and upon further dialysis of P-2 a second insoluble component separated. Repeating this process gave a third partition. The solubility of the dissociable component system at a given stage was a reflection of the relative amounts of soluble and insoluble proteins. Thus, the ratio of soluble to insoluble solids after the dialysis of P-2 was 1.2, while this ratio after the subsequent dialysis of the soluble component was 5.1.

It may be noted from Table I that the ratio of activity on hemoglobin to activity on ATEE is between 158 and 352 for pankrin fractions and between 14 and 18 for the two crystalline α-chymotrypsin preparations. The ratios of activity on hemoglobin to activity on TSAME are greater than 273 for pankrin and 29 and 41 for the two crystalline trypsin preparations. The very large spread in hemoglobin: TSAME for pankrin preparations indicates residual contamination with trypsin. The ATEE activities in pankrin may be the result of chymotrypsin contamination, but an inherent overlapping of specificities cannot be ruled out now. Fraction P-1r has 80% of the milk clotting activity of crystalline α-chymotrypsin but only 14% of the ATEE activity.

A preliminary digestion of corticotropin-A by fraction E-2 resulted in extensive cleavage of the molecule, as indicated by the appearance of at least 10 ninhydrin-positive spots on chromatograms of the digest.[15] Crystalline trypsin[16] and crystalline chymotrypsin[17-19] in similar experiments produced fewer cleavages of the peptide, indicating broader specificity for pankrin.

(15) W. F. White, The Armour Laboratories, personal communication.

(16) W. F. White and W. A. Landmann, THIS JOURNAL **77**, 1711 (1955).

(17) W. F. White and W. A. Landmann, *ibid.*, **76**, 4193 (1954).

(18) W. F. White, *ibid.*, **76**, 4194 (1954).

(19) W. F. White and W. A. Landmann, *ibid.*, **77**, 771 (1955).

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF ILLINOIS]

Pattern of Action of Crystalline Muscle Phosphorylase on Glycogen as Determined from Molecular Size Distribution Studies

BY J. LARNER, B. ROGER RAY AND H. F. CRANDALL

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The results of the action of crystalline muscle phosphorylase on rabbit muscle and liver glycogens have been studied. The sedimentation velocity diagrams from the ultracentrifuge have been analyzed according to the Baldwin–Williams procedure to yield distribution functions of the sedimentation coefficients; sedimentation and diffusion measurements on several fractions permitted transposition into molecular weight distributions. Liver glycogen showed a highly skewed distribution with molecular weights ranging from well below a million to over 100 million and with a broad maximum around 2 million. Muscle glycogen was less heterogeneous, containing species from several thousand up to 15 million and with a maximum around 2.5 million. Enzymatic degradations, carried out to various extents and under different pH conditions, showed that the highest molecular weight material most noticeably disappeared. The same general behavior was observed in every case. That is, a less heterogeneous sample resulted which had an increased proportion centered about approximately the same maximum as before degradation. Equilibration of glycogen and glucose 1-phosphate with phosphorylase shifted the molecular weight distributions in the same general manner as did degradation. The experimental results are consistent with the idea of greater probability of enzymatic action on the outer glucose chains of the largest molecules.

Introduction

The mechanism of action of crystalline muscle phosphorylase on the outer chains of branched polysaccharides of the glycogen–amylopectin class has been examined.[1] Analysis of the number of degraded outer chains susceptible to amylo-1,6-glucosidase action (units of limit dextrin configuration) during phosphorolysis has indicated that phosphorylase does not degrade by a "single chain" mechanism. It was also noted that the high yield of units of limit dextrin configuration from liver glycogen samples could be reduced by prior incubation (equilibration) of the glycogen with small amounts of glucose 1-phosphate and phosphorylase. This latter experiment was interpreted as indicating a redistribution or randomization of the glucose residues of the outer chains to produce a more uniform size distribution. In order to decide whether such a process could occur by an intermolecular mechanism, and also to determine whether phosphorolytic degradation had any degree of specificity with regard to molecular size of substrate, the partially degraded and the randomized glycogen samples have been examined ultracentrifugally. Sedimentation analyses have led to molecular weight distributions which indicate that in both cases the high molecular weight fraction of the polysaccharide is the preferential enzymatic substrate.

Experimental Details

Glycogen Samples.—Isolation of the glycogen samples both before and after enzymatic treatment has been discussed.[1,2] The samples studied by ultracentrifugal analysis are listed in Table I. With one exception, samples were reisolated after incubation with the enzyme by repeated ethanol precipitations after 1–2 minute treatment in 10–15% NaOH at 100°. The 21% degraded 117-1 was reisolated omitting the brief treatment with alkali.

Subfractions of glycogen samples were prepared by progressive addition of ethanol at 0° to solutions containing 2% glycogen and 2% NaCl. Precipitates were separated by centrifugation at 3°. They were dissolved in small volumes of water and dialyzed extensively against water at 3° to remove NaCl. Visually, fractions precipitating at lower ethanol concentrations were strikingly more opalescent than fractions precipitating at higher ethanol concentrations. Table II shows the ethanol concentrations used and the percentage distribution of the fractions. Recovery was about 99% in each case.

(1) J. Larner, *J. Biol. Chem.*, **212**, 9 (1955)

(2) B. Illingworth, J. Larner and G. T. Cori, *ibid.*, **199**, 631 (1952).

Enzymatic Treatment.—Crystalline muscle phosphorylase (10–12 times recrystallized) was prepared by the method of Green and Cori[3] and shown to be free of amylo-1,6-glucosidase (or α-amylase) by the method already described.[4] Methods previously discussed were used to estimate enzymatic degradation.[1]

Sedimentation.—A Spinco Model E Ultracentrifuge was used. Runs were made at 23–25°, with data being corrected to 20°, assuming the temperature of the run to be the average of the initial and final temperature of the thermojunction plugged into the rotor at the start and at the end. The Philpot–Svensson optical system utilized an inclined $^1/_{32}''$ wire. The rotor, drilled with a reference hole, permitted the use of twin cells equipped with wedge and plane windows. The majority of runs were carried out at speeds around 12,000 r.p.m.

The evaluation of the dependence of sedimentation coefficients on concentration was based upon coefficients calculated from the positions of the maxima of the refractive index gradients. Usually, ten exposures at 8 minutes were recorded and several measurements of each peak were made under a microcomparator. The data from a run were fitted to a least square line; the standard error of the slope was in every case less than 0.55 unit. For the calculations of distribution, gradient curves were enlarged 10-fold and traced onto graph paper. The middle of the gradient curves was estimated by eye.

