[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

The Kinetics of Hydrolysis of Ribonucleic Acid

BY JAMES E. BACHER[1] AND WALTER KAUZMANN

RECEIVED FEBRUARY 25, 1952

The non-enzymic hydrolysis of ribonucleic acid from yeast has been studied under a variety of conditions of *p*H, temperature and ionic strength. The titration curves of the hydrolysates and in some instances the contents of inorganic phosphate were determined. The content of secondary phosphate groups in each hydrolysate was found from the titration curve. From these data the kinetics of liberation of secondary phosphate groups were obtained. The entire content of diesterified phosphate hydrolyzes according to a first-order rate law under some but not all conditions. The salt effect and the dependence on the concentration of acid or alkali were examined. In acidic solution there is little or no salt effect, and the rate of hydrolysis is proportional to a power of the concentration of acid which is between one and two. The rate of hydrolysis in alkaline solution at constant ionic strength is proportional to the concentration of alkali. The salt effect in alkaline solution is compatible with the Brønsted relation for a reaction between two monovalent ions of the same sign. A catalytic effect by magnesium ion is obtained at *p*H 6 and 10. The mean enolic dissociation constant increases during hydrolysis, but the total titer of enolic groups in the samples does not change. The rate of change of the mean enolic dissociation constant is considerably greater than the rate of liberation of secondary phosphate groups under any condition of hydrolysis. Loss of acid precipitability during hydrolysis is more closely related to the shift in enolic *pK* than to the liberation of secondary phosphate groups. The nature of the internucleotide bonds is considered in relation to these results. The kinetics of hydrolysis of adenylic and cytidylic acids to 0.5 *N* potassium hydroxide were also examined.

The kinetics of hydrolysis of phosphate from ribonucleic acids and from nucleotides has been examined in some detail.[2] In acidic solution, the phosphate is hydrolyzed rapidly from the purine nucleotides and slowly from the pyrimidine nucleotides. A slight induction period was found in the liberation of phosphate. The reaction, diester phosphate–monoester phosphate, must contribute to that induction period. The general facts of the breakdown of the diester bonds have been known for a long time, namely, that ribonucleic acid is very unstable in alkali and unstable in acid, whereas most of the phosphate linkages of desoxynucleic acid are stable in alkali and unstable in acid. However, the actual kinetics of the liberation of the titratable secondary phosphate groups during the hydrolysis of nucleic acid have not been examined. If the hydrolysis of all of the secondary phosphate groups were to obey the same rate law under many conditions, it would seem reasonable to believe that all of the internucleotide linkages are of the same type.

Experimental

Three materials were used. One was purified from Schwarz ribonucleic acid from yeast, while the others were isolated from fresh baker's yeast.

Purification of Schwarz Ribonucleic Acid.—Twenty-five grams of RNA was suspended in ice-water and was dissolved by the slow addition of sodium hydroxide. The solution was deproteinized by the chloroform-gel method of Sevag, Lackman and Smolens,[3] and then was dialyzed at 5° for 60 hours. Seven grams of magnesium chloride was added, and ethyl alcohol was added to 8 vol. per cent. A small amount of dark precipitate was filtered off and discarded. The filtrate was adjusted to 35% alcohol, and the precipitate was filtered off. The filtrate contained 1 g. of nucleic acid per l., and was discarded. The precipitate was dissolved in 300 ml. of water, 5 g. of magnesium chloride and 200 ml. of alcohol were added and the precipitate was filtered off, redissolved, reprecipitated and then filtered. The precipitate was washed with increasingly concentrated alcoholic solutions, then with ether. The preparation is a white powder and has less than 2% protein by the biuret and Sakaguchi tests; recovery, 10 g.

Anal. N, 13.8; P, 8.4; labile phosphate,[2] 49% of the total phosphate.

The protein of the commercial material can be removed during the fractionation with magnesium chloride and alcohol. A neutral solution which is 4% in each of magnesium chloride and nucleate is brought to 8 volume per cent. in alcohol and the resulting precipitate is filtered off. The filtrate is brought to 35 volume per cent. in alcohol and the resulting precipitate is removed. Magnesium chloride is added to a 4% solution of the second precipitate and the process is repeated two or three times. The precipitates removed from the 8% alcoholic solutions can be reworked to increase the yield appreciably. These precipitates contain the protein and the pigment. Most of the dialyzable fragments of the commercial material are removed in the 35% alcoholic filtrates. The initial precipitate from the 8% alcoholic solution settles peculiarly into two distinct layers, the bottom layer being dark and the top layer quite light in color. The nitrogen and phosphate concentrations of the dried materials from the two layers are practically the same.

Isolation of RNA from Fresh Yeast.—It will be shown that magnesium ions interfere with the titration of nucleic acid and also exercise a catalytic effect on the hydrolysis at *p*H 10. These effects are discussed below. It was necessary to prepare nucleate free of magnesium. Present and previous[4] work shows that magnesium ion is tightly complexed by ribonucleate and that ribonucleate isolated by various procedures from yeast contains magnesium. The following procedure yields potassium nucleate free of magnesium.

Two pounds of fresh baker's yeast are defatted and then extracted with a 10% solution of sodium chloride according to the method of Clarke and Schryver.[5] This method of extraction was adopted because of the relatively high molecular weights obtained on such material[6] compared with those obtained for Schwarz RNA.[7] The extract is centrifuged. The supernatant liquid is chilled, and is adjusted to *p*H 1 with hydrochloric acid. The precipitate is centrifuged immediately and is ground into a solution of potassium acetate. Two volumes of alcohol are added and the resulting precipitate is dissolved in water. The steps starting with the precipitation by acid are repeated. The preparation has a few per cent. of protein, which is removed by the method of Sevag, *et al.*[3] The solution of potassium nucleate is finally dialyzed and lyophilized; yield 0.8 g.

Anal. N, 13.0; P, 7.7; labile phosphate,[2] 48% of the total phosphate.

Another preparation was obtained, based on the work of Henry and Stacey.[3] Two pounds of fresh baker's yeast was extracted on a rocking table with 800 ml. of water containing 25 g. of sodium lauryl sulfate for 24 hours. The

(1) College of Pharmacy, University of California Medical Center, San Francisco, Calif. Public Health Service Research Fellow of the National Institutes of Health, 1950-1952.

(2) For references, see J. E. Bacher and F. W. Allen, *J. Biol. Chem.*, **182**, 701 (1950).

(3) M. G. Sevag, D. B. Lackman and J. Smolens, *ibid.*, **124**, 425 (1938).

(4) C. Neuberg and I. S. Roberts, *Arch. Biochem.*, **20**, 185 (1949); H. Henry and M. Stacey, *Proc. Roy. Soc. (London)*, **133B**, 391 (1946); G. Junger, *Science*, **143**, 378 (1951).

