both similarities and differences (Table V). The mean molecular weights of the different polymers are about 10,000 except for the polymer described by Schiff in 1897.[9] In the cases reported, the polymer gave positive biuret tests as would be anticipated from the molecular weights.[38] Samples of polyaspartic acid prepared by Frankel and Berger and in this Laboratory gave negative ninhydrin tests, but the one described by Kovacs and coworkers gave a positive test. The analytical data for the polymer described in this paper already have been discussed. Such analyses are subject to possible misinterpretations due to the tenacity of water of hydration and also to the possibility of more than one polymeric form, *i.e.*, I and II.

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[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Countercurrent Distribution Studies with Ribonuclease and Lysozyme

BY T. P. KING AND LYMAN C. CRAIG

RECEIVED JANUARY 21, 1958

A search has been made for systems which will permit fractionation by countercurrent distribution of ribonuclease, lysozyme and chymotrypsinogen. Systems for the first two are reported in which there is no sign of denaturation. These proteins behave as nearly ideal solutes as far as countercurrent distribution is concerned.

Introduction

In the earliest attempts in this Laboratory to fractionate a protein by countercurrent distribution (C.C.D.) two types of system were found which would partition insulin.[1] One achieved partition in a useful range of partition coefficient by the use of salt as an important component of the system. The other contained a complexing agent, such as dichloro- or trichloroacetic acid which modified the hydrophilic nature of the molecule and thus increased partition in the alcohol phase. In neither type of system was there any evidence of denaturation or loss of biological activity even though the operation was carried out at 25° and required several days.

Since insulin is known to be a very stable protein even in an environment often quite different from that in which it occurs, the stability found was not considered representative of proteins in general. Indeed, it was soon found that most other proteins would not show anything like this stability. Nonetheless, up to the present time, a number of other proteins have been successfully distributed. These include adrenocorticotropins,[2] growth hormone,[2] lactogenic hormone,[2] lysozyme,[3] casein[4] and serum albumin.[5] Evidence was found in the serum albumin study that the complexing agent itself had a certain stabilizing effect at lower *p*H values where proteins tend to be more alcohol soluble.

This did not seem to hold true with most of the other proteins thus far studied. In many cases an initial partition ratio within a usable range was found but on carrying out the distribution, clearly defined bands were not obtained because of a progressive shift of the partition ratio (K). An inconsistency of the determined K and the position of the band on the pattern offered further evidence of slow transformation.

Aside from the question of denaturation, there is the question of adherence to ideal solute behavior. With serum albumin[5] an extremely narrow distribution band was found which was explained on the basis of solute binding. A similar behavior had been noted in the earlier insulin work[1] and somewhat later in the A.C.T.H. work.[2] If solute binding could produce a band too narrow, it could also produce one which would be too broad, an observation which usually denotes inhomogeneity in countercurrent distribution work.

It therefore would be very helpful to have an independent fractionation method available for cross checking the fractions, provided such a method had high resolving power and was suited to the small amount of substance available in the fraction. Ion-exchange chromatography provides such a method in the case of ribonuclease.[6] chymotrypsinogen[7] and lysozyme[8] and has been widely accepted as the most reliable method for studying the purity of these substances. For this reason these proteins have been chosen for one of the initial phases of our partition studies with proteins.

Experimental

The sample of ribonuclease used was obtained from Armour and Company, Lot 381-059. The lysozyme was also Armour material, Lot 381-187. The chymotrypsinogen was obtained from Worthington Biochemical Company.

The distribution equipment used included the previously described automatic 200-tube machine with 10-ml. capacity[9] and a new 1000-tube machine of similar construction but with 2-ml. capacity per phase in each cell.

Reagent grade ammonium sulfate, redistilled water and absolute ethanol were used to prepare the system which contained $(NH_4)_2SO_4$, H_2O and C_2H_5OH in the weight ratio, respectively, of 16.5:57.6:25.9. In practice it was found convenient to prepare this system by mixing three volumes of an ammonium sulfate stock solution (40 g. in 100 ml. of water), one volume of water and two volumes of ethanol. Slightly more lower phase was formed than the upper phase. A phase diagram of this three-component system at 25° was constructed and from the diagram it could be determined that the system used is near the critical point and the compositions of the upper and lower phases are, respectively,

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(2) C. H. Li, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 101.

