect the interpretation of the experiments reported in this paper. In further control experiments it was shown that there was no isotope exchange between O_2 and H_2^{18}O on shaking in the presence of notatin.

Experiments in $^{18}\text{O}_2$

Glucose monohydrate dissolved in normal water was placed in one leg of the gas-sample tube of Sprinson & Rittenberg (1948) and the dry notatin in the other leg. The solutions were degassed and the tube evacuated as described above. The tube and contents were kept in an ethanol- CO_2 bath and connected to the anodic part of an apparatus (Bentley, 1949) in which H_2^{18}O was electrolysed. When the required quantity of $^{18}\text{O}_2$ had been liberated, the tube was closed. The contents were allowed to thaw and the notatin dissolved by mixing. The tube was shaken at room temperature until the reaction was judged complete (3–4 hr.). The contents were frozen and a sample of the $^{18}\text{O}_2$ remaining in the tube admitted to the mass spectrometer for isotope analysis. The solution was washed into one leg of the tube and the H_2O_2 decomposed with catalase as described above. The O_2 obtained was analysed in the mass spectrometer.

Potentiometric and polarimetric experiments

Another series of experiments was carried out with the purpose of identifying the primary product of the action of notatin on glucose. In these experiments α - or β -glucose (anhydrous) was dissolved in water (with or without buffer); 0.2% (w/v) notatin and about $1 \times 10^{-8}\text{M}$ -catalase were then added and the solution made up to volume with water. The solution was then transferred to a conical flask (100 ml.) fitted with inlet and outlet tubes. O_2 was passed in a fairly rapid stream through the inlet tube whilst the flask was shaken mechanically. The temperature in all experiments was $30 \pm 0.2^\circ$, unless otherwise stated.

Polarimetric measurements were made with a 2 dm. tube at 19 – 20° . The pH was measured with a glass electrode using a Cambridge Instrument Co. portable pH meter.

^{*} One of us (R. Bentley) is very much indebted to Dr Rittenberg for a description of this tube in advance of publication.

Enzymic oxidation was followed either by estimating the remaining glucose by titration with hypiodite (Willstätter & Schudel, 1918) or by titrating alkalimetrically the total gluconic acid formed.

Total gluconic acid. As will be shown later, a lactone of gluconic acid is the first product of the enzymic reaction. It was therefore necessary, in order to estimate the combined and free gluconic acid and lactone, to boil the solution after each addition of alkali. The latter was added until the pink colour of phenolphthalein persisted for at least 1 min. The blank values obtained by titrating samples withdrawn at zero time were generally very small unless appreciable amounts of buffer were used. The values obtained by the two methods agreed generally within 3%. However, the alkalimetric method was found to be more reliable and figures for oxidation are based only on the total gluconic acid values, unless otherwise stated.

Free gluconic acid. This was estimated by titrating a sample with 0.05N-NH₃ at 1-3° to a pH of about 6.0-6.5, using B.D.H. Universal Indicator. The results obtained by this method were only accurate within about 5-10%, since hydrolysis of the lactone during the titration could not be completely avoided.

RESULTS

Isotope experiments

Experiments with H₂¹⁸O. Table 1 shows that there is no detectable isotope excess in the hydrogen peroxide produced in the notatin reaction, if ordinary oxygen and isotopically enriched water are used. The figures for oxygen uptake indicate that only about 80-90% of the total glucose has been oxidized. This is caused by the accumulation of hydrogen peroxide owing to the absence of catalase in the first phase of the experiment, and also by the absence of buffer.

Experiments with ¹⁸O₂. If the oxidation of glucose by notatin is carried out with isotopically enriched oxygen and ordinary water, the oxygen evolved from the hydrogen peroxide has the same isotope composition as the gaseous oxygen used (Table 2). Both sets of experiments prove conclusively that the oxygen atoms of the hydrogen peroxide produced in the notatin reaction, are not derived from the solvent, but from the oxygen of the gas phase.