(5) G. Clarke and S. B. Schryver, *Biochem. J.*, **11**, 319 (1917).

(6) I. Watanabe and K. Iso, THIS JOURNAL, **72**, 4836 (1950).

(7) J. E. Bacher and F. W. Allen, *J. Biol. Chem.*, **184**, 511 (1950).

suspension was centrifuged, and the optical density of the supernatant liquid was measured from 240 to 280 mμ. There were approximately 6 g. of the constituents of nucleic acid present in the extract, assuming a molar extinction coefficient of 10^4. Two volumes of alcohol were added. Only 15% of the ultraviolet-absorbing material was precipitated. This fact is in contrast to results obtained on saline extracts; although these contain considerably less ultraviolet-absorbing material, a much greater proportion of that material is precipitated by either alcohol or by acid than is precipitated from the detergent extracts. The net yields of nucleic acid have been about equal for the two procedures, and account for only a few per cent. of the total ultraviolet-absorbing material of the yeast.

The precipitate obtained by adding alcohol to the detergent extract is extracted with cold water and reprecipitated with two volumes of alcohol. When this precipitate is extracted with water it yields $^2/_3$ of the ultraviolet-absorbing material of the original precipitate. The solution is deproteinized by the method of Sevag, *et al.*,[3] then acidified and immediately centrifuged. The precipitate is dispersed in a solution of potassium acetate, which is then clarified by centrifugation. Two volumes of alcohol are added, and the precipitate is washed with increasingly alcoholic solutions and finally with ether; yield 0.3 g.

Anal. N, 13.2; P, 7.54; labile phosphate,[2] 49% of the total phosphate.

In agreement with the observations of Sevag and Smolens,[8] the ultraviolet-absorbing material of extracts is largely precipitated by barium or calcium chloride if it has been exposed to acid or to alkali. Otherwise, only a minor portion of the ultraviolet-absorbing material is precipitated by those salts. It is believed that the isolation and purification techniques generally employed actually fractionate the nucleic acids present in the cells, and that there are many molecular species of nucleic acid in a cell. It is consequently believed that analysis for the individual constituents of nucleic acid made on purified samples of nucleic acid do not necessarily represent the average composition of the ribonucleic acids of the tissue of origin.

The preparations were examined for their content of DNA by Dische's diphenylamine method.[9] The purified Schwarz nucleic acid gave a color equivalent to 0.6% DNA, and the others were less than 3% DNA.

Titrations of the hydrolysates were made in two ways, by titration with standard hydrochloric acid or potassium hydroxide, and by electrolytic generation of acid or base in the titration vessel. Potassium hydroxide containing less than 0.2% carbonate was obtained by a modification of Kolthoff's lime method.[10] The suspension of lime in potassium hydroxide solution is shaken for a few days before allowing it to settle, instead of 1 hour as recommended. Hard rubber or paraffined vessels were used since traces of silicate interfere in the colorimetric phosphate determination.[11]

For the coulometric titrations, a platinum wire was used as the electrode for the generation of acid or base. A bridge of agar in saturated potassium sulfate connected the solution being titrated to a copper electrode in dilute sulfuric acid. The current was delivered by five C-batteries, giving 225 volts. Current was measured by a milliammeter. One pole of a double pole switch was in the electrolytic circuit, while the other pole was connected to a timer which read to 0.1 second. The amount of reagent generated is proportional to the integral of the current times its duration, and therefore is proportional to the duration if the current is held constant. This is achieved by means of a 25,000 ohm, 100-watt resistance and a 4000-ohm slide wire resistor in series with the electrolytic cell. The resistance between the electrodes was 3 to 5 thousand ohms, depending on the salt concentration of the solution being titrated. Since the conductances of the solutions varied appreciably with pH, the slide wire resistor was used to maintain constant current during a titration. The current was generally about 7 milliamperes.

A Beckman model G pH meter was used for the pH measurements. The solutions were stirred magnetically during the titrations. The three electrodes and the salt bridge were mounted in a rubber stopper which sealed the titration vessel.

Aliquots of standard acid or base were titrated coulometrically with an accuracy of 0.5%.

For the acidic and alkaline hydrolyses, double-limbed tubes were used to preheat the reagents before mixing. To stop the reaction, the tubes were placed in ice-water and neutralized as quickly as possible. The hydrolysates were transferred to the titration vessel, and the volumes were adjusted to 8.0 ml. for the coulometric titrations, 17 ml. for titrations by a microburet. The samples of nucleic acid were 7 to 10 mg. in the former case, 30 to 50 mg. in the latter.

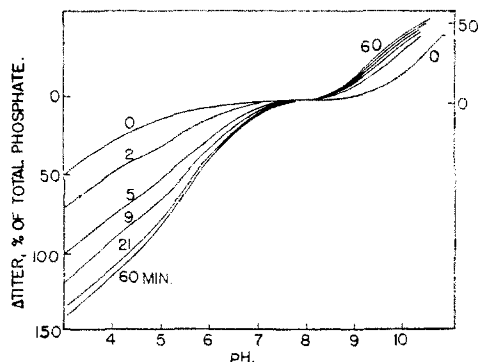


Fig. 1.—Titration curves of hydrolysates of magnesium nucleate in 0.48 N potassium hydroxide at 60°.

(8) M. G. Sevag and J. Smolens, *J. Biol. Chem.*, **140**, 833 (1941).
(9) Z. Dische, *Mikrochemie*, **8**, 4 (1930).
(10) I. M. Kolthoff and V. A. Stenger, "Volumetric Analysis," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1947.
(11) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

Results

Hydrolysis in Acid or Alkali.—Figure 1 shows a nest of titration curves obtained by hydrolysis in 0.48 N potassium hydroxide at 60.0°. The curves are zeroed at the only distinct inflection point, which occurs at pH 7.8, between the regions of the secondary phosphate groups and of the enolic groups. If the pK's of the titratable groups in the original nucleic acid do not shift as the hydrolysis proceeds and if the pK's of the liberated groups are constant during the hydrolysis, then the rate of change of titer at constant pH measures the rate of reaction. The difference between the titration curve of the original nucleic acid and that of a hydrolysate is the titration curve of the liberated groups unless there are consecutive reactions in which a titratable group is first formed, then modified or destroyed. In the sequence of reactions diester phosphate → monoester phosphate → inorganic phosphate, a titratable secondary phosphate group is first formed, and then its pK is shifted from 5.7 to 6.8. Under some conditions the concentration of monoester reaches a steady state and the difference curve will only show the formation of inorganic phosphate. If successive difference curves remain proportional along the axis of titer for all pH, then the pK's of the titratable groups are probably constant during the hydrolysis.