(3) E. Craenhals and J. Leonis, *Bull. soc. chim. Belges*, **64**, 58 (1955).

(4) P. von Tavel and R. Signer, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 237.

(5) W. Hausmann and L. C. Craig, THIS JOURNAL, **80**, 2703 (1958).

(6) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).

(7) C. H. W. Hirs, *ibid.*, **205**, 93 (1953).

(8) H. H. Tallan and W. H. Stein, *ibid.*, **200**, 507 (1953).

(9) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

10% $(NH_4)_2SO_4$, 56% H_2O, 34% C_2H_5OH and 24% $(NH_4)_2SO_4$, 60% H_2O, 16% C_2H_5OH. The *p*H of the upper and lower phases as measured with a glass electrode were 5.69 and 5.19, respectively.

By using 0.05 *N* ammonium hydroxide in place of water for the system, the upper and lower phases of the resulting system had a *p*H of 7.2. With 0.054 *N* sulfuric acid the system obtained had an upper phase of *p*H 3.5 and lower phase of *p*H 3.4.

The initial protein concentration for the distribution was usually 10–15 mg. for one ml. of upper plus one ml. of lower phase. The protein was dissolved in water first, the ammonium sulfate stock solution then added, and to the mixture, cooled to 20°, ethanol was added. After equilibration, enough pre-equilibrated upper phase was added to give equal volumes of each phase and the solution then scattered in five to fifteen tubes. The distribution was carried out at 25 ± 2° with the machine set for a 60 second settling time and with six shakes for equilibration. About 780 transfers could be done in a 24 hr. period.

The distribution was analyzed by optical density at 280 mμ in a cuvette with an optical path of 1 cm. The solution in the cuvette tended to become cloudy during analysis due to a slight evaporation or change of temperature but could be cleared by addition of a droplet (0.01 ml.) of water. For the recovery of solute, the pooled fraction was concentrated at 25° on a rotary evaporator to one third of its volume and the turbid solution was allowed to stand at 5° overnight. The precipitated protein was collected by centrifugation, dissolved in a small volume of water and dialyzed in Visking 23/32 seamless cellophane tubing to remove salt at 25°. This tubing has been found to be impermeable to ribonuclease under these conditions. The solution was centrifuged to remove some lint, then lyophilized.

A sample of ribonuclease weighing 440 mg. was distributed in the 200 tube machine. At 196 transfers analysis gave the pattern shown in Fig. 1a. The major band, tubes 68 to 108, upon recovery yielded 330 mg. The majority of this fraction (305 mg.) was redistributed in the 1000 tube machine by recycling to 3550 transfers as shown in the pattern of Fig. 1b. From tube 1401 to 1490, 210 mg. of almost pure ribonuclease A could be recovered. This material containing 7.2% moisture had an optical density value at 280 mμ of 0.62 for C = 1 mg./ml. in water and a ninhydrin color yield of 0.60 μmole leucine equivalents per mg. Using the spectrophotometric assay of ribonuclease,[10] the specific activity of this fraction showed no significant increase as compared to the starting material. The yield from tubes 1301–1352 was 8 mg., from 1353–1400 was 17 mg., from tubes 1491–1525 was 16 mg. and from tubes 1526–1550 was 5 mg.

Titrimetric assays with cyclic 2′,3′-cytidylic acid of the major fraction, kindly performed by Dr. M. Uziel, indicated that the specific activity of this sample was the same as that of ribonuclease A prepared by column chromatography and was 15% higher than the starting material.

After correcting for approximately a 10% loss of material due to manipulations during each stage of purification, it could be estimated that the original sample contained about 70% of ribonuclease A by weight.

