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THE ISOLATION AND CHARACTERIZATION OF SPLEEN RIBONUCLEIC ACID

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ELLIOT VOLKIN AND CHARLES E. CARTER

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THE ISOLATION AND CHARACTERIZATION OF SPLEEN RIBONUCLEIC ACID*

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Although mammalian desoxyribonucleic acids have been extensively studied[1], the preparation and characterization of high molecular weight ribonucleic acids from mammalian tissues have received little attention. In view of the apparent dependence of the composition of ribonucleic acids upon the method of preparation as well as the source[2,3,4], certain criteria appear necessary to the assessment of physical and chemical descriptions of ribonucleic acid preparations; first, the preparative procedure and source; second, a statement of the physical-chemical homogeneity and particle size of the ribonucleic acid; third, a description of the degradation of the ribonucleic acid by ribonuclease in order to determine how many susceptible bonds are present in the molecule; and fourth, a statement of the purine and pyrimidine composition of the ribonucleic acid. A preparation of ribonucleic acid obtained from beef spleen by a procedure involving the separation of the ribonucleic acid by high concentrations of guanidine has been studied with respect to these criteria.

The ribonucleic acid isolated by Cohen and Stanley[2] from tobacco mosaic virus exhibited a molecular weight of 300,000, and ribonucleic acids of large particle size have been isolated from bacterial surfaces by Stacey et al[5]. In general, the preparations which have been studied by physical-chemical techniques were of smaller particle size. An estimated molecular weight of yeast ribonucleic acid of about 17,000 was reported by several investigators[3,4], while an alkaline extracted sample of tobacco mosaic virus ribonucleic acid was found to have a molecular weight of 37,000[3]. The native molecular character of ribonucleic acid is presumably not reflected in the latter descriptions because of the degradation involved in the preparative procedures.

* A revision of section by same title appearing in ORNL-244, Biol. Div. Quart. Report for the period ending May 15, 1949.

Ribonucleic acid isolated from beef spleen by the procedure described below was essentially homogeneous in the analytical ultracentrifuge, and exhibited a sedimentation constant (S_{20},W) of 3.09 S which, in the absence of diffusion and partial specific volume data, might indicate a molecular weight of from 30,000 to 50,000. The difference between the molecular weight of the virus nucleic acid prepared by Cohen and Stanley[2] and beef spleen ribonucleic acid may be due to inherent structural differences or to factors introduced in the isolation procedure.

Ribonuclease liberated 1.5×10^{-6} equivalents of titratable acid per mg. of spleen ribonucleic acid under conditions which, in similar experiments with commercial yeast ribonucleic acid, yielded 7×10^{-7} equivalents of titratable acid per mg. Presumably the spleen ribonucleic acid preparation had approximately twice as many linkages susceptible to nuclease degradation as yeast ribonucleic acid. This may be due to differences in molecular weight or configuration. The mononucleotide products of ribonuclease degradation of yeast ribonucleic acid, determined by ion exchange and chromatographic procedures, proved to be predominantly uridylic and cytidylic acids (Carter and Cohn)[6], in agreement with the data of Schmidt[7]. The nuclease degradation of spleen ribonucleic acid follows a qualitatively similar pattern. A study of the nuclease degradation of mammalian ribonucleic acids will be reported elsewhere.

The composition of spleen ribonucleic acid has been determined by ion exchange analysis of the constituent mononucleotides following alkaline degradation of the nucleic acid. Since the classical statement of the tetranucleotide structure of yeast ribonucleic acid by Levene[8] several investigators have presented evidence which was not in agreement with such a simplification. Gulland et al.[1], by titrimetric and elementary analysis concluded that a

tetranucleotide structure was untenable and Vischer and Chargaff[9], employing techniques of quantitative paper chromatography, found molar proportions of the basic constituents of ribonucleic acids which did not support a tetranucleotide structure. Although the evidence presented in this paper for the composition of spleen ribonucleic acids is at variance with the equimolecular proportions of the nucleotides required by the Levene hypothesis, judgment regarding the ultimate validity of the tetranucleotide structure of ribonucleic acids may be withheld until the influence of preparative methods on the analytical composition of the nucleic acid has been studied more extensively.