In 0.5 N potassium hydroxide the release of secondary phosphate groups is about 600 times faster than the formation of inorganic phosphate. In 0.5 N hydrochloric acid the ratio of rates is about 15. In the pH region 4 to 6 the rates are approximately equal and the difference curves no longer correspond to a single ionization constant. Furthermore, although the difference curves obtained from the alkaline hydrolysis (Fig. 2) level off below pH 4, those obtained under other conditions do not quite level off here. Consequently the clearest interpretation can be obtained from hydrolysis in alkaline solution. Figure 2 shows the difference curves obtained from Fig. 1. The curves fit the titration curves computed for a group whose pK is 5.7. If the titratable secondary phosphate groups of the original nucleic acid also have pK 5.7, then the amount of such groups is given by twice the titer at pH 5.7. The original magnesium nucleate has 15% of its total phosphate in monoester form while the potassium nucleate has 10%.

A rate curve is obtained by plotting change of titer at constant pH, measured from the titration curve of the original nucleic acid, *vs.* time. The data are fitted to a first-order curve by trial and error, adjusting both the half-life and the total amount of reaction. Since it is an average

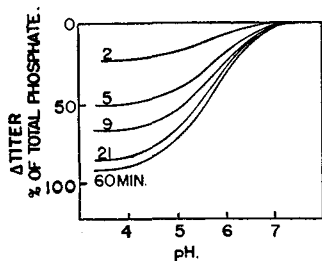


Fig. 2.—Difference curves obtained from Fig. 1.

from several titrations, the value so obtained for the total amount of reaction is accepted as a more reliable estimate than that obtained from the final difference curve. Figure 3 shows first-order rate curves fitted to the data of five hydrolyses in acid and alkaline solutions. The asymptotic values range from 85 to 90% of the total phosphate of magnesium nucleate. This, together with the monoester originally present accounted for 100 to 105% of the total phosphate. In Fig. 3 each curve has a separate origin on the scale of ordinate so that the curves do not overlap on the graph. Each point on a rate curve represents the change of titer above the corresponding zero-time point, and the changes of titer are given by difference curves such as are shown in Fig. 2. It is seen that the first two or three points of each curve, excluding the zero-time point, extrapolate to the zero-time point. Consequently, there is no evidence for either an induction period or for a small fraction of phosphate groups more labile than the rest under these conditions of hydrolysis. In the former case the extrapolation would be to a point on the scale of ordinate below the zero-time point, and in the latter case the extrapolation would be above the zero-time point. The liberation of secondary phosphate groups seems to be kinetically uniform under the conditions of Fig. 3.

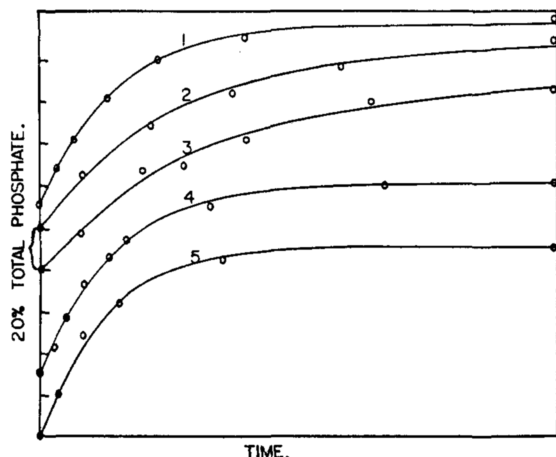


Fig. 3.—Hydrolysis of magnesium nucleate. Ordinate is the change in titer at *p*H 4.0. One scale division equals 20% of the total phosphate: curve 1, 0.48 *N* HCl at 100°, full time scale = 15 min.; curve 2, 0.48 *N* HCl at 60°, 120 min.; curve 3, 0.19 *N* KOH at 70°, 25 min.; curve 4, 0.49 *N* KOH at 60°, 60 min.; curve 5, 0.48 *N* KOH at 60°, 60 min.

Cavalieri[12] recently showed that in 0.4 *N* alkali at 37°, the mononucleotides are liberated from Schwarz ribonucleic acid in a manner which required at least two rate constants for each mononucleotide, although the four pairs of constants were similar. Magnesium nucleate was hydrolyzed at 35° and at 45° in 0.5 *N* potassium hydroxide; the results are shown in Fig. 4. It is seen that at these lower temperatures there is a segregation into two rates, since the data do not fit simple first-order curves.

Ammonia is slowly liberated during the hydrolysis of nucleic acid. The effect on the titration curves can, however, be neglected, since in 0.5 *N* alkali at 100° the amount released increases nearly linearly with time, with 11% of the total nitrogen being liberated in 200 minutes. In 0.5 *N* acid at 100°, 1.3% is liberated in 150 minutes, and at *p*H 6 and 100°, 3.4% is liberated in 60 hours.

(12) L. F. Cavalieri, THIS JOURNAL, 73, 4899 (1951).

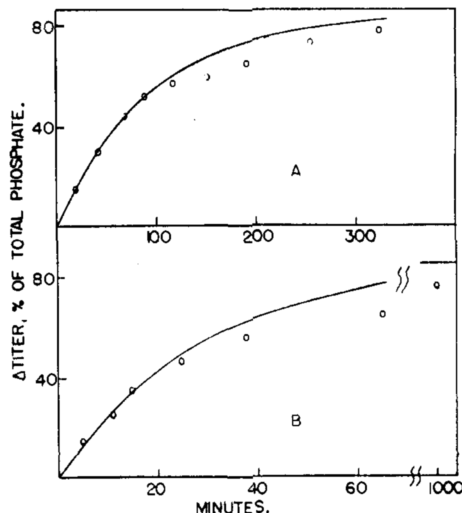


Fig. 4.—Hydrolysis of magnesium nucleate in 0.50 *N* potassium hydroxide (A) at 35.0° and (B) at 45.0°. Total reactant is 85% of the total phosphate. First order curves are constructed to fit the initial rate.

Hydrolysis at *p*H 2.—The secondary phosphate groups are definitely not liberated according to the first-order rate law during hydrolysis in 0.010 *N* acid. These results are presented in Fig. 5. There is a partition into at least two phosphate fractions. Furthermore the two samples of nucleic acid have different ratios for the fraction of the phosphate which hydrolyzes rapidly to the fraction which hydrolyzes slowly. This ratio for the magnesium nucleate is also different from the ratio obtained in the low temperature, alkaline hydrolyses described in the preceding section.

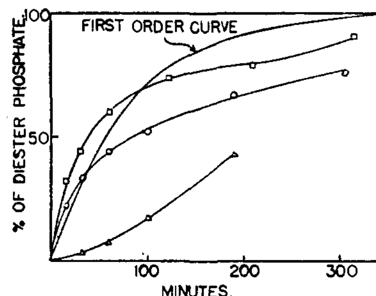


Fig. 5.—Rate of formation of titratable secondary phosphate groups in 0.01 *N* hydrochloric acid at 100°: from potassium nucleate, □; from magnesium nucleate, O. Liberation of inorganic phosphate from magnesium nucleate, Δ. A first-order curve is shown for comparison.