Diffusion.—Diffusion coefficients were calculated from schlieren photographs of free-boundary experiments carried out at 0° in a Frank Pearson Associates electrophoresis apparatus. The low temperature was chosen to avoid bacterial action[5] during the runs, which required about 24 hours. The two arms of a microcell of Tiselius type were used independently. Boundaries were introduced by placing solution into one arm of the dry cell and then floating water on top by means of a very fine capillary pipet. A boundary was sharpened by slowly withdrawing solution from below.

The coefficients were evaluated by the height–area method. There was some skewness of the curves in the direction of the solution. The average area in each run was obtained by reading the individual enlarged tracings with a planimeter. The plots of $1/H^2$ *vs.* t were satisfactorily linear over the entire time interval of 12–24 hours. The experimental coefficients were corrected to 20° using the customary temperature and viscosity ratios.[6]

Experimental Results

Sedimentation Coefficients.—The coefficients were determined at several concentrations for the original liver glycogen (117-1), and the original muscle glycogen (M-1), and also for the high and low fractions obtained from each of these by ethanol precipitation. The results, in Svedberg units, are tabulated in Table III.

It is apparent that there exists a definite concentration dependence of the sedimentation coefficient of the order of 15–20% per 1% change in concentration. The concentration dependence of s was important to the estimation of the distributions of sedimentation coefficients. Furthermore, certain analytical forms for the dependence were to be preferred for calculation. For the two fractions of liver glycogen

$$s = s_0 - Bs^2c^2 \qquad (1)$$

where B has the value 1.611×10^{10} when c is in wt. % and s is in sec. This particular form is arbitrary, but chosen so as to have real roots for all values of s_0 and c. The concentration dependence of the fractions of muscle glycogen is conveniently given by the expression

$$s = s_0(1 - Kc^2) \qquad (2)$$

where K has the value 0.152. The values of s_0, from least squaring the data, are included in the table.

Diffusion Coefficients.—These are tabulated in Table IV for the same glycogen samples as were studied in sedimentation. An approximately linear increase of D with concentration was found for the samples of muscle glycogen. Considerable difficulty was met in obtaining reproducible diffusion results. Part was due, probably, to inherent features of the height–area method

TABLE I

IDENTIFICATION OF RABBIT GLYCOGEN SAMPLES

Sample	pH during Degradation	% degradation	Ref. to figure
Liver glycogen 117-1 (Original)	..	...	2, 3
Irreversibly degraded (phosphoglucomutase present)	6.5	10.2	3
Reversibly degraded	7.0	12.6	3
	7.3	21.0	Table II
E-1 (Equilibrated 117-1, 3% addn. of glucose)	..	...	2
Reversibly degraded	7.4	14.0	2
E-2 (Equilibrated 117-1, 7% addn. of glucose)	..	...	2, 4
Reversibly degraded	7.0	12.9	4
	7.6	12.3	4
	7.6	15.8	4
Muscle glycogen M-1 (Original)	..	...	5
Reversibly degraded	7.6	12.1	5
	7.6	16.3	5

TABLE II

FRACTIONATION OF GLYCOGEN SAMPLES

Sample	Fraction 1 % ethanol	Fraction 1 % of total	Fraction 2 % ethanol	Fraction 2 % of total
Original 117-1	15.8	76	50.0	24
Original 117-1 after 21% degradation	13.3	33	50.0	67
Original M-1 and M-2	20.0	70	50.0	30

TABLE III

SEDIMENTATION COEFFICIENTS OF RABBIT GLYCOGENS

Original Concn., %	s^w_{20}	Fraction 1 Concn., %	s^w_{20}	Fraction 2 Concn., %	s^w_{20}
Liver glycogen 117-1					
0	99	0	109	0	70
0.250	98	0.308	109	0.272	71
.500	97	.616	101	.500	70
.750	93	.924	96	.750	66
1.000	88	1.332	82	1.235	62
Muscle glycogen M-1					
0	64	0	76	0	61
0.250	62	0.291	74	0.250	60
.500	58	.500	73	.500	61
.750	51	.706	72	.750	55
1.000	44	1.000	67	1.000	49

(3) A. A. Green and G. T. Cori, *J. Biol. Chem.*, **151**, 21 (1943).

(4) G. T. Cori and J. Larner, *ibid.*, **188**, 17 (1951).

(5) In early experiments it was found that a glycogen solution stored for one week at 4° gave high and erratic diffusion coefficients. Later samples were kept frozen in Dry Ice until time for use.

(6) Unpublished experiments by J. J. Scholz in this Laboratory have established that this customary type of correction is quite satisfactory over the range of 0–25° for dextran fractions.

TABLE IV

DIFFUSION COEFFICIENTS OF RABBIT GLYCOGENS[a]

Original Concn., %	D_{20}^{w}	Fraction 1 Concn., %	D_{20}^{w}	Fraction 2 Concn., %	D_{20}^{w}
		Liver glycogen 117-1			
0.250	17	0	13	0	15
0.750	17	0.308	12	0.272	16
1.000	21	.616	14	.500	14
		.924	12	.750	15
		...	..	1.235	16
		Muscle glycogen M-2[b]			
		0	11	0	16
		0.250	13	0.250	16
		.500	15	.500	22
		.750	20	.750	18
		1.000	19	1.000	23

[a] D_{20}^{w} in cm.^{2} sec.$^{-1}$ $\times 10^8$. [b] The data refer to fractions obtained from a second preparation of muscle glycogen, M-2, isolated exactly as was the original, M-1. The first preparation had apparently become bacterially degraded. The sedimentation coefficients of the new sample are within 5% that of the original.

and another part to the small amounts of material available. On occasion the results were erratic; bacterial action was thought responsible in one series. Several complications may be involved in the measurement of diffusion coefficients of very large molecules.[7,8] We estimate the data to be reliable to $\pm 10\%$.

Average Molecular Weights.—These were calculated by application of the Svedberg equation using the limiting s and D values from Tables III and IV. $\bar{V}$ was taken as 0.65.[9] The values are

Liver glycogen 117-1	Original	4.0×10^6
	Fraction 1	5.4×10^6
	Fraction 2	3.0×10^6
Muscle glycogen M-1	Fraction 1	4.6×10^6
	Fraction 2	2.7×10^6

The molecular weight calculated in this manner, often designated as $M_{s,D}$, is a rather poorly defined average for a heterogeneous material and is dependent upon the particular methods of observation used. Kinell and Ranby,[10] Miller and Hamm,[11] and Williams and co-workers[12] and others have discussed this and other molecular weight averages. The values of M in each series can be fitted by the equations $M = 569s^2$ and $M = 761s^2$ for the liver and muscle types, respectively.