EXPERIMENTAL

Method of Preparation of Calf Spleen Ribonucleic Acid. - The method of isolation of ribonucleic acid from a spleen homogenate consisted of (a) the removal of desoxyribonucleic acid as a nucleic acid-protein complex[10], (b) the precipitation of the ribonucleic acid from a 4-M guanidine hydrochloride solution in which the large bulk of protein remains soluble, and (c) further purification of the ribonucleic acid by chloroform extraction[11].

This procedure was found to be applicable to a number of mammalian tissues, the ribonucleic acid composition of which will be presented in another publication. The method of preparation of ribonucleic acid from calf spleen is presented below in detail:

Frozen calf spleen (200 g.) was cut in small pieces and blended for eight to ten minutes with 2 volumes per gram tissue of 0.15 M NaCl-0.03 M phosphate buffer, pH 6.8. The homogenate was filtered through 4 thicknesses of cheese cloth, then centrifuged at 3000 g. for sixty minutes. By this process the desoxyribonucleic acid was removed in the form of an insoluble nucleic

acid-protein complex as described by Mirsky and Pollister[10]. All operations were carried out between 2-6°:

To the supernatant solution, enough solid guanidine hydrochloride was added, with rapid stirring, to make the solution 4 molar with respect to guanidine hydrochloride.

The solution was then placed in a 38° water bath for one hour, after which it was immediately chilled at 0-2° and allowed to stand at this temperature for one hour. Under these conditions most of the protein of the splenic extract remains soluble, while a gelatinous precipitate forms which contains ribonucleic acid and a small amount of protein. The precipitate was washed with a solution of 4 M guanidine hydrochloride in pH 6.8 saline-phosphate buffer, and dissolved in water. The turbid solution was allowed to dialyze exhaustively against water after which insoluble denatured protein was removed by centrifugation. The supernatant solution containing the ribonucleic acid was further purified by adjusting the acidity of the solution to pH 4.2 with acetic acid and precipitating the nucleic acid with 2 volumes of cold ethanol. Upon redissolving in water, additional protein remained insoluble and was removed by centrifugation.

The final trace of protein was removed by twice extracting the aqueous solution of nucleic acid with chloroform-octyl alcohol[11]. A solution of nucleic acid in water was shaken with an equal volume of a 5:1 chloroform-octyl alcohol mixture for one hour. The mixture was centrifuged and the upper aqueous layer containing the nucleic acid removed. The ribonucleic acid was then reprecipitated by adjusting the solution of pH 4.2 with acetic acid and adding 2 volumes of alcohol. The precipitate was washed with alcohol and ether and dried in a vacuum desiccator, or lyophilized.

The dried ribonucleic acid was a white hygroscopic powder which readily dissolved in water to give a water-clear solution. From 200 g. of whole spleen, a yield of about 500 mg. of purified ribonucleic acid was obtained. The nucleic acid preparation gave an intense orcinol reaction[12] while even the most concentrated solutions failed to give a reaction with diphenylamine reagent[13], indicating that all of the nucleic acid was of the ribose type. Analysis of the spleen ribonucleic acid revealed 8.74% phosphorus and 15.2% nitrogen; N/P = 1.74.

Spectrophotometric Assay. - Figure 1 shows the ultraviolet absorption spectra of (a) nucleic acid, precipitated once with alcohol but not purified by chloroform extraction, and (b) the final product, purified by chloroform extraction. It can be seen that the absorption curve of the purified product is quite characteristic of a nucleic acid. The presence of protein in the impure material can be readily detected by the markedly increased absorption at wave lengths below 240 mμ.

Sedimentation in the Analytical Ultracentrifuge. - The degree of molecular homogeneity of the ribonucleic acid preparations was determined by means of the analytical ultracentrifuge. Figure 2 shows the sedimentation-velocity photographs of the nucleic acid preparations described above, before purification by chloroform extraction (A), and after chloroform extraction (B). The conditions of the experiments are given in the text of the figure. The photographs of the impure nucleic acid (A) show the presence of a major component with a sedimentation constant $S_{20,W} = 3.11$, along with about 40% of faster sedimenting material. Since the spectrophotometric assay of this material indicated the presence of about 85% ribonucleic acid it may be inferred that the faster moving components are protein-nucleic acid complexes. Photographs of the purified material (B) indicate that only about 5% of the nucleic acid preparation is a faster

Figure 1.

The ultraviolet absorption curves of spleen ribonucleic acid preparations.