The *p*H of these hydrolysates at *p*H 2 falls a few hundredths of a unit during the course of the reaction. This effect should increase the values of the specific rate constants if they are changed at all.

Hydrolysis at *p*H 4 and 6.—Under these conditions the *p*H drifts downward about 0.4 of a unit during unbuffered hydrolyses. The rates of hydrolysis at *p*H 4 and 6 are practically equal, however, so that exact buffering of the hydrolysates in this *p*H range is not necessary. The nucleic acid itself is an adequate buffer.

It is necessary to consider in detail the difference curves obtained from the titration of hydrolysates at *p*H 6. Figure 6 shows some examples. The mid-points of curves 1 and 2 occur at *p*H 6.5 and 6.8, which indicates that they represent principally inorganic phosphate. These indications were confirmed by the analyses for inorganic phosphate. Curve

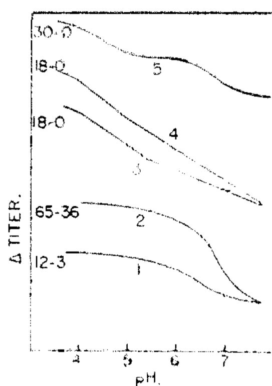


Fig. 6.—Difference curves from hydrolysates at *p*H 6 and 100°. The label *x*-*y* refers to the hours of hydrolysis of the two titration curves used to obtain the difference curve.

5, Fig. 6, shows an increase in inorganic phosphate and in some group which titrates around *p*H 4. Still another type of difference curve is illustrated by curves 3 and 4. These curves are believed to represent production of inorganic phosphate (*pK* 6.8), of monoester phosphate (*pK* 5.7), and of a group which titrates around *p*H 4. A group titrating at *p*H 4 also appears on prolonged alkaline or acidic hydrolysis. The group appears in an irregular manner, particularly at *p*H 6, and varies from sample to sample and from one run to another. The appearance of the group complicates the estimation of titratable secondary phosphate.

This group either may be a new group produced by the hydrolysis, or it may appear through a shift in the *pK* of a pre-existing group. If the latter were the case, the group in question would almost certainly have to be an amino group. Yet Fig. 2 shows that alkaline hydrolysis forms mononucleotides quantitatively without any great shift in the *pK*'s of their amino groups. The group titrating around *p*H 4 appears in the alkaline hydrolysates long after degradation to mononucleotides is complete, and the same is true to a lesser degree for acidic hydrolysates. Consequently it is believed that a new group is formed. It might arise from the oxidation of guanine or of the aldehydic group of ribose, or from the hydrolysis of the purine bases. It might be significant that after 3 or 4 days of hydrolysis at *p*H 6 the solutions became discolored.

It is believed that the slightly high recoveries of total secondary phosphate groups obtained in acidic or alkaline hydrolyses, as measured by the change of titer at *p*H 4.0, is a consequence of the relatively slow appearance of the new group. The liberation of secondary phosphate at *p*H 6 is particularly difficult to follow since the rate of formation of the new group is comparable to the rate of hydrolysis of diester phosphate. This is especially true of the hydrolysis of potassium nucleate, which is very slow.

Figure 7 shows the liberation of secondary phosphate as estimated from the change of titer at *p*H 5.0 or 5.2, and of inorganic phosphate at *p*H 6 and 100°. It is assumed that the unhydrolyzed nucleates had titratable secondary phosphate groups equal to 15% of the total phosphate, and the data for the release of secondary phosphate groups are plotted from the ordinate equal to 15% of the total phosphate as a base line. Then the vertical distance at any time between corresponding curves for secondary phosphate groups and for inorganic phosphate measures the amount of monoester phosphate present at that time.

Three conclusions emerge from the data of Fig. 7: (1) The average rate constant for the hydrolysis of all monoester phosphates is at least as great as the average rate constant for the hydrolysis of the diester phosphates of these hydrolysates.

(2) Small amounts of magnesium ion catalyze the hydrolysis of the diester phosphates. The ratio of the concentration of magnesium to that of total phosphate in different hydrolysates was varied from 0.5 to 1.5 without producing any further acceleration of the hydrolysis. Some of the hydrolysates of potassium nucleate contained added potassium sulfate; the rates which were obtained were the

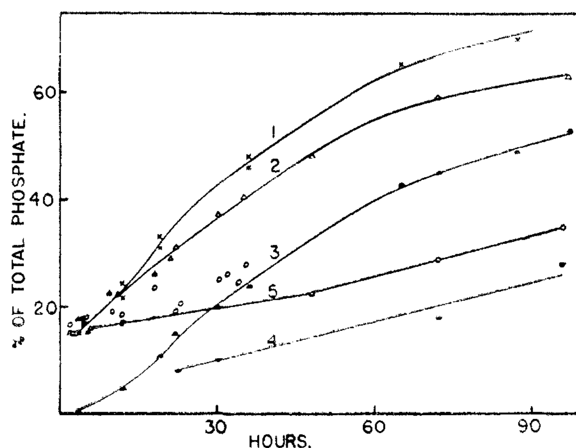


Fig. 7.—Hydrolyses at *p*H 6 and 100°. Secondary phosphate groups from magnesium nucleate, 1; from potassium nucleate in the presence of magnesium sulfate, 2; from potassium nucleate, 5. Inorganic phosphate from nucleate in the presence of magnesium ion, 3; from potassium nucleate, 4. Curve 5 is drawn through one run of the hydrolysis of potassium nucleate.

same as those without potassium sulfate. Consequently the increased rate of hydrolysis of potassium nucleate in the presence of magnesium sulfate is not due to sulfate ion or to the increase in ionic strength.

(3) There seems to be an induction period for the appearance at *p*H 6 of secondary phosphate groups from the magnesium nucleate prepared from commercial (Schwarz) ribonucleic acid. A definite induction period was obtained in duplicate runs at 100° (curve 1, Fig. 7), while two runs at 70° were inconclusive. The data for the hydrolysis of potassium nucleate, which was prepared from fresh yeast, does not seem to show any induction period in the presence of magnesium sulfate (curve 2, Fig. 7). The data for potassium nucleate are too scattered to permit any conclusions (curve 5, Fig. 7). It is possible that the difference between the two samples is fictitious, and is a result of difficulty in the interpretation of the titration curves.