The relative rates of oxidation of α - and β -glucose

Fig. 1 shows the results obtained with α - and β -glucose respectively. In these experiments the glucose was added last to the solution in order to minimize the effect of mutarotation of the glucose. It appears that the enzyme acts on both forms, although the β -form is oxidized about 1.3 times more quickly than the α -form. It cannot be assumed that α -glucose is first converted to β -glucose by spontaneous mutarotation and then attacked by the enzyme, since the rate of mutarotation of glucose under the conditions used is too slow, compared with that of the enzymic oxidation. Moreover, the shape of the curve does not indicate that the rate of oxidation of α -glucose increases with time. It was considered possible that the enzyme catalyses the mutarotation of glucose. This was tested in the following manner:

Anhydrous α -glucose (0.54 g.) was dissolved in 25 ml. of water which had been boiled to remove O₂ and then quickly cooled to 20°; to 10 ml. of this solution was added 0.5 ml. of 0.2% notatin and 7.5 ml. of water, whilst the control was made up to the same volume with water. Both samples

Table 1. Atom % excess ¹⁸O in hydrogen peroxide formed on oxidation of glucose by notatin with ¹⁸O₂ in H₂¹⁸O

(Experimental error of isotope analysis for ¹⁸O in samples of normal or nearly normal abundance = ±0.003 atom %.)

Exp. no.	Glucose monohydrate (mg.)	Notatin (mg.)	H ₂ ¹⁸ O (ml.)	Atom % excess ¹⁸ O in water	Duration of exp. (hr.)	O ₂ uptake at N.T.P. (ml.)	Atom % excess ¹⁸ O in H ₂ O ₂
1	75	0.19	1.0	1.01	2.5	6	0.002
2	100	0.5	2.0	1.01	3.0	10	0.003
3	100	0.5	2.0	1.01	4.0	9.5	0.003

Table 2. Atom % ¹⁸O in hydrogen peroxide formed on oxidation of glucose by notatin with ¹⁸O₂ in H₂¹⁸O

(Experimental error of isotope analysis in samples of about 0.6 atom % excess = ±0.005%. α -Glucose monohydrate was used in Exps. 1 and 2 and anhydrous β -glucose in Exp. 3.)

Exp. no.	Glucose (mg.)	Notatin (mg.)	Water (ml.)	¹⁸ O ₂ evolved (calc.) at N.T.P. (ml.)	Temp. (°)	Duration of exp. (hr.)	Atom % excess ¹⁸ O of		
							Electrolytic oxygen	Oxygen remaining at end of incubation	Oxygen from peroxide decomposition
1	100	0.5	1	8.6	18	3.5	0.603	0.563*	0.563
2	100	0.5	1	10.2	18	3.75	0.591	0.593	0.587
3	100	0.5	1	8.6	37	1	—	0.579	0.580

* This dilution was due to an air bubble accidentally admitted during electrolysis. The presence of air was confirmed by an ion beam in the spectrometer at mass 40, showing the presence of argon.

were transferred to polarimeter tubes and the changes of optical rotation observed for 4 hr. The following $[\alpha]_D$ values were obtained for the control solution: +92.5° (22.5 min.); +87.5° (31.5 min.); +68.5° (74.5 min.); +55.4° (300 min.). The sample containing notatin had the following $[\alpha]_D$ values: +76.2° (23 min.); +69.1° (32 min.); 55.5° (75 min.); +51.7° (300 min.). There thus seemed to be a definite difference between the two solutions. However, it was found

Fig. 1. The relative rates of oxidation of α - and β -glucose by notatin. Anhydrous α - or β -glucose (600 mg.) was added to 60 ml. solution containing M-acetate buffer pH 5.7 (4 ml.), catalase (0.5 ml.) and notatin (0.4 mg.). Oxidation was at 22° for the times indicated by the abscissae; 0.1N- I_2 (10 ml.) and 0.1N-NaOH (15 ml.) were then added to 5 ml. samples. After standing for 5 min., the I_2 not used up was titrated with 0.1N- $Na_2S_2O_3$ after the addition of 2.5 ml. 5N- H_2SO_4 .

that the notatin had brought about the oxidation of about 3–4% of the glucose. The specific rotations of gluconic acid and the δ -lactone are smaller than that of α -glucose and this will account for a small part of the difference. Moreover, the formation of free gluconic acid and the consequent change of pH is bound to affect the non-enzymic mutarotation of glucose. However, the slight oxidation found cannot explain the results obtained.