X = Crude preparation, before chloroform extraction.
Concentration = 0.08 mg. per cc.

O = Purified preparation, after chloroform extraction.
Concentration = 0.07 mg. per cc.

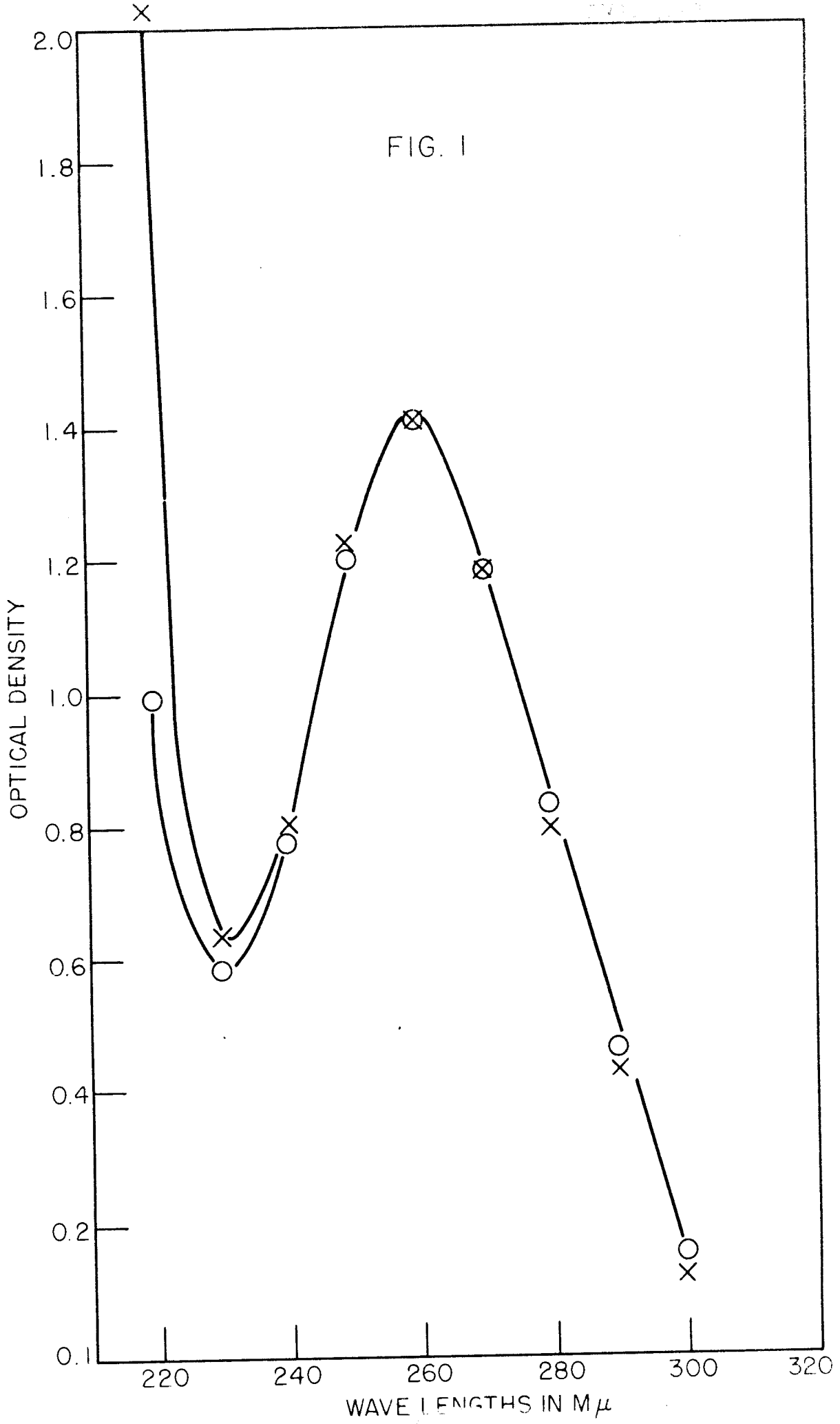
FIG. 1
OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.1
220
240
260
280
300
320
WAVE LENGTHS IN Mμ

Figure 2.

Analytical ultracentrifuge photographs of spleen ribonucleic acid preparation.