A sample of lysozyme weighing 150 mg. was used to obtain the distribution pattern in the 1000 tube machine as shown in Fig. 3a. After 1000 transfers an effluent series was collected until 1390 transfers had been reached. In the effluent series from tube 1160 to 1190 there was a visible amount of precipitate. The cut of tubes 515 to 575 gave 95 mg. of white fluffy solid upon recovery. The redistribution pattern of this fraction (75 mg.) at 3420 transfers obtained by recycling in the 1000 tube machine is shown in Fig. 3b. The cut of tubes 1290 to 1365 gave 50 mg. of pure lysozyme A. After correcting for loss during recovery, the original sample could be estimated to contain 65% of lysozyme A by weight. The pure recovered lysozyme containing 7.1% moisture, has an optical density value at 280 mμ of 2.5 for C = 1 mg./ml. in water and a ninhydrin color yield of 0.34 μmole leucine equivalents per mg. Its lytic activity assayed according to the procedure given by Tallan and Stein[8] showed no significant increase in specific activity over that previously reported. The sample of dried *Micrococcus lysodeikticus* was kindly supplied by Dr. H. H. Tallan.

(10) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1940).

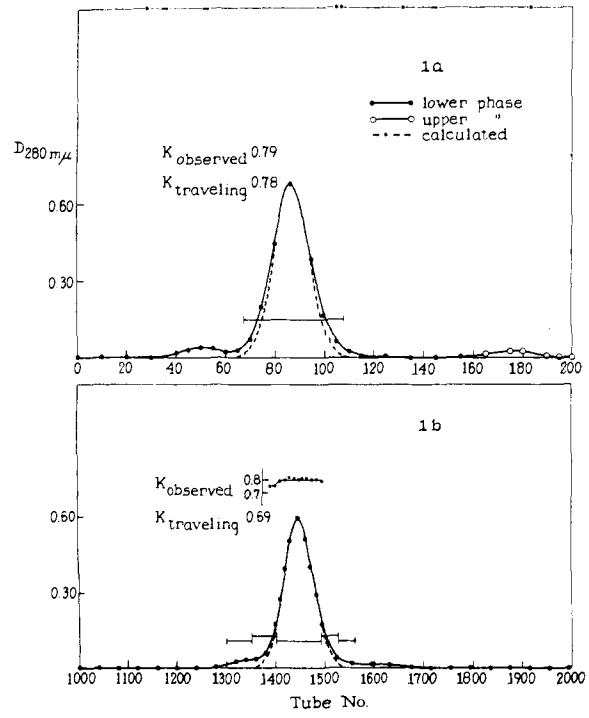

Fig. 1.—Distribution patterns of ribonuclease: (a) Armour sample after 196 transfers; (b) fraction 68–108 after 3550 transfers.

Chromatographic analyses of ribonuclease and lysozyme were done following the procedures of Hirs, Moore and Stein[6] and Tallan and Stein,[8] as shown in Figs. 2 and 4.

Discussion

Wherever possible in countercurrent distribution it is advisable to choose systems which will permit weight analysis. Partly for this reason the earlier part of this study was concerned with a study of the effect of volatile complexing agents such as trichloroacetic acid. Several recent findings seemed to indicate that the fluorinated fatty acids form even more tightly bound complexes with proteins[11] and have a pronounced solvent effect.[12] It seemed advisable to investigate their effect in systems suitable for countercurrent distribution.

Perfluorobutyric acid (PFBA) was found to have a strong partitioning effect for ribonuclease in the *sec*-butyl alcohol system, but like trichloroacetic acid with insulin the partition ratio value was strongly dependent on the concentration of the acid. A concentration of 0.0093 *M* PFBA was found to give a reasonable K for ribonuclease. However, after 200 transfers, analysis revealed two major bands, one band containing about one third of the sample with a partition ratio of 0 and another broad band containing two thirds of the sample with traveling partition ratio near 6. When the distribution was carried out in a system containing 0.0075 *M* PFBA, the same two bands resulted but the relative amount of the two bands was altered. These results indicated that some type of transformation was taking place.