Molecular Weight Distributions.—These were determined by a study of the progressive spreading of the sedimentation peaks that took place during a sedimentation experiment. Analysis led to frequency plots of sedimentation coefficients which were then transformed into molecular weight frequency curves.

(7) N. Gralen, "Sedimentation and Diffusion Measurements on Cellulose and Cellulose Derivatives," Almquist and Wiksells, Uppsala, 1944.

(8) C. O. Beckmann and J. L. Rosenberg, *Ann. N. Y. Acad. Sci.*, **46**, 329 (1945).

(9) D. J. Bell, H. Gutfreund, R. Cecil and A. G. Ogston, *Biochem. J.*, **42**, 405 (1948).

(10) P. Kinell and B. G. Ranby, "Advances in Colloid Science," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1950, Chapt. 5.

(11) L. E. Miller and F. A. Hamm, *J. Phys. Chem.*, **57**, 110 (1953).

(12) J. W. Williams, Winifred M. Saunders and Jean Cicirelli, *ibid.*, **58**, 774 (1954).

Williams and co-workers,[12] among others, have discussed the interpretation of sedimentation peaks so as to yield a significant picture of the distribution by mass, or shape, of the particles. The method applied was that of Baldwin and Williams.[13] From a set of 6 or 8 sedimentation peaks photographed during a single sedimentation run at 1% concentration an apparent distribution function, $g^*(s)$, was calculated for each of 15–20 values of s on each of the enlargements. This function is taken as

$$g^*(s) = \frac{(dn/dx) \times x\omega^2 t}{\int (dn/dx)\, dx}$$

where dn/dx is the height of the curve at the distance x and $\int (dn/dx)dx$ is the total area under the curve at time t. The distance x is calculated to be the position of a species with a coefficient of (s) after time t. To reduce the labor of computation and the possibility of arithmetical error, the calculations were encoded for solution by Illiac, the University of Illinois digital computer.

More to examine the general behavior of the data than to eliminate a possible diffusional contribution, the values of $g^*(s)$ were plotted against $1/xt$ and a graphical extrapolation made to $1/xt = 0$ to yield $g(s)$. The best fits appeared to be given by straight lines and all extrapolations were done on this basis from a least square treatment of all the points. To give an idea of the character of the data, several representative extrapolations for $g(s)$ are shown in Fig. 1 for M-1 at 1% concentration.

The concentration dependence of $g(s)$ was eliminated by carrying out an analytical extrapolation to zero concentration according to the method introduced by Baldwin.[14] First, we calculated values of dc/dx using the relationship

$$\frac{dc}{dx} = \frac{g(s)c_0}{x_0\omega^2 t(\exp(s\omega^2 t))}$$

where c_0 is the original glycogen concentration and t is chosen as the mid-point of the run. From the plot of dc/dx *vs.* x, a table of values was made up, choosing values of x at equal intervals. For each value of x a parameter $r/\omega^2 x$ was calculated from

$$\left(\frac{r}{\omega^2 x}\right)_{x_i} = \frac{\ln x_j - \ln x_0}{\omega^2 t}$$

This parameter is equal to s_i at the point x_i and values for $(s_0)_i$ and for s_i at other points in the cell may be calculated from it by using the expression for the concentration dependence of s.

The incremental concentration of a component i was calculated from the relationship

$$[\Delta C_i]_{(x_{i+1} - x_i)} = \frac{[C_i]_{x_i}[\Delta S_i]_{(x_i + (x_{i+1} - x_i))}}{[r/\omega^2 x - S_i]_{(x_i + 0)}}$$

and the increments were summed in the expression

$$C_i = [dc/dx]_{x_i} \Delta x - \sum_{k=1}^{i-1} [\Delta C_k]_{(x_i - x_{i-1})} + \sum_{m=i}^{n} [\Delta C_i]_{(x_m - (x_{m-1} - x_m))}$$

The last expression gives the concentration of the ith component beyond the boundary region where

(13) R. L. Baldwin and J. W. Williams, THIS JOURNAL, **72**, 1325 (1950).

(14) R. L. Baldwin, *ibid.*, **76**, 402 (1954).

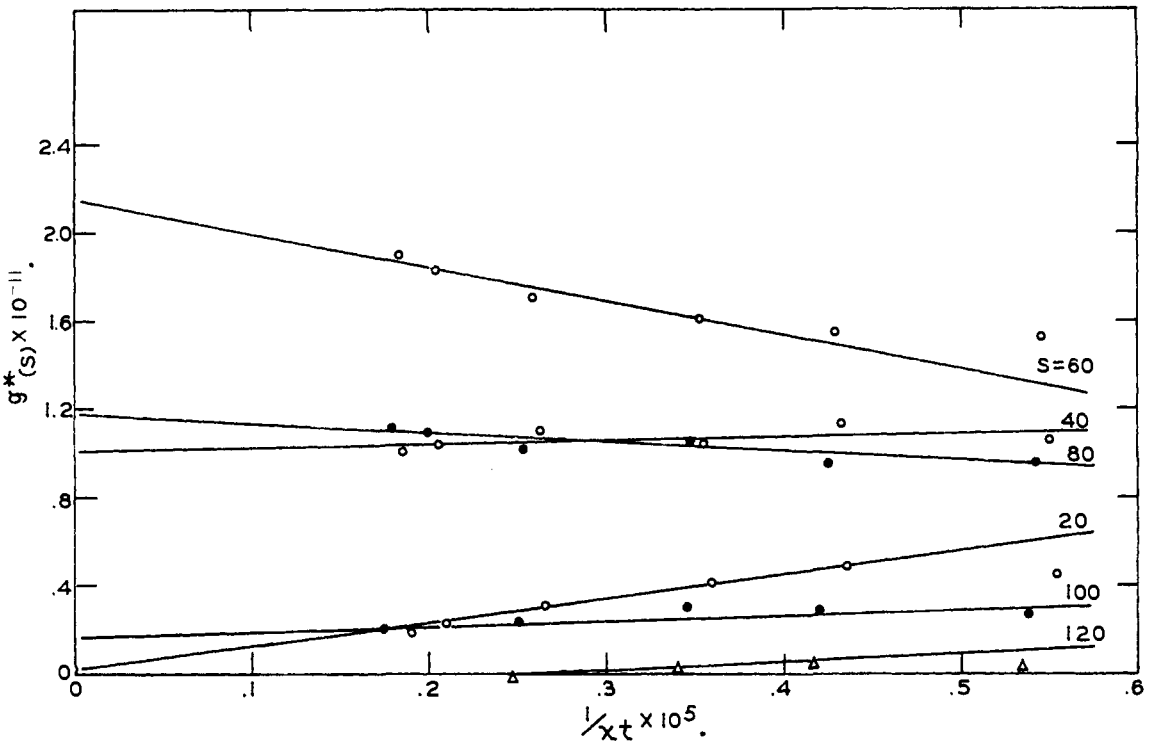

Fig. 1.—Extrapolation of apparent distribution function of sedimentation coefficient to infinite time; rabbit muscle glycogen (M-1) at 1% concentration.