A. The mean ultracentrifugal field was 185,500 g. Photographs were taken at 8-minute intervals. The inclined bar in the Philpot-Svensson optical system was at a 65° angle for the first three pictures and at a 45° angle for the last two. The concentration was 12 mg. per cc. dissolved in 0.2 M NaCl, 0.05 M phosphate buffer, pH, 6.7.

B. The mean centrifugal field was the same as in A, while the photographs were taken at 16-minute intervals and the angle of the inclined bar was 65° throughout. The concentration was 15 mg. per cc., dissolved in the same buffer as A.

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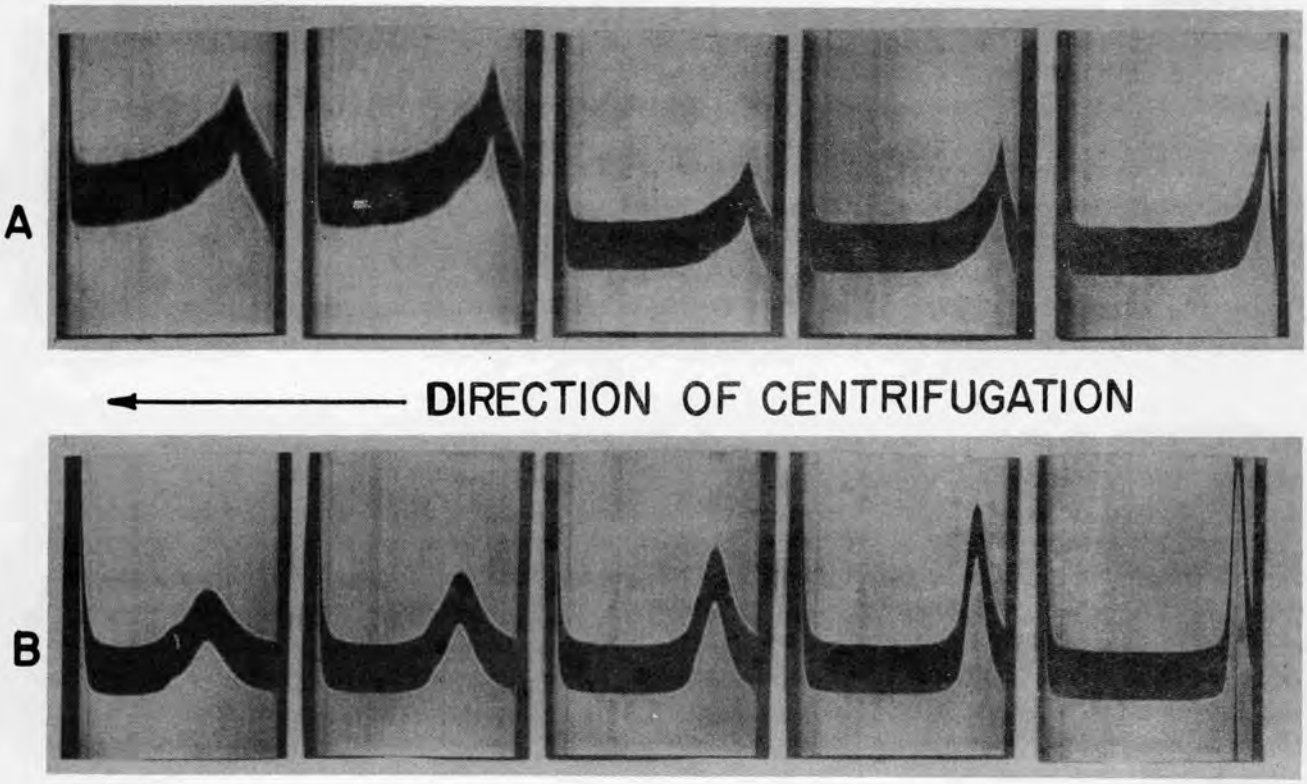

FIG. 2

Analytical Ultracentrifuge Photographs of Spleen Ribonucleic Acid Preparations

A. This mean ultracentrifugal field was 185,500 g. Photographs were taken at 8-minute intervals. The inclined bar in the Philpot-Svensson optical system was at a 65° angle for the first three pictures and at a 45° angle for the last two. The concentration was 12 mg per cc, dissolved in 0.2 M NaCl 0.05 M phosphate buffer, pH 6.7.