There is thus suggestive, but no conclusive evidence that notatin catalyses the mutarotation of glucose and that α -glucose is converted enzymically to β -glucose before oxidation can take place.

The nature of the primary product of oxidation

Comparison between the development of acidity and the disappearance of glucose. Gluconic acid is a

moderately strong acid. Thus gluconic acid solutions prepared by treatment of calcium D-gluconate hydrate with the calculated amount of oxalic acid had the following pH values: 0.001M, 3.18; 0.01M, 2.71; 0.1M, 2.25. A 0.1M solution half neutralized with 0.1N-NaOH had a pH of 3.66. The pK of gluconic acid must therefore be about 3.6–3.7.* It is to be expected therefore that the formation of a small amount of free gluconic acid will, in the absence of buffering, reduce the pH to below 3.5. The pH optimum for notatin is about 5.6 (Keilin & Hartree, 1948a) and the rate of oxidation falls off rapidly from pH 4 to 3.0 to become quite small at higher acidities. Müller (1928) had already found that a decrease of pH and increase of base binding occurs in the notatin reaction and this retards the action of the enzyme. It was thought, therefore, that gluconic acid itself is the first product of this oxidation. However, it can be shown that the development of acidity is not part of the oxidation process, but occurs later and is almost certainly not catalysed by the enzyme. This is clearly shown by the following experiments.

Oxidation with a large amount of enzyme, but without buffer. β -Glucose (80 mg.) was added to a solution (total vol. 15 ml.) containing 2.4 ml. 0.2% notatin and 0.5 ml. catalase. Oxidation was allowed to proceed for 8 min.; by that time 84% of the glucose had been oxidized. The initial pH was 5.4 and at the end of the oxidation 3.55. The solution was now cooled to 20° and left to stand under N_2 . The pH changes were as follows: 15 min. after the addition of enzyme, 3.15; 20 min., 2.99; 40 min., 2.75; 60 min., 2.55. There was no further oxidation during the second part of the experiment.

Oxidation in the presence of a small amount of buffer. In this experiment the amount of base in the acetate buffer was equivalent to 10% of the glucose used. β -Glucose (0.54 g.) was dissolved in a solution (total vol. 30 ml.) containing 3 ml. 0.12N-acetic acid, 3 ml. 0.1N-NaOH, 0.3 ml. catalase solution and 3 ml. 0.2% notatin. Oxidation was allowed to proceed for 0.5 hr. During that time the pH had fallen from 5.58 to 4.29 and 94% of the glucose had been oxidized. The solution was again cooled to 20° and the pH changes noted for the following 15 hr. During the second part of the experiment a further 3% of the glucose was oxidized. The pH changes were as follows. Times (in hr.) from the beginning of the experiment are given in brackets: 4.15, (0.8); 4.00, (1.1); 3.88, (1.5); 3.78, (1.75); 3.69, (2.1); 3.55, (2.7); 3.38, (4.0); 3.27, (5.0); 3.08, (8.0); 3.02, (16.0).

In this experiment the pH did not fall below 3.0 owing to the buffering effect of the gluconate ion; sodium acetate and gluconic acid must have reacted to give acetic acid and sodium gluconate.

* May, Weisberg & Herrick (1929) found a value of 3.78 for the pK of gluconic acid.

Measurement of free and total gluconic acid. Table 3 shows that the primary product is a substance which does not titrate as an acid under the conditions used for the estimation of free gluconic acid, but can be estimated under the more vigorous conditions employed for 'total' gluconic acid. The most reasonable assumption is that this substance is a lactone. The relative proportion of free gluconic acid is small in the beginning of the oxidation, but increases rapidly, after most of the oxidation is finished. This is in accordance with the pH measurements reported above. The converse, of course, applies to the lactone which is measured by the difference between total and free gluconic acid.