the total concentration and the concentration of each component is a function of time only. Here $(dc/dx)\ \Delta x$ is the increase in total concentration over the interval Δx. The term $\Sigma[\Delta C_k]$ is the correction for the changes in concentration of components up to i. The final term $\Sigma[\Delta C_i]$ gives the change in concentration of component i to any other point in the cell. The calculation is quite tedious unless the analytical expression for the concentration dependence is very simple. Also, arithmetic errors accumulate quite rapidly. For these reasons the Illiac was utilized.

Plots of $g(s_0)$ against s_0 comprise sedimentation frequency functions. To transform to distributions as molecular weights the expression $f(M) = g(s_0)\ ds/dM$ was applied. Here ds/dM could be calculated from the relationships already given. The molecular weight frequency functions for the several glycogen samples described in Table I are presented in Figs. 2 through 5. (All the curves have been normalized.) The samples derived from liver glycogen showed extended tails of material on the high molecular weight end. The calculated upper limit, which is of course only qualitative at most, is indicated in each legend by the number at the right.

Discussion

In two previous studies on rabbit glycogens sedimentation coefficients were determined. Bridgman[15] found s_{20} values ranging from 61 to 100 for rabbit liver glycogens prepared and treated in different ways. His sample IIIB, extracted with 50% potassium hydroxide, having an s of 100 appears most similar to our sample 117-1 with an s of 97 at $^1/_2$%. Bridgman stated that any concentration dependence was less than the rather large variations found in the s values (up to 10 units). Bell and coworkers[9] gave s^w_{20} values of 81 for liver glycogen and 58 for muscle glycogen, both at 1% in molar sodium chloride. These are seen to compare to our values of 88 and 44 units at 1% in water.

Bridgman reported diffusion coefficients in the range of 5 to 11 $\times$ 10^{-8} cm.^{2} sec.$^{-1}$ for samples of liver glycogen. Bell, *et al.*, reported values of 12.1 and 12.7 for liver samples and 15.5 for a muscle sample.

The sedimentation and diffusion results reported in Tables II and III show that an ethanol fractionation yields two fractions considerably different in average behavior. Undoubtedly the properties reflect the great molecular heterogeneity of the original samples, but also include various interaction effects between solute particles and perhaps solvent which are functions of the size and shape of the particles themselves. To illustrate, the sedimentation coefficient of the original M-1 is smaller than for either of the two fractions at concentrations of 1%. As already noted, corrections for concentration dependence of the liver and muscle glycogens are best fitted by different expressions.

The indicated proportionality between the limiting sedimentation coefficient and the square root of the molecular weight is consistent with results from other polymer systems in which the molecules behave as random swollen coils entrapping solvent.[12,16,17] We would point out that our observed relationship is, at best, relatively crude. Indeed,

(15) W. B. Bridgman, THIS JOURNAL, **64**, 2349 (1942).

(16) P. J. Flory, "Principles of Polymer Chemistry," Cornell Univ. Press, Ithaca, N. Y., 1953, Chapt. 14.

(17) J. J. Scholz, Ph.D. Thesis, University of Illinois, 1954.

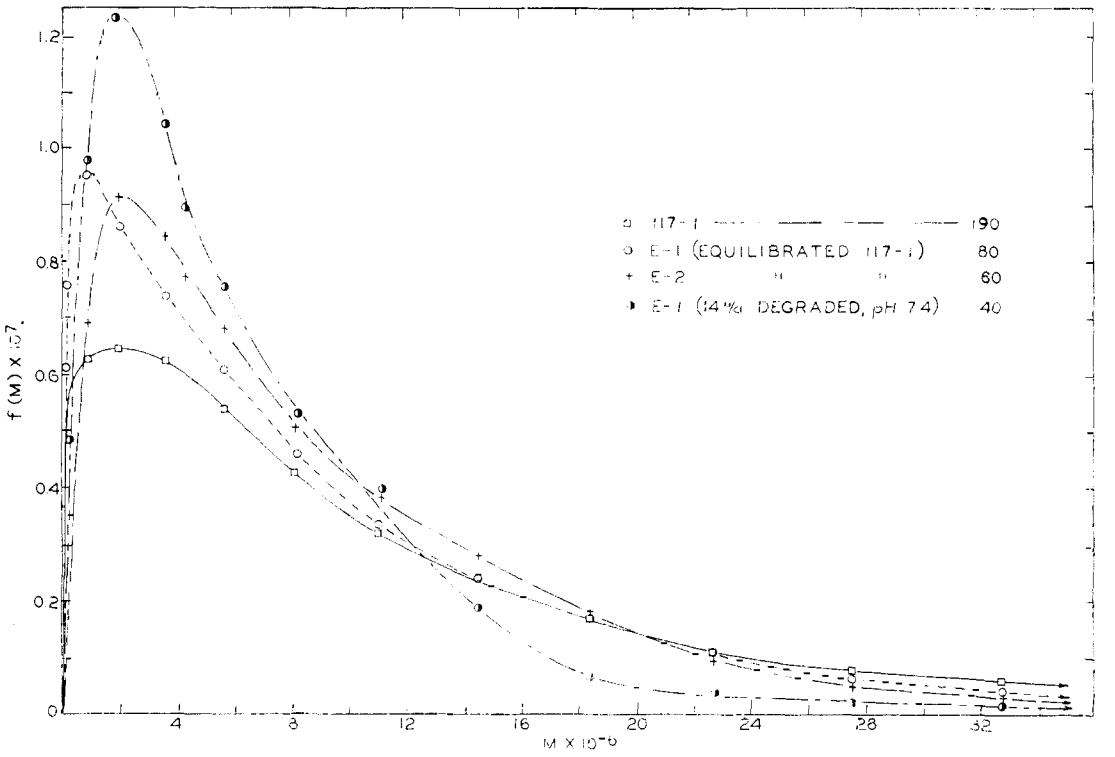

Fig. 2.—Molecular weight distributions of rabbit liver glycogens: original (117-1), after equilibration (E-1 and E-2), and following degradation of E-1.