Hydrolyses in Slightly Alkaline Media.—These hydrolyses were conducted in a jacketed titration vessel through which water at 70° was pumped. The rate of consumption of alkali to maintain constant *p*H was measured. The reaction was stopped after different lengths of time by cooling to 24°, and then the titration curves of the reaction mixtures were determined. The liberated secondary phosphate groups were estimated from the titration curves. They usually accounted for approximately one-half of the alkali required to maintain constant *p*H at 70°. The enol groups, which become more acidic during a hydrolysis, accounted for the remainder. The enol groups also become more acidic by approximately one *pK* unit during the temperature rise from 24 to 70°.

The Beckman No. 8990 electrodes are quite stable at 70°. In periods of 3 to 5 hours at 70° the calibration generally did not drift more than 0.05 *p*H unit. In one run of 36 hours duration the calibration drifted 0.2 *p*H unit. The system can therefore be maintained at constant *p*H at 70° for a few hours, then cooled and the titration curve can be determined at room temperature without removing the electrodes from the solution. Finally the meter can be recalibrated and the titration curve can be corrected for whatever slight drift in calibration may have occurred. The titration curve of nucleic acid at 70° can only be measured between *p*H 4 and 8, since outside of this range the hydrolysis of secondary phosphate groups is too rapid.

The results obtained can only be considered exploratory. Protection against carbon dioxide was incomplete, since blank hydrolysates, which were simply solutions of potassium sulfate adjusted to *p*H 10, consumed alkali. From the titration curves of the blanks, it appeared that some silicate was dissolved from the glass vessel. Figure 8 shows the rate of consumption of alkali at constant *p*H in the *p*H range 8.4 to 10.5, with the *p*H measured at 70°. The rate

increases with increasing pH, as does the rate of release of secondary phosphate groups, from pH 9.0 to 10.5.

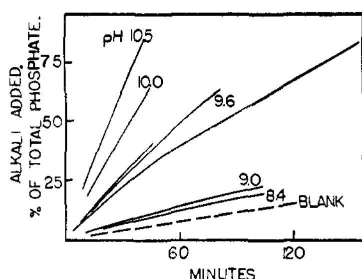


Fig. 8.—Consumption of alkali at constant pH and 70° by magnesium nucleate. Three curves are shown at pH 9.6.

The rate of release of secondary phosphate groups is much greater here than at pH 6. It is also much greater than would be expected from the results in alkali described above if the rate is proportional to the hydroxyl ion concentration. At pH 9.6, measured at 70°, the secondary phosphate appears to be liberated roughly according to a first-order law, with a rate constant of 0.4 hour^{-1}.

The influence of magnesium ion is shown in Fig. 9. Potassium nucleate was hydrolyzed alone and in the presence of magnesium sulfate. Magnesium definitely increases the rate of consumption of alkali and the rate of appearance of secondary phosphate groups.

Differential Effects.—The salt effect and the dependence on the concentration of catalyst were studied in approximately 0.1 N acid or base. The method of comparison is illustrated as follows. One hydrolysis tube contained 1 ml. of a solution of nucleate and 2 ml. of 0.17 N potassium hydroxide. A second tube contained 1 ml. of nucleate, 1 ml. of 0.17 N potassium hydroxide and 1 ml. of 0.17 M potassium chloride. A third tube contained 1 ml. of nucleate, 1 ml. of 0.17 N potassium hydroxide and 1 ml. of water. The first tube was hydrolyzed for one-half as long as the other two tubes. After hydrolysis the contents of all tubes were neutralized, adjusted to equal concentrations of salt, and then titrated. If there were no salt effect and if the rate were proportional to the concentration of alkali, then the three titration curves should be the same. The procedure was repeated for different extents of hydrolysis.

At constant ionic strength, the rate is found to be proportional to the concentration of hydroxide ion up to 0.3 N. At constant alkalinity and at ionic strengths up to 0.3, the variation of the rate with ionic strength (μ) is in satisfactory agreement with the Brønsted relation $\log (k/k_0) = z_a z_b \sqrt{\mu}$ with $z_a = z_b = 1$. Rates measured in 0.48 and in 0.81 N alkali were also proportional to the concentration of alkali. The foregoing information is compatible with a reaction between hydroxyl ion and a negatively charged phosphate ester.

The effects in acid are more complicated. At an ionic strength of 0.3, the rates in 0.05, 0.10 and 0.30 N acid are in the proportion 1:3.0:10.8. The rates per unit concentration of acid in 0.47 and 0.81 N acid have the ratio 1:2.1. The rate thus appears to vary more rapidly than the first power of the hydrogen ion concentration. In 0.04 N acid, the rate decreases with increasing ionic strength. However, the effect is much less than is required by the Brønsted theory. A salt effect was not detectable in 0.15 N acid.

The Effect of Hydrolysis on the Enol Groups.—The titration curves of Fig. 1 show pronounced changes in the titration of the enol groups as the hydrolysis proceeds. A complete study of these changes would have required titration of all hydrolysates to pH 12. The titrations were performed on solutions from 0.002 to 0.005 M in total nucleotide phosphate so that the titration of the solvent was relatively large above pH 11. Most of the titrations were therefore only extended to about pH 10.5. Nonetheless, rate data are obtainable because of the following considerations. If the total amount of titratable enol groups does not change during hydrolysis, and if the only change is a shift in the pK of the enol groups during the hydrolysis, then the rate of change of pH at constant titer above the inflection point measures the rate of change of pK. In the ideal case, then, the titration curve of the enol groups would move parallel

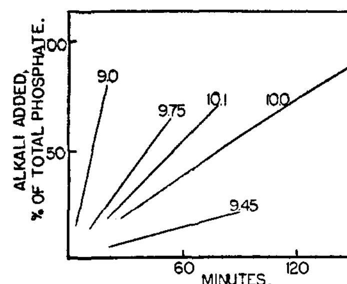


Fig. 9.—Consumption of alkali at 70° by solutions of potassium nucleate. The pH of each run was maintained at the constant value shown by each curve in the graph. The samples contained 16.4 μmole phosphate. The solution at pH 9.75 also contained 29 μmole magnesium sulfate, while the solution at pH 9.0 contained 290 μmole magnesium sulfate.

to the axis of pH without any change in size or shape. Wiener, *et al.*,[13] found that this condition was approximately fulfilled during hydrolysis of Schwarz' ribonucleic acid by ribonuclease.

The magnesium nucleate used in the present studies is not titratable above pH 11 because the establishment of equilibrium between the magnesium nucleate complex and magnesium hydroxide occurs very slowly. After the addition of each increment of base, the pH slowly drifted back, and each reading required tens of minutes to reach an apparently stationary value. Attempts to remove the magnesium completely from the nucleate were unsuccessful. The material was dialyzed at 5° and at neutral pH for two days in dilute potassium chloride and for two more days against distilled water. The titratability was not improved. A solution of the nucleate was cooled to 0° and the pH was adjusted to 12. After 45 minutes at 0°, the solution was centrifuged. One aliquot was titrated immediately, while another was hydrolyzed in 0.5 N alkali for 6 minutes at 100°, then titrated. Some downward drift in pH occurred, but considerable time was allowed for equilibration near the end-points, at pH 8 and 12. The hydrolysate appeared to have about 15% more enol groups than the unhydrolyzed sample, but the increased titer was probably due to a small amount of magnesium ions in the hydrolysate. The mid-point of the titration curve changed from pH 10.2 to 9.5.