B. The mean centrifugal field was the same as in A, while the photographs were taken at 16-minute intervals and the angle of the inclined bar was 65° throughout. The concentration was 15 mg per cc, dissolved in the same buffer as A.

moving component. The sedimentation constant of the major component was $S_{20,W} = 3.09$ S.

The Nucleotide Composition of Spleen Ribonucleic Acid. - Mild alkaline degradation of ribonucleic acids results in the formation of monoribonucleotides. These components may be resolved and quantitated by the anion-exchange procedure described by Cohn[14]. In addition to the mononucleotide product, alkaline degradation under the conditions employed results in the formation of small amounts of adenosine. The low affinity of this nucleoside for the anion resin affords a sharp separation from the nucleotides.

For analysis the ribonucleic acid was dissolved in 0.5 N NaOH at a concentration of 20 mg. per ml. and kept at 37° C. for 17 hours. The digest was then partially neutralized by the addition of one-half volume of 0.5 N HCl and diluted to 100 ml. This solution was run through the anion-exchange resin (Dowex-1, bed size 8 cm. by 0.72 cm.^{2}, which had previously been converted to the chloride form) followed by 100 ml. of water, 20 ml. of 0.05 N NaCl and 10 ml. of 0.01 N HCl. The effluent collected to this stage contains adenosine which was identified by paper chromatography[15] and by ultraviolet spectrum. This component was then quantitated in the spectrophotometer at pH 3.0 employing the molecular extinction coefficient of 13,900. Since a rigorous ion-exchange analysis of this preliminary wash fraction was not performed, very small amounts of other ribosides and bases may be present. However, these could not be detected by paper chromatography.

The elution of mononucleotides by increasing concentration of HCL follows the procedure described by Cohn[15]. All ribonucleic acids examined in this laboratory which have been isolated from mammalian tissues or microorganisms, have been found to contain two isomeric guanylic acids[14,15]. Thus, in the fractionation scheme employed, six nucleotide components are

separated as illustrated in Figure 1. Ninety-seven per cent of the nucleic acid phosphorus analyzed was recovered in the nucleotide fractions.

The composition of spleen ribonucleic acid (Figure 3) is similar to that of pig liver ribonucleic acid reported by Chargaff, et al.[16] and to rat liver ribonucleic acid[17]. The high content of cytidylic acid and guanylic acid is characteristic of these preparations.

The Action of Crystalline Ribonuclease on Beef Spleen Ribonucleic Acid. - In Figure 4 the time course of the enzymatic degradation of purified spleen ribonucleic acid by crystalline ribonuclease is shown. The enzymatic hydrolysis of spleen ribonucleic acid liberated a total of 1.5×10^{-6} equivalents of H^+ ions per milligram of nucleic acid, which was twice the amount found when the enzyme acted on commerical yeast ribonucleic acid[6]. This difference may be attributed to either the inherent structural differences of the two nucleic acids, or to the fact that spleen ribonucleic acid by virtue of its higher molecular weight and presumably less degraded state possesses more susceptible bonds per mg. of substrate.

Previous work in this laboratory[15] confirmed Schmidt's finding[7] that pyrimidine nucleotides are the principal mononucleotide products of the enzymatic hydration of yeast ribonucleic acid by crystalline ribonuclease. The degradation of spleen ribonucleic acid by this enzyme also results in the formation of uridylic and cytidylic acids, identified by ion exchange analysis[14] and paper chromatography[15], and little or no purine mononucleotide.

SUMMARY

A new method of preparation of ribonucleic acids from mammalian tissue involving their precipitation by 4-M guanidine hydrochloride is described.

Employing this method, a purified ribonucleic acid from calf spleen has

Figure 3.

Anion-exchange chromatography of spleen ribonucleic acid mononucleotides. Conditions for the adsorption and elution of the mononucleotides are described in the text. The milligram yields of the individual mononucleotides and adenosine isolated from the column are summarized at the right of the figure. From these quantities the percent composition and molar ratio of the mononucleotides in spleen ribonucleic acid have been calculated.