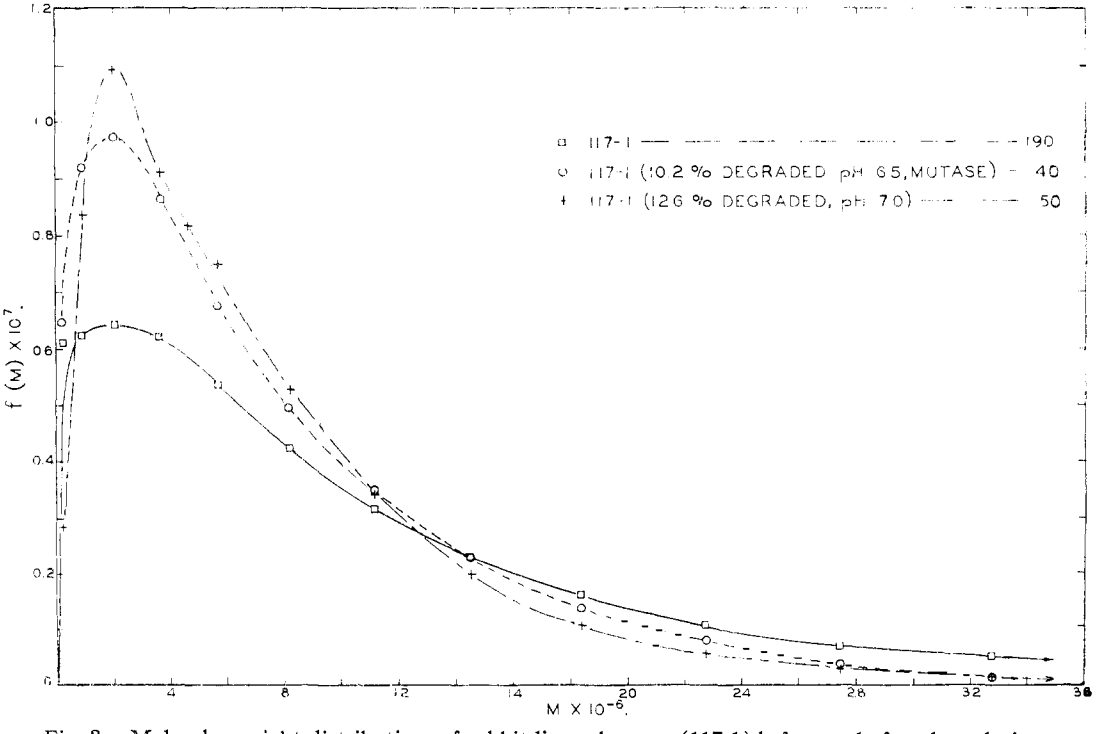

Fig. 3.—Molecular weight distributions of rabbit liver glycogen (117-1) before and after degradation.

we should expect a more precise correspondence with the weight average molecular weight rather than with $M_{s,D}$. It may sometime be desirable to transpose the molecular weight distribution curves presented here back into sedimentation distribution curves if a more accurate relationship between molecular weight and sedimentation coefficient becomes available.

A few comments may be made about the application to these samples of the Baldwin–Williams

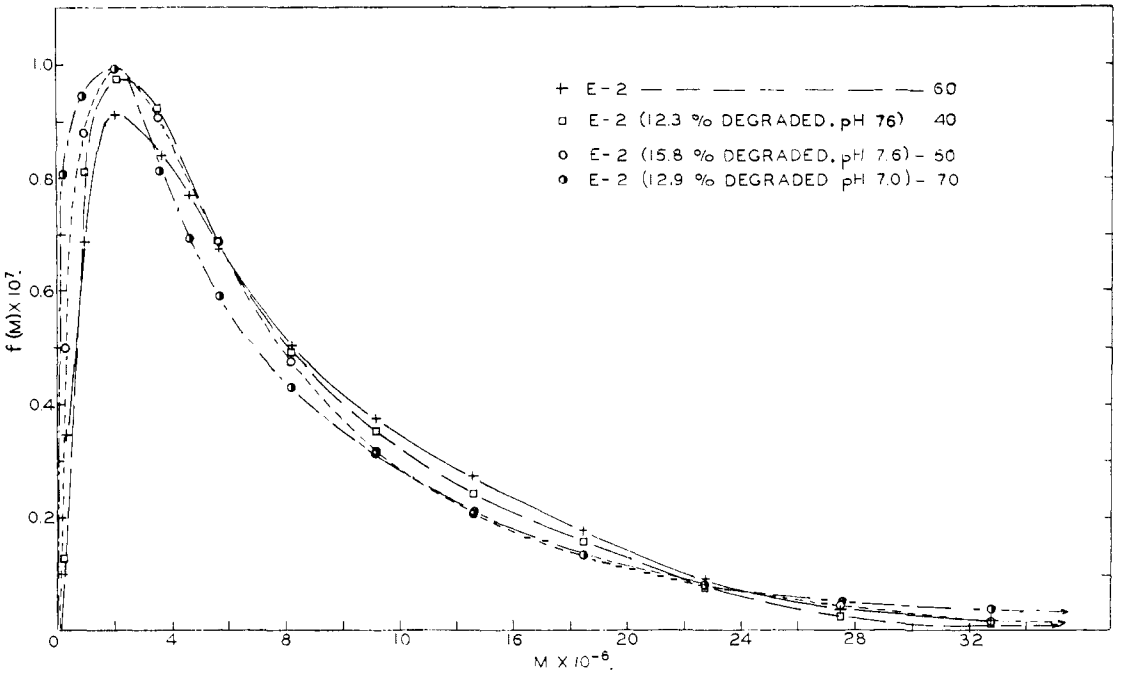

Fig. 4.—Molecular weight distributions of equilibrated liver glycogen (E-2) before and after degradation.