The potassium nucleate isolated from fresh yeast offered no such difficulty and there was no significant increase in total enol groups during hydrolysis. The mid-point of the titration curve was shifted from pH 10.8 to 9.5 by complete alkaline hydrolysis. The shape of the titration curve was not appreciably changed, so the rate of change of pH at constant titer above the inflection point should be a semi-quantitative measure of the rate of change of pK. This change follows approximately the first-order rate law. In acidic or alkaline hydrolyses, the rate of the change of pK is 2.5 to 3 times faster than the corresponding rate of liberation of the secondary phosphate groups. The ratio of these two rates of change at pH 6 is 4 or 5.

Hydrolysis of Mononucleotides.—The rates of hydrolysis of phosphate from adenylic and cytidylic acids in 0.5 N potassium hydroxide were measured at 70° and at 100°. The nucleotides were obtained from Nutritional Biochemicals Corporation. Each sample is probably a mixture of the isomers isolated by Cohn,[14] which is the product to be expected from alkaline hydrolysis of nucleic acid. The samples behaved as expected[2] during acidic hydrolysis. Glass and stainless steel vessels could not be used, since they gave appreciable blank values in the colorimetric determination for phosphate. Nickel crucibles were inert, but inconvenient. Teflon tubes were used.

The first-order rate constants for cytidylic acid are 0.0112 hour^{-1} at 70° and 0.108 hour^{-1} at 100°, expressed with natural logarithms. The corresponding constants for adenylic acid are 0.024 and 0.19 hour^{-1}. The energies and entropies of activation computed from the above rate con-

(13) S. Wiener, E. L. Duggan and F. W. Allen, *J. Biol. Chem.*, **185**, 163 (1950).

(14) W. E. Cohn, This Journal, **72**, 2811 (1950).

stants are practically the same. The mean values are 18 kcal. and −31 e.u.

It was previously shown that the pronounced difference between the rates of hydrolysis of phosphate from purine and pyrimidine mononucleotides in acidic solution is directly related to the stabilities of the riboside bonds.[2] In alkaline solutions both purine and pyrimidine riboside bonds are stable, and the rates of hydrolysis of phosphate are nearly equal and similar to the rates for simple alcoholic esters of phosphate such as ethyl or glyceryl phosphates.

Energies and Entropies of Activation.—Let M be the concentration of titratable secondary phosphate groups and D be the concentration of total diester phosphate. Then the rate constants are expressed as $k = \mathrm{d}M/D\mathrm{d}t$. In 0.50 N hydrochloric acid at 60 and 100°, constant values of k, 1.9 and 26 hour^{-1}, respectively, are obtained leading to $\Delta H^{\ddagger}$ = 16 kcal./mole and $\Delta S^{\ddagger}$ = −26 e.u. In 0.01 N acid the kinetics are complicated (Fig. 5). The comparison of rates was made by superposition on a graph of the runs at 70 and 100°, and estimating the ratio of the two time scales. For the first part of the reaction $\Delta H^{\ddagger}$ = 22 kcal./mole and $\Delta S^{\ddagger}$ = −15 e.u. The same procedure was used at pH 6. For the hydrolysis of potassium nucleate in 0.03 M magnesium sulfate at pH 6, $\Delta H^{\ddagger}$ = 27 kcal. and $\Delta S^{\ddagger}$ = −12 e.u. The rates in 0.50 N potassium hydroxide were measured at four temperatures. Figure 10 shows a plot of ln k *vs.* $1/T$. The values of the rate constants at the two lower temperatures were taken from the first-order curves of Fig. 4. The rate constants of Fig. 10 therefore apply to the entire hydrolysis of secondary phosphate groups at 60 and 70°, but those at 35 and 45° only apply to the hydrolysis of approximately one-half of the phosphate. Figure 10 implies that the four rate constants apply to the same mechanism. For this mechanism at an ionic strength of 0.5

$$\log k - \log(\mathrm{KOH}) = 14.2 - 4340/T$$

giving $\Delta H^{\ddagger}$ = 19 kcal. and $\Delta S^{\ddagger}$ = −15 e.u. Tentative measurements indicate that at constant ionic strength $\Delta H^{\ddagger}$ does not vary appreciably with a change in the concentration of base, and that $\Delta H^{\ddagger}$ increases with decreasing ionic strength at constant alkalinity.

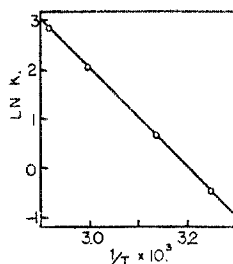


Fig. 10.—Temperature dependence of the rate of hydrolysis of secondary phosphate groups in 0.50 N potassium hydroxide.

As a conservative estimate, the relative errors in the rate constants may be taken as 10%. Then the relative error in the ratio of constants measured at different temperatures is 14%. Since

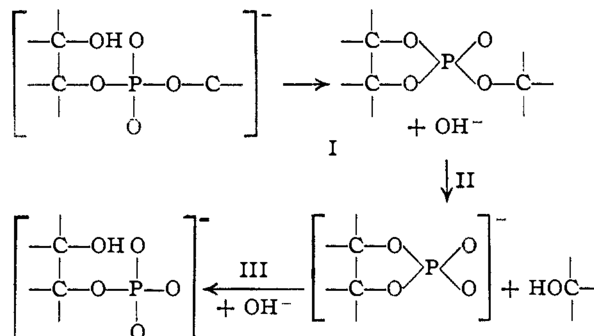
$$\frac{\mathrm{d}\,\Delta H^{\ddagger}}{\Delta H^{\ddagger}} = \frac{\mathrm{d}\ln(k_1/k_2)}{\ln(k_1T_2/k_2T_1)}$$

then for a temperature interval which gives $k_1/k_2 = 10$, the relative error in $\Delta H^{\ddagger}$ is approximately 6%. The errors in the values of $\Delta S^{\ddagger}$ are roughly ±3 e.u.