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(12) A. L. Koch, W. A. Lamont and J. J. Katz, *Arch. Biochem. Biophys.*, **63**, 106 (1956).

In the case of chymotrypsinogen in this type of system it was noted that a satisfactory K could be obtained immediately after mixing the components but that there was a progressive shift of K on standing. This shift of K could be reduced or prevented by adding sufficient acetic acid to the system. Thus in a system made by equilibrating 2-butanol with an aqueous solution, 0.00014 M with respect to PFBA and 0.5 M with respect to acetic acid, a sample of chymotrypsinogen gave one well defined band with a K of 0.7 after 200 transfers. The position of the band was in good agreement with the observed partition ratio values across the band, indicating stability during the run. However, attempts to recover the protein free of the complexing agent have not thus far been successful. During the process of removing the 2-butanol by evaporation in the rotary evaporator the protein complex separated as needles which had little or no solubility in water, 0.1 N sulfuric acid or 0.1 N sodium hydroxide. It is possible that the problem here is that of learning the proper conditions for removal of the PFBA.

In the above case it is further possible that the acetic acid has acted as a stabilizer against denaturation just as caprylate ions or acetyltryptophan[5,13] was found to do for serum albumin. Another example of this type is the stabilization of ribonuclease against reversible denaturation in 8 M urea by phosphate, arsenate and sulfate ions in the order of their decreasing effectiveness.[14]

With ribonuclease a similar system but with a much higher concentration of PFBA (0.0093 M PFBA and 0.5 M acetic acid), only one major band with a traveling K of about 3 was obtained. However, the band was much too broad for a single solute and K determinations across the band revealed discrepancies. Furthermore, ribonuclease recovered from the band revealed gross inhomogeneity by the ion-exchange column.[6]

An attempt to use the "salting out" phenomenon in order to achieve partition was much more promising for ribonuclease. Systems of this type have been extensively used by Porter.[15] Herbert and Pinsent[16] used a two phase system made from a concentrated ammonium sulfate solution and ethanol for the preliminary purification of catalase from the cell lysates of *M. lysodeikticus*. This bacterial catalase with a reported molecular weight of 230,000 probably is the largest protein ever purified by liquid–liquid partition. In our own work when a mixture of ethanol, water and ammonium sulfate in a weight ratio of 25.9:57.6:16.5, respectively, was chosen for the system, it was found that the protein ribonuclease gave a partition ratio of 0.8 and behaved almost as an ideal solute as far as the partition isotherm is concerned.

The distribution pattern of ribonuclease in this system at 196 transfers is shown in Fig. 1a. Here the major band contained 87% of the total optical density units used but showed apparent deviation from the theoretical curve. The recovered material was found chromatographically to contain 90% of ribonuclease A in contrast to the starting material which contained 72% of component A.

Redistribution of the recovered material to 3550 transfers is shown in Fig. 1b. For easier comparison this pattern was plotted on an abscissa much reduced from that used in pattern 1a, a recommended procedure to show the width of a distribution curve relative to the increasing number of transfers applied.[17] The close agreement between the curve of the major component and the theoretical curve is apparent. The major component totaled 81% of the optical density units used for this second distribution, and it may be estimated that the original sample approximated 73% of the A component. This same value is obtained from the weight of sample recovered.

Several cuts were taken from this distribution and examined chromatographically (Fig. 2). The center cut contained 88% of the material under the theoretical curve, but it was revealed by chromatography that a small amount, not more than 3% of the total ninhydrin color, traveled in front of component A. This was due to the overlapping bands as evidenced by the chromatograms of the neighboring cuts. If a smaller cut (about 70% of the band) had been taken, even the small amount of impurity could have been greatly reduced.

Comparison of the various chromatograms in Fig. 2 confirms the steps of purification. The top chart, a, is the original sample. The second, b, shows the purification reached on the first stage, Fig. 1a. The third, c, shows the first cut on the left of Fig. 1b to be mainly the small impurity at the base and to the right of the main component in 2a but to have also another component too far to the left to be the main component of ribonuclease. The fourth, 2d, shows the second cut from the left in 1b to be mostly the main band but also partly the second component in the above fraction. A similar result is given in 2f for the fourth cut of 1b. The last cut of 1b is shown by 2g to be a mixture of two components and free of the main component. By contrasting the results of the column and C.C.D., it seems clear that the preparation contains six minor components in addition to the main one present in approximately 72%.