Table 3. Comparison of the rate of formation of free and total gluconic acid during the oxidation of glucose by notatin

(α -Glucose (0.540 g.) was dissolved in 60 ml. water containing 0.41 mg. notatin and 0.5 ml. catalase. Oxidation proceeded at 24°. 3 ml. samples were withdrawn at suitable intervals and titrated for free and total gluconic acid as described in the text. The very small blank values were subtracted.)

Time after addition of glucose (min.)	Percentage of glucose originally present converted into		Ratio of total/free gluconic acid
	(a) Free gluconic acid	(b) Total gluconic acid	
10	0.5	10.9	21.8
20	1.0	17.9	17.9
30	1.6	24.0	15.0
60	5.1	42.4	8.3
90	9.3	52.5	5.6
128	19.0	59.3	3.2
160	35.0	75.1	2.1
1080	84.2	100.1	1.18

Polarimetric measurements

The experiments so far reported indicated that notatin converts glucose to a lactone or some other neutral substance which is slowly converted to gluconic acid. A more definite identification of this primary product was achieved by following the changes in optical rotation. δ -Gluconolactone has an initial $[\alpha]_D$ of +66.2° (Nef, 1914; Isbell & Frush, 1933) which falls over 24 hr. to +8.8°, whilst γ -gluconolactone reaches an equilibrium value much more slowly. Fig. 2 shows that the primary product of the notatin reaction resembles δ -gluconolactone very closely; the slight differences between the mutarotation curves found here and those reported by Nef (1914) or by Isbell & Hudson (1932) are caused by the fact that in experiments with pure δ -lactone all the lactone is present at zero time, while in the enzymic experiments it is being formed from glucose during the first 20–30 min. of the experiment. The small amount of glucose left at the end of the experiment (6.5 and 7 % respectively)

explains the relatively high equilibrium figure after 24 hr., which was +12.1° instead of +8.8° found by the other workers.

Fig. 2. Comparison of the mutarotation of the product of the oxidation of β -glucose by notatin with the mutarotation of δ - and γ -gluconolactone. Curve A gives the $[\alpha]_D$ values (calculated in terms of gluconolactone) of the oxidation product. Curve B is the mutarotation curve of δ -gluconolactone, and curve C is that of γ -gluconolactone. The data for the two lactones were obtained from the figures given by Nef (1914), Isbell & Hudson (1932) and Isbell & Frush (1933). The oxidation was carried out as follows: Anhydrous β -glucose (600 mg.) was added to 30 ml. of a solution containing notatin (4.8 mg.) and catalase solution (0.4 ml.). Oxidation proceeded for 25 min. at 30°, when 93 % of the glucose had been oxidized. The solution was then filtered and the rotation measured.

DISCUSSION

The isotope experiments prove conclusively that notatin catalyses the transfer of hydrogen from glucose to the oxygen of the gas phase. The fact that there was no isotope exchange between $H_2^{18}O$ and $^{18}O_2$ on shaking with notatin is confirmatory evidence that this enzyme does not produce a direct 'activation' of molecular oxygen, similar to the 'activation' of hydrogen produced by the enzyme system of *Proteus vulgaris* (Hoberman & Rittenberg, 1943).

At first, the most likely substrate of notatin appeared to be the hydrate of the aldehyde form of glucose—which could be dehydrogenated to the free gluconic acid in the manner suggested by Wieland (1932). However, Goto & Titani (1941) have shown that at room temperature, but not at 100°, the rate of exchange of glucose with $H_2^{18}O$ is very slow and that mutarotation takes place without exchange of oxygen. Since reversible hydration of glucose necessarily involves oxygen exchange on C_1 , it follows that such an uncatalysed hydration is slow at room temperature. Thus, if the actual substrate of dehydrogenation were a glucose aldehyde hydrate,

it would have to be assumed that notatin, in addition to its oxidizing action, also catalyses the conversion of ring forms of glucose to the open aldehyde form and/or the hydration of the aldehyde. Such a rather complicated hypothesis becomes unnecessary in view of the experiments reported now.