Discussion

Internucleotide Linkages.—Part X of the series on nucleotides from the laboratory of A. R. Todd[15] has some fundamental suggestions and information on the internucleotide linkages. The essential ideas are these: Ribose nucleosides have three hydroxyl groups available for phosphorylation at C_2', C_3' and C_5', while desoxyribose nucleosides have hydroxyl groups available at C_3' and C_5'. In the ribose polynucleotides one phosphate may form a cyclic derivative by esterification at both C_2' and C_3' of one ribose moiety, with a third linkage to a different ribose. The evidence is that the third linkage is to the C_5'-position.[16] The polydesoxynucleotides cannot form the cyclic triester structure. One bond of triester phosphates hydrolyses readily in either acid or in alkali, while the second and third do not. The difference in stability toward alkali for the two types of polynucleotides is nicely accounted for by this theory. Another pertinent property of derivatives of 2-hydroxyethyl phosphate is that the cyclization will only occur if no more than one of the acidic groups of phosphate is ionized. Thus either α- or β-glyceryl phosphate will form an equilibrium mixture only in acid, while either structural isomer of methyl glyceryl phosphate will form a mixture in either acidic or alkaline solution. Finally, it seems probable, from the summary of evidence given by Todd, that an equilibrium mixture of, for example, the glyceryl phosphates in acidic solution will have a small proportion of the cyclophosphate compared to the monoester forms. The equilibrium is possible because equilibrium among the isomers is established very rapidly compared to the rate of hydrolysis of glyceryl phosphate.

An application of the above ideas to the hydrolysis of nucleic acid should start with hydrolysis in alkaline solution. It is assumed that formation of the cyclic triester structure is essential. Consider the sequence of reactions



Reaction II is considered essential. The product of reaction III is the substance which is measured by the analytical procedure. The salt effect may occur in reaction I if reaction I has a transition state in which hydroxyl ion has coupled with the reactant. Such a coupling of hydroxyl ion and the diester phosphate seems likely in view of the fact that isomerization of α-glycerylphosphorylcholine requires heating in acidic or alkaline solution.[17] The salt effect may occur in reaction I if the rate-determining step is I, II or III. If the salt effect occurs in reaction III, then III is rate-determining, in which case I may also have a salt effect. The entropy of activation for alkaline hydrolysis is 11 e.u. higher than for acidic hydrolysis. The relatively high entropy of activation is more easily understood if the rate-determining step were the fission of a ring, rather than the formation of a ring. Reaction II should be much faster than III, so it is not likely that II is rate-determining.

In the region of a few tenths normal acid, the rate

(15) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(16) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).

(17) E. Baer and M. Kates, *J. Biol. Chem.*, **185**, 615 (1950).

of hydrolysis of the secondary phosphate bonds in ribonucleic acid depends on a power of the acid concentration greater than one but less than two and it has little or no dependence on ionic strength. Since desoxynucleic acid hydrolyzes almost as rapidly in acid as does ribonucleic acid, the cyclic triester form in the latter may not be important during its acidic hydrolysis. The acidic group of diester phosphate in ribonucleic acid should have approximately *p*K 1.[18] If the specific rate of hydrolysis were greater for the undissociated form than for the dissociated form of diester phosphate, and if the rates for each form were proportional to the concentration of acid, then the over-all rate for both forms would show the observed behavior.

If k_1 = rate constant for undissociated diester phosphate, k_2 = rate constant for dissociated diester phosphate, K = dissociation constant of the diester phosphate, and (P) = concentration of diester phosphate, then

$$\frac{d(P)}{dt} = (H^+)(P)\left[\frac{k_1(H^+)}{K + (H^+)} + \frac{k_2 K}{K + (H^+)}\right]$$

Three such pairs of rate constants may be applicable, one for the direct hydrolysis of diester phosphate, and one for each of reactions I and III if they occur in acidic solution. The hydrolysis of the undissociated form should also show almost no salt effect. Furthermore, the experimental results show that a small salt effect may exist in 0.04 *N* hydrochloric acid, whereas none was observed in 0.15 *N* acid. This information is compatible with reactions involving both dissociated and undissociated diester phosphate.

The hydrolysis at *p*H 6 is believed to be a much more indiscriminant type of breakdown than occurs in acid or alkali. At *p*H 6 the nearly equal rates of hydrolysis of diester and monoester phosphates indicate that the bonds may hydrolyze in any order, and that the intermediary products of hydrolysis may be considerably different from those obtained in acid or alkali. The following observations strongly support this suggestion: The acid precipitability of hydrolysates obtained at *p*H 6 disappears when about 15% of the diester phosphate has been hydrolyzed, whereas in acidic or alkaline hydrolysates the precipitability in acid does not disappear until 30 to 40% of the diester phosphate has been hydrolyzed. Additional support comes from the fact that during acidic or alkaline hydrolysis the ratio of the rate of shift for the mean *p*K of the enol groups to the rate of hydrolysis of diester phosphate is approximately one-half of the ratio of rates obtained at *p*H 6. Consequently the loss in acid precipitability is parallel to the shift in the mean *p*K of the enol groups.

The kinetics of hydrolysis at *p*H 2 and at low temperatures in alkaline solution were found to be complex. This does not, however, mean that the internucleotide bonds are not all of the same type, since it is possible that the specific rate constant for a particular internucleotide linkage might vary with the length of the polynucleotide chain in which the linkage exists or with its distance from the end of the chain. Other secondary structural effects are readily conceivable. It is difficult to see why such possibilities would disappear when the temperature at which the hydrolysis is conducted is raised 20°. On the other hand, if two types of linkage exist with different values of $\Delta H^{\pm}$ for hydrolysis in 0.50 *N* alkali, the two rates may coincide at 65°, but would then be different at other temperatures. The same possibilities exist for hydrolysis at *p*H 2.

The rapid change in the mean *p*K of the enol groups during hydrolysis to a final value equal to that of pure guanylic or uridylic acid shows that the polynucleotide molecules undergo changes other than merely the hydrolysis of the phosphate linkages. The ratio of the rates of the two types of change during hydrolysis is low enough to produce a decided induction period in the hydrolysis of diester phosphate if the shift in the *p*K of the enol groups were a necessary preliminary reaction to the hydrolysis of diester phosphate. The absence of an induction period shows that the two processes are not kinetically consecutive. The increase in acid strength of the enol groups is 1.3 *p*K units for potassium nucleate, which is equivalent to an average of 1800 cal. additional binding energy for the enolic protons in the polynucleotides. The value is small enough to be caused by any one of a number of effects. It may be that the change in the *p*K is caused by some sort of unfolding of the polynucleotide whereby the enol groups, and perhaps the pyrimidine rings to which they are attached, can become more strongly solvated.

A process which may be closely related to the shift in *p*K of the enol groups and the loss in acid precipitability was observed by Vandendriessche.[19] The volume of an enzymic digest of ribonucleic acid shows an initial rapid expansion followed by a slower contraction. The contraction seems to be related to the liberation of secondary phosphate groups. The initial dilatation might well result from an unfolding of the nucleic acid after which the enol groups become stronger acids and the acid precipitability is lost.