The chromatography of crystalline ribonuclease[6,15] has been found to give, in addition to component A, another enzymatically active minor peak. Tanford and Hauenstein[18] had isolated this material from a different Armour lot which was rich in this material and studied its physical properties. They concluded that the only difference between this material and component A is in its amide content.

Hakim[19] has reported that these two ribonuclease fractions obtained by chromatography possessed different specificities for the hydrolysis of cyclic cytidylic and guanylic acids. A closer study of

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(14) M. Sela and C. B. Anfinsen, *Biochem. Biophys. Acta*, **24**, 229 (1957).

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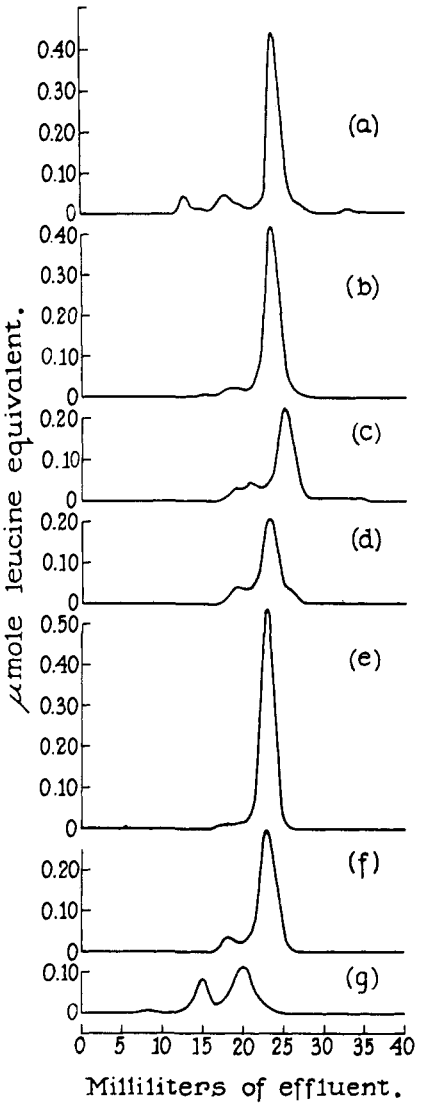

Fig. 2.—Chromatography of ribonuclease samples: (a) Armour sample; (b) fraction 68–108 in Fig. 1a; (c) fraction 1301–1351 in Fig. 1b; (d) fraction 1352–1400 in Fig. 1b; (e) fraction 1401–1490 in Fig. 1b; (f) fraction 1491–1525 in Fig. 1b; (g) fraction 1526–1550 in Fig. 1b.

the specificities of the fractions obtained by C.C.D. will be of interest.

The distribution of lysozyme was studied in a similar manner. A sample of lysozyme containing 91% of the A component as determined by column chromatography gave a distribution pattern at 1390 transfers shown in Fig. 3a. Under the theoretical curve there was 92% of the total optical density units used. Its center cut upon redistribution to 3420 transfers gave close fit to a theoretical distribution. Material recovered from a cut representing 86% of the band gave on column chromatography a single peak in the position of lysozyme A (Fig. 4c), but its lytic activity showed no significant increase when compared to the starting material.