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FIGURE 3

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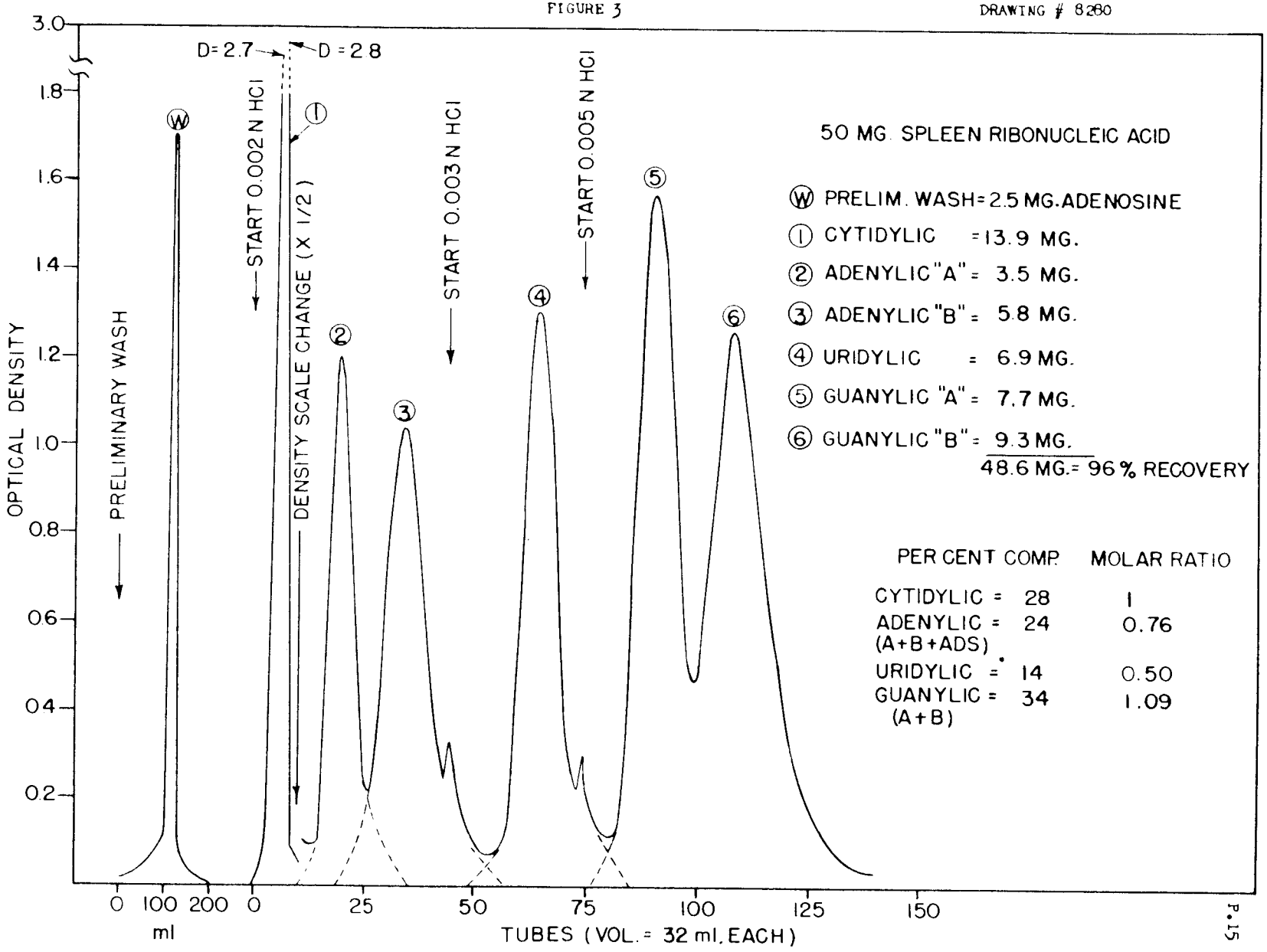

Figure 4.

The enzymatic degradation of spleen ribonucleic acid by crystalline ribonuclease.

Forty mg. of ribonucleic acid were dissolved in 2.5 cc. of water and the solution adjusted to pH 7.5 with dilute NaOH. Then 2 mg. of solid crystalline ribonuclease were added to the solution under stirring. As the reaction progressed the solution was maintained at pH 7.5 by the addition of 0.01 N NaOH from a 1.0-ml. burette graduated in divisions of 0.01 ml. The additions of 0.01 N NaOH with time are shown in the figure.