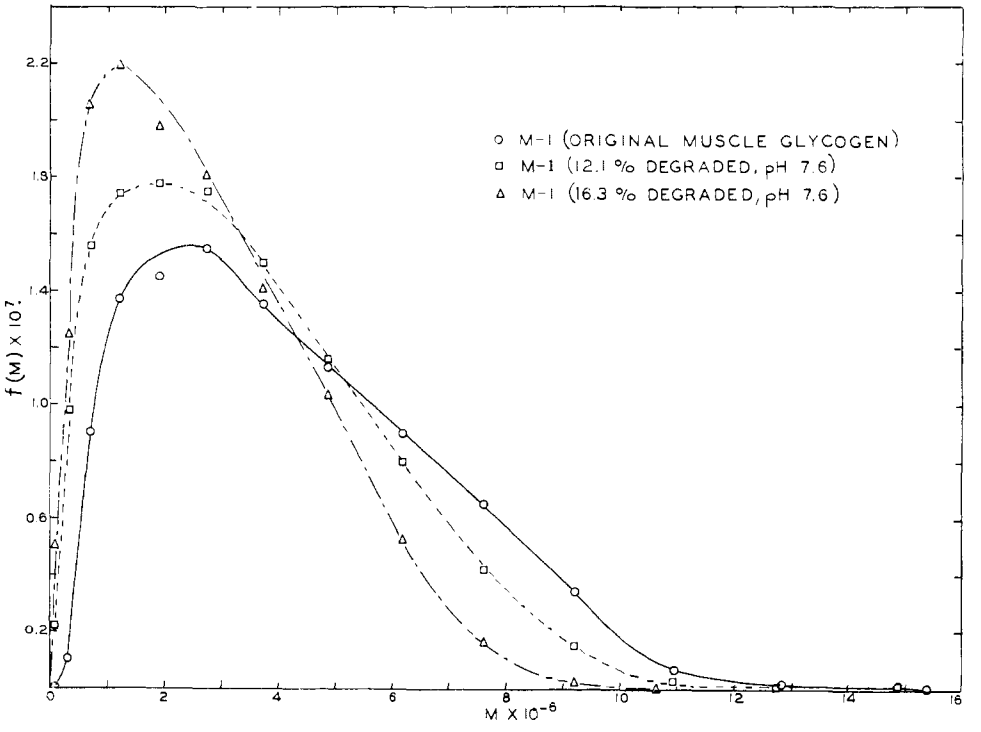

Fig. 5.—Molecular weight distributions of rabbit muscle glycogen (M-1) before and after degradation.

method for estimating the distribution of sedimentation coefficients. In the first place, the correction of $g^*(s)$ for diffusion may very well be unnecessary because of the high molecular weight. On the other hand, the samples appear to show an appreciable fraction of material below one million. Also, the sedimentation runs were rather short, and the effect of diffusion is maximum at the start.

If diffusion and concentration effects are negligible then plots of $g^*(s)$ *vs.* $1/xt$ should be horizontal straight lines instead of the curved lines which have been reported for systems in which diffusion was appreciable. It was of interest to us to test the behavior of our polymer samples. As shown by the typical data in Fig. 1, the lines are best plotted straight, but they do tend to slope so as to cut off

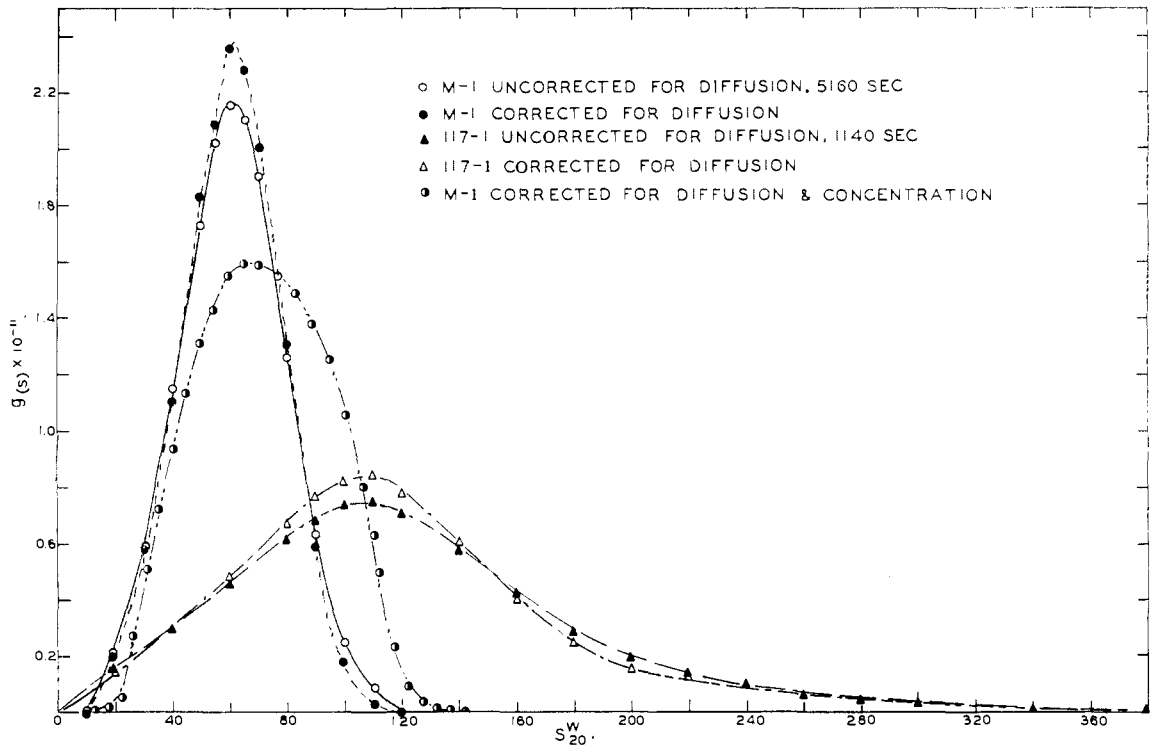

Fig. 6.—Sedimentation frequency functions for glycogen sample M-1 and 117-1 corrected for diffusion and concentration.

the ends of the distribution as happens in the previously studied cases in which diffusion was important. One explanation lies in the concentration dependence of s; the $g^*(s)$ assigned to any calculated value of s should properly refer to a larger s value; this could increase or decrease the slope of $g^*(s)$ as the calculations progressed from one side of the distribution to the other and act as a partial correction for concentration dependence. If so, the analytical corrections applied to $g(s)$ to obtain $g(s_0)$ might tend to overcorrect. The complex problem of concentration dependence is treated in the valuable contribution of Baldwin.[14]

The magnitudes of the different treatments applied to the sedimentation frequency functions can be judged from Fig. 6 where the functions for the original liver and muscle glycogens are reproduced. In each pair of curves, the broader curve is obtained when $g^*(s)$, at 1% concentration, is plotted and the sharper curve when the extrapolated value $g(s)$ is plotted. It can be seen that the effect of the $1/xt$ extrapolation is relatively small; the molecular weight distributions presented here would not be greatly displaced nor would their relative characteristics be significantly affected by its omission. However, the correction of $g(s)$ for concentration dependence to yield $g(s_0)$ is relatively large. This can be seen in Fig. 6 where $g(s_0)$ *vs.* s for the muscle glycogen is also plotted.