From the experience reported in this paper it would appear that the inherent selectivity of this system is not particularly high since large numbers

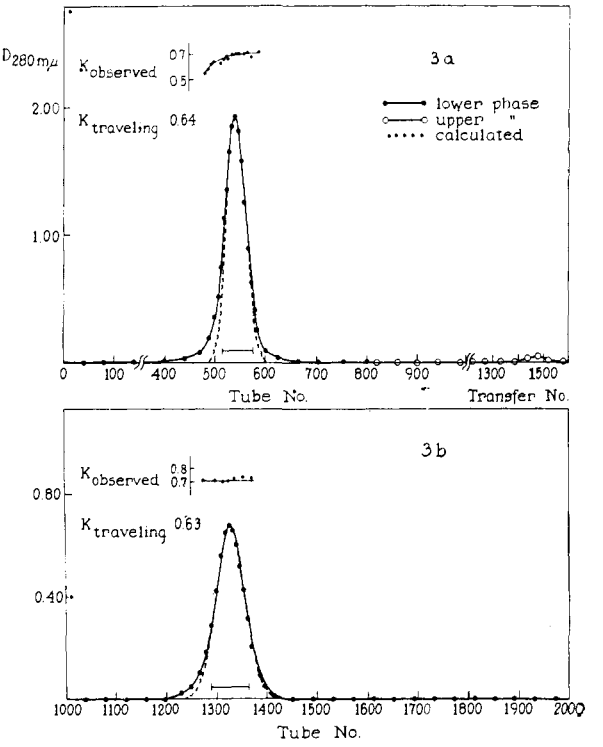

Fig. 3.—Distribution patterns of lysozyme: (a) Armour sample after 1390 transfers; (b) fraction 515–575 after 3420 transfers.

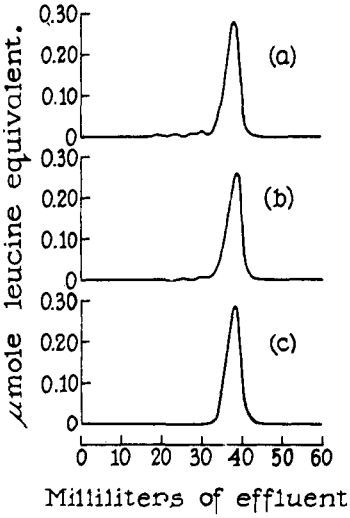

Fig. 4.—Chromatography of lysozyme samples: (a) Armour sample; (b) fraction 515–575 in Fig. 3a; (c) fraction 1291–1364 in Fig. 3b.

of transfers are required to separate the components of the proteins studied. Possibly by using this mixture with its composition further removed from the critical point of the phase diagram, a more selective system might be obtained. Adjustment of the *p*H of the system did not bring an increased selectivity for the fractionation of ribonuclease. The K of ribonuclease A increased to 1.5 in this system at *p*H 7.2 and remained the same at *p*H 3.4 with an accompanying decrease in solubility of the protein. However, the apparent lack of specificity

is offset by the good adherence to ideality, thus permitting high numbers of transfers to be applied.

In this system both chymotrypsinogen and bovine serum albumin had K values of infinity. By addition of varying amounts of 1-propanol to the system, useful K's could be obtained with both proteins. With bovine serum albumin in the system of ethanol, 1-propanol, ammonium sulfate solution (40 g. in 100 ml. of water), water with volume ratios of 1.1:0.9:2:2, a K value of 1.6 was obtained; however, on standing, the K value gradually decreased. This shift was prevented by substitution of 0.01 N sodium caprylate in place of water in the above system; sodium caprylate was originally discovered to be a stabilizing agent for serum albumins against heat or urea denaturation.[13] Preliminary results obtained in this Laboratory showed promise for the fractionation of serum albumins in this solvent system.

With chymotrypsinogen in the system containing ethanol, 1-propanol, ammonium sulfate solution and water with volume ratio of 1.5:0.5:2.5:1.5, a K value of 1 was also obtained. On standing, precipitation took place and the precipitate was found to be no longer soluble in water, an indication that the cause was denaturation rather than the solubility of the protein in the system. Substitution of ethyl cellosolve for ethanol did not prevent precipitation though this solvent was successfully used by Porter[15] for the partition chromatography of chymotrypsinogen.