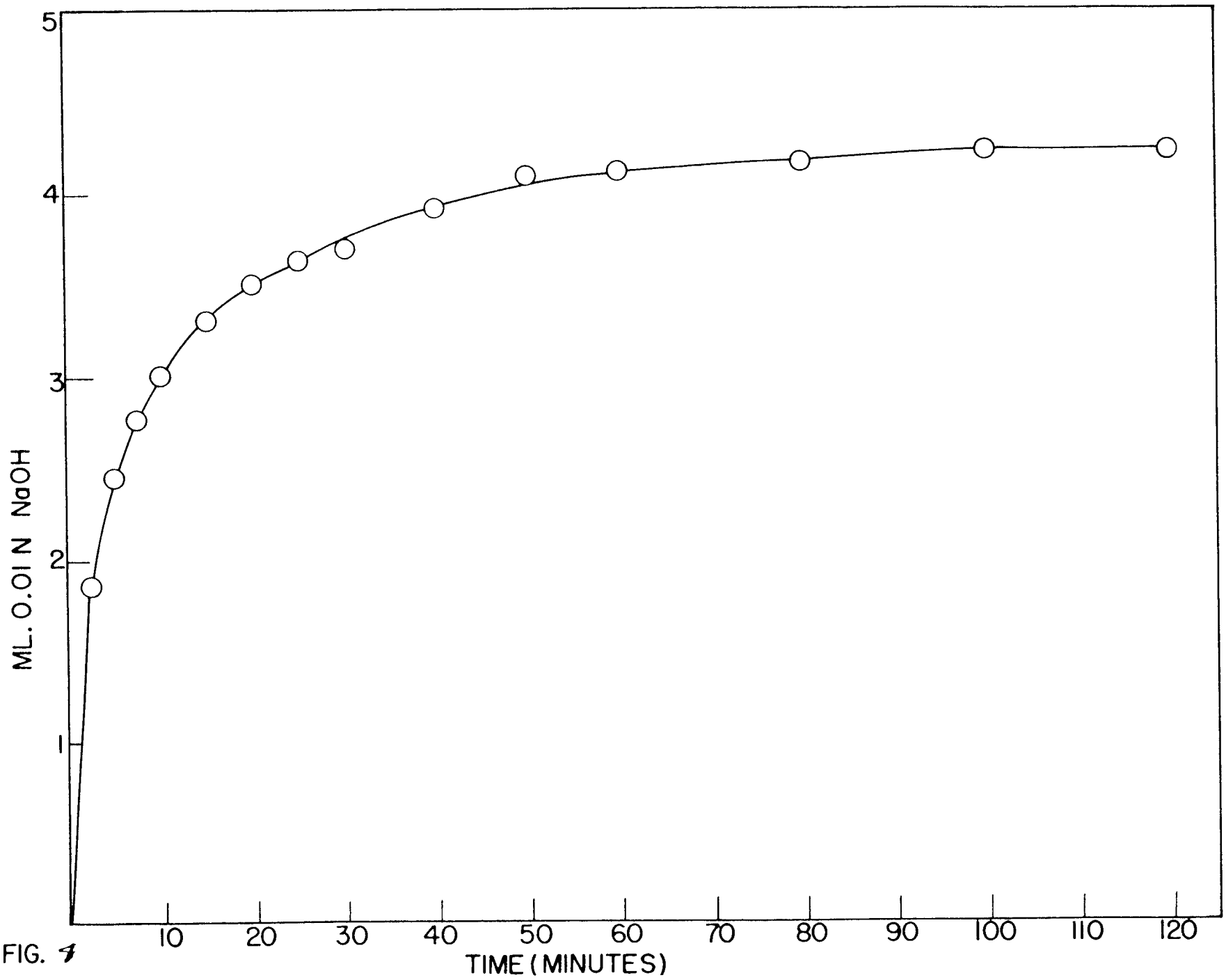

FIG. 4

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been prepared which is essentially homogeneous in the analytical ultracentrifuge and under the conditions employed exhibited a sedimentation constant of 3.09 S.

The composition of the spleen ribonucleic acid, determined by ion-exchange analysis of the constituent mononucleotides, was found to be 28% cytidylic acid, 24% adenylic acid, 14% uridylic acid and 34% guanylic acid.

The enzymatic degradation of spleen ribonucleic acid by crystalline ribonuclease liberated a total of 1.5×10^{-6} equivalents of H^+ ions per milligram of nucleic acid.

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