Changes in polysaccharide molecular weight distributions after enzymatic treatment will be discussed with regard to, first, the effect of equilibration, and second, the effect of degradation.

Equilibration.—Shown in Fig. 2 is the molecular weight distribution of original liver glycogen, sample 117-1. It is obviously very heterogeneous with particles of the order of 200 million[18] yet with an appreciable fraction below 1 million. The characteristics of the two equilibrated samples, E-1 and E-2, are shown for comparison. Equilibrations were done by incubating portions of 117-1 with small amounts of glucose 1-phosphate in the presence of relatively large amounts of crystalline muscle phosphorylase under conditions such that phosphate equilibrium was attained rather rapidly. Incubation was then continued for a time. Sample E-1 represents a 3% increase in weight or an increase of an average of $^1/_2$ glucose unit per outer chain; sample E-2 represents a 7% increase in weight or an increase of an average of 1 glucose unit per outer chain.

As is apparent, a pronounced redistribution of molecular weight occurred during equilibration. There was, relatively, a large loss of high molecular weight species and an increase in the 1–8 million range with a sharper maximum at around 2 million. The changes observed appeared to be progressive in nature and indicative of a redistribution of molecular size. The results are compatible with an intermolecular equilibration, *i.e.*, the transfer of glucose from one to another molecule in a manner as to increase the size of small molecules and to decrease the size of the large molecules. They provide no information on whether a simultaneous intramolecular redistribution occurred in addition. These results support the recent enzymatic findings

(18) The mathematical treatment consisted of an incremental analysis of the experimental sedimentation peaks in which 20–30 equal increments were selected. The upper limit, in each case, was taken as one increment beyond the last one to have a finite height. The limits are uncertain to, perhaps, 20% and even more in the case of the largest s values due at least in part to the proportionality between s^2 and M.

interpreted as decreased asymmetry of outer chain size upon equilibration.[1]

Degradation.—Changes resulting from enzymatic degradation are presented in Figs. 2, 3, 4 and 5. Experiments were done with liver and muscle glycogen samples, and with equilibrated liver glycogen samples. Other variations of conditions include degradation at different *p*H values (Figs. 3 and 4) and irreversible degradation in the presence of excess phosphoglucomutase (Fig. 3). The general behavior observed is the disappearance of the high molecular weight tail with a shift of the curve to the left and a sharpening of the maximum around 2 million. This is most pronounced in the case of 117-1 (Fig. 3). In the equilibrated samples E-1 and E-2, the high molecular weight species present in the starting material have already been reduced by prior equilibration, so that the disappearance of this fraction during degradation is decreased. For E-1 the changes are clearly evident (Fig. 2); for the more extensively equilibrated E-2 degradation is accompanied by very small shifts at most (Fig. 4).

That the enzymatic attack is a preferential degradation of the high molecular weight species, and not a preferential resynthesis of the low molecular weight species, is shown by the experiment done in the presence of excess phosphoglucomutase (Fig. 3). Under these irreversible conditions the changes in the high molecular weight material were similar to those observed in the cases of reversible degradation.

The results with the samples of muscle glycogen are given in Fig. 5. The original sample M-1 differs from the original liver material in that it is more homogeneous without an extended high molecular weight tail and with a lower average molecular weight. Degradation produces changes similar to those for liver glycogen with respect to the large species. Here again on degradation, there is an increase of species in the 1–2 million range.

The changes which occur in the small molecular species are not as clearly evident since the distribution curves are compressed in this region.[19] The observed changes are as follows: reversible degradation of 117-1 and E-1 is accompanied by a decrease; irreversible degradation of 117-1 and reversible degradation of E-2 and of M-1 are accompanied by an increase. One interpretation of these results is that a simultaneous equilibration involving the low molecular weight species accompanies reversible degradation. However, either extensive prior equilibration or irreversible degradation will preclude observing this process with the present method of analysis.

It is to be noted that degradations were carried out at *p*H values over the range 6.5 to 7.6. There were no apparent differences in the character of the results.

In any study of the physical properties of biological polymers isolation methods are of great importance. In the present work the original polysaccharides 117-1 and M-1 were isolated following digestion for several hours in concentrated alkali. Presumably, they do not represent the state of the polymer in the cell.[20] The present work concerns the effects of enzymatic treatment on these isolated polysaccharides. After enzymatic treatment, the samples were re-isolated in high yield either by brief alkaline hydrolysis (1–2 minutes, 10–15% NaOH) followed by ethanol precipitation or else directly by ethanol precipitation without alkaline treatment.

That the changes we observe are due to enzymatic activity rather than to the method of re-isolation is shown by two types of controls. First, a sample of 117-1 containing phosphorylase was divided into two equal portions and the glycogen re-isolated from one portion by the alkali method and from the other by ethanol alone. In both fractions over-all recovery was 87%. Using the twin-cell technique, the ultracentrifugal patterns were simultaneously obtained for the two re-isolated samples. The patterns were identical. Furthermore, when the alkali re-isolated sample was compared with original 117-1 using the twin-cell technique, the sedimentation curves were again identical. Second, an experiment was performed in which 117-1 was reversibly degraded 21%, re-isolated without alkali, and fractionated. As shown in Table II only 33% precipitated in 13–15% ethanol as compared to 76% for the starting material indicating that the high molecular weight fraction had been drastically reduced.

It is known that phosphorylase attacks the terminal glucose units of the outer chains of glycogen liberating glucose 1-phosphate. If the enzyme attack were random with regard to molecular size the reduction in size of a molecule would be proportional to the number of non-reducing end groups which, in turn, is proportional to the molecular weight. The molecular weight distribution of the degraded sample would, therefore, be related to the distribution of the original sample by

$$M' = (1 - f)M$$

where M' and M are the molecular weights after and before degradation, respectively, and f is the degree of degradation. The high molecular weight tail will be reduced and the maximum of the distribution shifted to the left. However, the decrease in large species will only be equal to the per cent. of degradation, perhaps 10 to 12%, whereas the observed decrease was in the range of 30 to 40%. In addition, the maximum is too far to the left. Clearly, this mechanism is not compatible with the observed results.