The potassium nucleate isolated from fresh yeast is different in some respects from the purified commercial material used in this study. The shift in the *p*K of the enol groups is 1.3 and 0.7 units, respectively. The titratable secondary phosphate in the potassium nucleate is only 10% of the total phosphate, whereas the value for the commercial material is 15%.. The titration curve of potassium nucleate which was stored in solution at 5° and *p*H 6 showed appreciable changes, particularly for the enol groups, in 3 days, whereas there was no change in the titration curve of the purified commercial material during storage for 13 days under the same conditions. These differences may mean that the commercial material is simply more degraded, but that the internucleotide linkages are essentially the same in the two materials. However, the curves of Fig. 5 seem to indicate a more important difference.

Catalysis by Magnesium Ion.—Catalysis by magnesium ions of the hydrolysis of phosphate bonds at *p*H around 10 is not unexpected in view of

(18) W. D. Kumler and J. J. Eiler, THIS JOURNAL, 65, 2355 (1943).

(19) L. Vandendriessche, *Compt. rend. trav. lab. Carlsberg, Ser. chim.* 27, 342 (1951).

previous work.[20,21] Apparently the ions of metals which catalyze the hydrolysis of phosphate bonds are most effective at the *p*H at which precipitation of the insoluble hydroxide begins. It was previously suggested[21] that supersaturation of the hydroxide may be involved. On the other hand, the active catalyst may be ionic species such as $La(OH)_2^+$ or $Mg(OH)^+$. Catalysis of the hydrolysis of the secondary phosphate bonds of nucleic acid at *p*H 6 by magnesium ions is, however, not explained by these suggestions. The formation of a complex with magnesium ions very probably involves the phosphate groups of nucleic acid, and the small catalytic action at *p*H 6 may be due to changes in the phosphate ester bonds which may occur when the magnesium ion is complexed by the nucleate. It would be interesting to see if simple diester phosphates which do not complex magnesium ions also exhibit a catalyzed hydrolysis at *p*H 6 and 10. Catalysis would be expected at *p*H 10, but not at *p*H 6.

The potassium and magnesium nucleates have the same rates of hydrolysis in acidic and in alkaline solution. In 0.5 *N* base the activity of magnesium ion must be extremely low. On the other hand, acid can be used to displace magnesium from magnesium nucleate, as was discussed in the section on the preparation of potassium nucleate. In either case, acid and base are such effective catalysts that the effect of magnesium ion is negligible at high or low *p*H.

(20) E. Bamann, *Angew. Chem.*, **52**, 185 (1939).

(21) J. E. Bacher and F. W. Allen, *J. Biol. Chem.*, **188**, 59 (1951).

PRINCETON, NEW JERSEY

[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

Elastoviscous Properties of Polyisobutylene. VI. Relation between Stress Relaxation Modulus and Dynamic Modulus

BY ARTHUR V. TOBOLSKY

RECEIVED FEBRUARY 25, 1952

An idealized distribution function for the complete elastoviscous behavior of polyisobutylene is presented. From this function an exact expression for stress relaxation modulus as a function of time and temperature can be derived. It is shown to agree very closely with experimental values. An exact expression for the dynamic modulus is also calculated from the idealized distribution function and is available for experimental test.

Introduction

In Part V of this series,[1] the complete relaxation curve $E_{r,T}(t)$ for unfractionated polyisobutylene was presented. Empirical functions describing major portions of $E_{r,T}(t)$ were given, as were the corresponding distributions of relaxation times. The existence of a glassy, rubbery and transition region of elastoviscous behavior was demonstrated.

In this paper a complete idealized distribution of relaxation times giving an excellent fit with the relaxation of stress data $E_{r,T}(t)$ obtained thus far is given. The distribution of relaxation times consist of two discrete portions, the short time portion being molecular weight independent, and the long time portion being molecular weight dependent. From the idealized distribution of relaxation times analytical formulas for the relaxation modulus $E_{r,T}(t)$ and the dynamic modulus $E'_T(y)$ are derived.

The Idealized Distribution of Relaxation Times.—The distribution of relaxation times $D(\tau)$ for any (sufficiently high) molecular weight and for any temperature will be given the idealized representation

$$\frac{298}{T} D(\tau) = 0 \quad \tau < \tau_1 \qquad (1)$$

$$\frac{298}{T} D(\tau) = M(\tau/\kappa)^{-3/2} \quad \tau_1 < \tau < \tau_2 \qquad (2)$$

$$\frac{298}{T} D(\tau) = 0 \quad \tau_2 < \tau < \tau_3 \qquad (3)$$

$$\frac{298}{T} D(\tau) = \frac{298}{T} E_0(\tau/\kappa)^{-1} \quad \tau_3 < \tau < \tau_m \qquad (4)$$

$$\frac{298}{T} D(\tau) = 0 \quad \tau > \tau_m \qquad (5)$$

In the equations $D(\tau)$ has the units dynes/cm.^{2} per hour and τ is expressed in hours. The parameters appearing in these equations have the values

$$M = 10^{3.95};\ E_0 = 7.2 \times 10^5;\ (\tau_1/\kappa) = 10^{-12.5}$$
$$(\tau_2/\kappa) = 10^{-5.4};\ (\tau_3/\kappa) = 1.39 \times 10^{-25}\ \bar{M}_v^{3.30} \qquad (6)$$
$$(\tau_m/\kappa) = 1.53 \times 10^{-19}\ M_v^{3.30}$$

where $\bar{M}_v$ is the viscosity average molecular weight and κ is a tabulated function of temperature which has the value unity at 25°.[1-3] For polyisobutylene sample N.B.S. the following values were used

$$\tau_3 = 10^{-4.34};\ \tau_m = 10^{1.70} \qquad (6a)$$

A plot of the idealized distribution of relaxation times for polyisobutylene (sample N.B.S.[4]) is shown (solid line) in Fig. 1. The plot is in the form $D'(\log \tau)$ *versus* $\log \tau$. The relation between $D'(\log \tau)$ and $D(\tau)$ is: $D'(\log \tau) = 2.303\ \tau\ D(\tau)$. As can be seen in the figure $D'(\log \tau)$ consists of a wedge and a box. Change of temperature merely adds a constant to the logarithmic relaxation time scale plotted along the abscissa. Changing the molecular weight at a fixed temperature leaves the position of the wedge unaltered, but causes a horizontal translation of the box along the abscissa, so that for larger molecular weights the wedge and the box are separated more widely.

(1) A. V. Tobolsky and J. R. McLoughlin, *J. Polymer Sci.*, **8**, 543 (1952).

(2) R. D. Andrews, N. Hofman Bang and A. V. Tobolsky, *ibid.*, **3**, 669 (1948).

(3) R. D. Andrews and A. V. Tobolsky, *ibid.*, **6**, 221 (1951).

(4) The abbreviation N.B.S. stands for National Bureau of Standards.