While it is an often observed fact that most proteins are denatured readily by organic solvents at room temperature, we feel that it is worthwhile to search for agents that will modify the labile nature of proteins. Operationwise it is easier to carry out a fractionation at room temperature than at lower temperatures. Our attempt to find a stabilizer for chymotrypsinogen in the system described above has thus far been unsuccessful. Among the compounds tried have been caprylic acid, mandelic acid, glycine, cholesterol, sodium citrate, phosphates and β-phenylpropionic acid, a known reversible inhibitor for the chymotrypsins. Work along these lines is being continued.

Acknowledgments.—The technical assistance of Mrs. Judith O'Brien and Miss Gerty Walker is gratefully acknowledged. We also wish to thank Dr. Mayo Uziel for helpful discussions on ion-exchange chromatography.

NEW YORK 21, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLUMBIA UNIVERSITY, COLLEGE OF PHYSICIANS AND SURGEONS]

A Comparison of the Rate of Mutarotation and O^{18} Exchange of Glucose[1]

BY D. RITTENBERG AND CHARLES GRAFF[2]

RECEIVED FEBRUARY 6, 1958

The effect of pH on the exchange reaction between the aldehyde oxygen of glucose and the oxygen of water has been measured. The rate is at a minimum at pH 4 and rises rapidly below pH 3 and above pH 6.

The exchange reaction between aldehydic oxygen atoms and the oxygen atom of water has long been known, and various aspects of this phenomenon have been reported. Cohn and Urey[3] demonstrated that acetone readily exchanges its oxygen with that of water, and Titani and Goto[4] have shown that glucose exchanges one-sixth of its oxygen, presumably the oxygen atom on carbon atom one, with the solvent, water. During the course of another investigation we observed that the rate of exchange between glucose-1-O^{18} and water was erratic when the pH was uncontrolled. Cohn and Urey had shown in the case of acetone that the exchange reaction is both acid and base catalyzed. Accordingly, we undertook a study of the effect of pH on the rate of the exchange reaction between glucose-1-O^{18} and water. We hoped, further, that a determination of the glucose–water exchange kinetics might permit the re-evaluation of existing theories as to the mechanism of glucose mutarotation.

Experimental

The rate of exchange reaction was followed in the system normal water–glucose-1-O^{18}.

Glucose-1-O^{18}.—Glucose-1-O^{18} was prepared by heating a solution of glucose containing 29.2 atom per cent. excess O^{18} water for 18 hours at 100°. The water was removed by distillation *in vacuo* and the sirupy residue further dried over fresh P_2O_5. The anhydrous sirup was dissolved in a minimum of normal water and alcohol added. Scratching of the cold solution induced crystallization after some hours. The crystalline glucose was dried and analyzed for O^{18}.[5] It contained 4.12 ± 0.03 atom per cent. excess; somewhat less than one-sixth of the isotope concentration of the water. Another sample of glucose was prepared by this procedure. It contained 3.51 ± 0.03 atom per cent. excess O^{18}.

To determine the rate of the exchange about 18 mg. of glucose-O^{18} (0.1 mM) was weighted into a tube shown in Fig. 1A and then about 90 mg. of a buffer solution (5 mM) carefully added. The tube was sealed off and kept in a thermostat for a known time after which it was cooled in Dry Ice–alcohol to stop the reaction. The frozen sample tube was transferred to a vacuum system in which the water from the glucose solution could be distilled to another tube similar to that shown in Fig. 1A; CO_2 was added to a pressure of 10 cm. (~0.02 mM), the tube sealed off and kept at 100° for 3 hours to equilibrate the O^{18} concentration in the CO_2 and the water. Separate tests showed equilibration to be complete in this period. The tube was transferred to

(1) This work was supported by contracts to Columbia University from the Office of Naval Research, Department of the Navy (ONR 26602), from the Atomic Energy Commisstion (AT (30-1) 1803) and from the National Institutes of Health (E-1461). Reproduction of this article in whole or in part is permitted for any purpose of the United States Government.

(2) Holder of a U.S.P.H.S. Medical Student Part Time Research Fellowship of the National Institutes of Health.

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(4) Toshizo Titani and Kokiti Goto, *Proc. Imp. Acad. Tokyo*, **15**, 298 (1940); **16**, 398 (1940).

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