If the degree of degradation f is now made proportional to the molecular weight it is possible to reproduce more closely the experimental findings. Here the tail and the maximum are adequately reproduced, but the intermediate portion of the curve is slightly elevated. It appears that a more extensive treatment is required accurately to describe the experimental curves. A physical interpretation of a proportionality between f and M would suggest some sort of "trapping" or "holding" mechanism whereby the probability of repeated enzymatic attack is higher per outer chain for a large polysaccharide molecule. It should also be

(19) Using samples M-1 and 117-1, the effects of degradation in the low molecular weight range were accentuated by plotting the difference in the unnormalized $g^*(s)$ functions before and after degradation.

(20) A. Lazarow, *Anat. Rec.*, **84**, 31 (1942).

mentioned that the variation in outer chain size as a function of molecular weight has not as yet been established. If this were the case, it might directly bear upon the results. It is hoped that a more complete understanding of our observations will follow from a continuation of these studies.

Our results indicate that the high molecular weight glycogen species are enzymatically more readily attacked when incubated with the phosphorylase. It has been shown recently by Stetten and Stetten[21] that the turnover of liver and muscle glycogen *in vivo* follows the pattern proposed by previous enzymatic studies *in vitro*,[22] the outer chains being metabolically most active. The work reported here would suggest that if the present enzymatic studies may be similarly extended to the whole animal, it is the outer chains of the high molecular weight species which are subject to most rapid turnover, so far as phosphorylase action is concerned.

We would point out that the present experiments do not rule out the possibility of a splitting of a weak linkage in these polysaccharides by either an enzymatic or non-enzymatic reaction.

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URBANA, ILLINOIS

(21) M. R. Stetten and D. Stetten, *J. Biol. Chem.* **213**, 723 (1955).
(22) J. Larner, *ibid.*, **202**, 491 (1953).

[CONTRIBUTION FROM THE SECTION ON PHYSICAL CHEMISTRY, NATIONAL INSTITUTE OF MENTAL HEALTH]

The Fractionation of Ribonucleic Acids on ECTEOLA-Cellulose Anion Exchangers[1]

BY D. F. BRADLEY AND A. RICH

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Cellulose treated with epichlorohydrin and triethanolamine has been investigated as an anion exchanger for fractionating ribonucleic acid (RNA). RNA is adsorbed from 0.01 *M* neutral phosphate buffer and eluted with sodium chloride solutions of increasing concentration. Elution profiles differ significantly for RNA's from different sources as well as from the same source isolated by different methods. RNA's with higher mean sedimentation coefficients are eluted at higher salt concentrations and the fractions eluted at these higher salt concentrations have higher mean sedimentation coefficients. However, the fractions are nearly as heterogeneous with respect to sedimentation coefficient as the starting material indicating that sedimentation velocity of the adsorbed RNA is not the only factor determining the elution process.

Introduction

Many lines of evidence have accumulated which point to an intimate association between ribonucleic acid (RNA) and protein synthesis. If such a relationship exists, it is most probable that there exists an inherent heterogeneity in intracellular RNA which reflects the large variety of proteins which are synthesized in a single cell. This study was prompted by a desire to resolve some of this heterogeneity by developing methods for fractionating RNA.

In addition to this inherent heterogeneity, there is also a superimposed artifact heterogeneity which arises during the isolation of the RNA from the living organism. At the present time, we cannot clearly separate these two sources of heterogeneity.

For this study, we have adopted an ion-exchange adsorbent of cellulose treated with epichlorohydrin and triethanolamine (ECTEOLA). Bendich, Fresco, Rosencranz and Beiser[2] have used this material to fractionate desoxyribose nucleic acid (DNA) and this has stimulated us to try various forms of ECTEOLA on RNA. In this study, we have fractionated several RNA's from different sources prepared by different methods, and have measured the heterogeneity of the resultant fractions.

(1) Presented in part at the 128th National Meeting of the American Chemical Society at Minneapolis, Minn., Sept. 11, 1955.
(2) A. Bendich, J. R. Fresco, H. S. Rosencranz and S. M. Beiser, THIS JOURNAL, **77**, 3071 (1955).

Experimental

Samples of ECTEOLA were generously contributed to this study by Drs. Peterson and Sober of the National Cancer Institute.[3] The ECTEOLA was treated with 1.0 *M* NaOH, 1.0 *M* KH_2PO_4 and 0.01 *M* neutral phosphate buffer in succession before every run.

Samples of RNA were generously contributed to this study by Drs. E. Volkin of the Oak Ridge National Laboratory (calf liver),[4] S. A. Morell of the Pabst Laboratories (yeast-A), F. W. Allen of the University of California (yeast-C),[5] C. A. Knight of the University of California (tobacco mosaic virus (TMV) RNA),[6] E. Mihalyi of the National Heart Institute (muscle)[7] and E. L. Grinnan and W. A. Mosher of the University of Delaware (rat liver).[8] A commercially available preparation from Nutritional Biochemicals was also used (yeast-B). Solutions of the RNA's in 0.01 *M* neutral phosphate buffer were analyzed for RNA concentration from their optical absorption at 260 mμ, assuming O.D. (1 cm. cell, 1% solution) = 200. The fractionations were all performed at room temperature, 22–24°, unless otherwise indicated.

In the kinetic experiments various volumes of ECTEOLA suspension and RNA solution were mixed, shaken and centrifuged after a measured contact time. Adsorption from salt solution was performed by adding solid NaCl to the RNA solution prior to mixing with the ECTEOLA. Desorption into salt solution was performed by mixing the ECTEOLA and RNA solutions, shaking for 20 minutes, adding solid NaCl to the mixture, shaking and centrifuging

(3) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).
(4) E. Volkin and C. E. Carter, *ibid.*, **73**, 1516 (1951).
(5) A. Crestfield, K. C. Smith and F. W. Allen, *J. Biol. Chem.*, **216**, 185 (1955).
(6) C. A. Knight, *ibid.*, **197**, 241 (1952).
(7) E. Mihalyi and D. F. Bradley, in preparation.
(8) E. L. Grinnan and W. A. Mosher, *J. Biol. Chem.*, **191**, 719 (1951).