The fact that the development of acidity is not directly associated with the enzymic process and can be made to take place almost entirely after the oxidation has stopped, shows that the primary product is not gluconic acid. This is also shown by the comparison of the formation of 'free' and 'total' gluconic acid. From the polarimetric experiments it can be deduced that the neutral primary product is δ -gluconolactone. It thus follows that the substrate of notatin is glucopyranose and not the free aldehyde,

about 35 times faster with β - than with α -glucose (Isbell & Pigman, 1933). Oxidation with hypochlorite, on the other hand, is faster with α - than with β -glucose (Jeannes & Isbell, 1941).

*Detailed mechanism of the
hydrogen transfer*

In the reaction under discussion, two protons and two electrons are transferred from glucopyranose through the flavoprotein to molecular oxygen. This could occur in two successive steps, each involving the transfer of one electron; this mechanism may be pictured as follows (leaving out the intermediate reactions involving the prosthetic group of the enzyme):

hyde, or its hydrate, or one of the two glucofuranoses. If the aldehyde were the substrate, the primary product would be gluconic acid, whilst the oxidation of a furanose would have yielded the γ -lactone.

The results reported here may suggest that notatin acts on both α - and β -glucopyranose, though the rate is definitely faster with the β -isomeride. However, suggestive evidence was obtained that notatin catalyses the mutarotation of α -glucose, and it is very probable that mutarotation catalysed by notatin precedes oxidation.* Keilin & Hartree (1948a) have recently examined the substrate specificity of notatin in some detail. They have found that mannose and xylose are oxidized at about 1% of the rate found for glucose, and galactose is also attacked, albeit more slowly. It is of interest that the action of bromine on glucose in a buffered, weakly acidic medium also yields δ -gluconolactone and not gluconic acid (Isbell & Hudson, 1932; Isbell, 1932). However, in this reaction the rate is

* Keilin & Hartree (private communication) have independently discovered the effect of notatin on the mutarotation of α -glucose and have made this problem the subject of an extensive investigation.

In the first step the glucopyranose loses a proton and an electron to give two free radicals, $\text{HO}_2^{\bullet}$ and a radical derived from glucose; the latter then loses a second electron to form a carbonium ion which is stabilized by resonance with an oxonium ion. A prototropic reaction between the oxonium ion and the peroxide anion then completes the oxidation. Riboflavin and flavoproteins can exist in the form of fairly stable free radicals (Haas, 1937; Michaelis & Schwarzenbach, 1938), and preliminary experiments carried out here appear to show that notatin can catalyse the reaction between titanous chloride and iodine, presumably by the mechanism suggested by Shaffer (1933, 1936). It can therefore be assumed that notatin may be an intermediate in reactions involving the transfer of one electron by being itself transformed into a free radical. However, there is no evidence for even the transient existence of a free radical derived from glucose, and the known facts are equally compatible with the assumption that the oxidation of glucose by notatin occurs by an ionic mechanism.

According to this concept the essential step in the reaction is the transfer of a hydride ion, i.e. a proton with two electrons, from the glucopyranose to mole-

cular oxygen, and thus the reactions (1), (2) and (3) are telescoped into one:

This is followed by reaction (4) as above. The shift of a hydride ion has been postulated as the mechanism of other redox reactions such as a Cannizzaro reaction (viz. Hammett, 1940). No facts are available which would enable us to decide which of the two interpretations is correct. In any case, the distinction may be somewhat unreal, since the ionic mechanism may be considered to represent an extreme case of the free radical mechanism in which reactions (2) and (3) are infinitely fast compared with reaction (1).

SUMMARY

1. By using water and molecular oxygen labelled with ^{18}O it is shown that the oxygen atoms of the hydrogen peroxide produced in the oxidation of glucose by notatin are derived from molecular

oxygen. It is concluded that notatin catalyses the transfer of hydrogen from glucose to oxygen.

2. A comparison of the rate of oxidation of glucose by notatin and the development of acidity shows that the first product of the enzymic action is a neutral substance. Polarimetric measurements indicate that this is δ -gluconolactone which changes by a non-enzymic reaction into gluconic acid.

3. The mechanism of the transfer of two hydrogen atoms of glucose to oxygen is discussed. Two alternative interpretations are proposed, one involving a free radical mechanism and the other a simultaneous transfer of two electrons as the rate-determining step.

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