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The Preparation of Sodium Ribonucleate with the Use of Sodium Dodecyl Sulfate[1]

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The following paper describes a method of preparing sodium ribonucleate from a variety of tissues with the use of sodium dodecyl sulfate. The product from normal adult tissues is protein free, as shown by the negative biuret and Sakaguchi reactions, and also by the absence of detectable amino acids in a hydrolysate of the material. The molecular weight of the isolated product was relatively high as estimated on the basis of viscosity and sedimentation behavior. The ultraviolet absorption curve, with values of $E^{1\%}_{1\,cm.}$ between 190 and 195, also indicates that the product is relatively undegraded. The yield of the purified material is about 50% as based on the preparation from rat liver.

This paper describes a method for the preparation of sodium ribonucleate from a variety of tissues, using sodium dodecyl sulfate as the extracting and dissociating agent. This reagent has been used in the preparation of sodium desoxyribonucleate[2] and this work is therefore a further extension of the use of the reagent to the preparation of pure sodium ribonucleate.

The use of sodium dodecyl sulfate as a protein denaturant seems to have been first mentioned by Bull and Neurath.[3] Subsequently Bawden and Pirie[4] found that preparations of bushy stunt virus could be destroyed by incubation with sodium dodecyl sulfate. Similarly Sreenivasaya and Pirie[5] found that sodium dodecyl sulfate disintegrated tobacco mosaic virus with the subsequent release of the nucleic acid. In the method described in this paper for preparing ribonucleic acid, the ribonucleoproteins are first separated from the desoxyribonucleoproteins of the homogenate by the use of physiological saline, which keeps the latter material insoluble.[6] The ribonucleoproteins are then precipitated from the sodium chloride solution by lowering the *p*H to 4.5 in the cold. The ribonucleic acid is next gradually dissociated from the ribonucleoprotein complex by means of sodium dodecyl sulfate. This detergent appears to block the action of enzymes which may partially degrade ribonucleic acid.

The dissolved nucleic acid is precipitated in the presence of molar NaCl by adding two volumes of ethanol. A second treatment with the detergent is employed for further deproteinization, and finally a high speed centrifugation in the cold at a *p*H of 4.5 completes the process of deproteinization.

In most of the recently developed methods for the extraction of ribonucleic acid, the alkaline extractions commonly used in the past for preparing a low molecular weight product from yeast[7,8] have been avoided, since the sensitivity of ribonucleic acid to alkali is now widely appreciated. A ribonucleic acid prepared by heat treatment of tobacco mosaic virus without the use of alkali had a calculated molecular weight of 300,000,[9] this being the highest molecular weight ribonucleic acid thus far obtained. Similar high molecular weight materials have been obtained from other viruses by Markham, *et al.*[10] Ribonucleic acid also has been prepared from pancreas tissue without the use of alkali by Hammarsten,[11] Levene and Jorpes,[12] Kerr and Seraidarian,[13] and Bacher and Allen[14]; and also recently from liver tissues[15,16] and various animal tissues and yeast.[17,18]

Very recently the preparation of ribonucleic acid from a variety of tissues has been accomplished with the use of guanidine hydrochloride.[19] It has been claimed that the use of the latter reagent results in the isolation of a ribonucleic acid quite free of protein and of a relatively high molecular weight, judging by sedimentation in the ultracentrifuge. A further modification of this procedure has been used by Grinnan and Mosher[20] with the incorporation of a short heat treatment of the ribonucleoprotein.

An advantage claimed for methods involving the use of guanidine hydrochloride is that a product of relatively high molecular weight is obtained. Such methods do not however avoid the difficulty of still requiring treatment of the partially purified nucleic acid with the chloroform and octyl alcohol mixture of Sevag, *et al.*,[21] to remove residual protein.

It is quite apparent that the method of preparation of ribonucleic acids is of paramount importance when studies of the nature of this material are being made. It is also of special importance in certain types of work that the ribonucleic acids be as free as possible from contaminating protein. In the method described in this paper a product is obtained from normal adult tissues which has no detectable protein. The yield is about 50% when starting with rat liver.

(1) This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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

Experimental Work and Results

Method of Preparation of Ribonucleic Acid.—The tissue is placed on ice as soon as possible after death of the animal. After transportation to the laboratory, the tissue is frozen and stored at −15° until used. Fifty grams of the frozen liver is chopped into small pieces and placed in the Waring blendor with 200 ml. of ice-cold salt–citrate solution used in the preparation of the DNA.[2] (This solution consists of 0.9% NaCl in a solution of 0.01 *M* Na citrate.) The mixture is blended for 5 minutes at 0–3°. The homogenate thus produced is then spun in a refrigerated centrifuge (0–3°) at 2500 r.p.m. for 30 minutes to remove the insoluble desoxyribonucleoproteins.[6] The supernatant is decanted and is centrifuged again at 2500 r.p.m. in the cold to remove any residual desoxypentose nucleoproteins. The supernatant again is decanted and to it is added with stirring in the cold enough 1 *N* HCl to lower the *p*H to 4.5. The heavy precipitate is then centrifuged at 2500 r.p.m. for 15 minutes in the cold. The red supernatant is discarded. The sediment is mixed with sufficient 0.9% NaCl to make a total volume of 100 ml. Nine ml. of the stock solution of sodium dodecyl sulfate (5% purified sodium dodecyl sulfate in 45% ethanol—see reference 2) is added with stirring. Ten per cent. NaOH is added carefully to the mixture to raise the *p*H to 7, and the stirring is continued for 3 hours, as in the preparation of DNA.[2] After this period sufficient solid NaCl is added to make the concentration 1 *M* with respect to the NaCl. The slightly viscous solution is centrifuged in the Servall centrifuge for 30 minutes at 14,000 r.p.m. The clear supernatant is decanted and the nucleic acid which is present is precipitated by adding two volumes of 95% ethanol. A white flocculent precipitate forms which is centrifuged down and washed with ethanol and acetone, and finally is dried in the air.

The dry material is dissolved in 100 ml. of water, and 9 ml. of the sodium dodecyl sulfate solution is added with stirring. The solution is stirred for 2 hours at room temperature. At the end of the time, sufficient solid NaCl is added to make the concentration 1 *M* with respect to the NaCl. The solution is centrifuged in the Servall centrifuge (14,000 r.p.m.) for 30 minutes. The supernatant is then decanted and the nucleic acid is precipitated by adding two volumes of ethanol. The white flocculent precipitate is centrifuged and washed with ethanol, acetone, and finally is dried in the air.

The white powder from the above procedure is dissolved in 30 ml. of water and the solution is cooled to approximately 0°. Sufficient NaCl is added to make the solution 0.9% with respect to this salt, and sufficient 0.1 *N* HCl is then added carefully to lower the *p*H to 4.5. The solution is centrifuged in the cold at 14,000 r.p.m. for 1 hour. The supernatant is decanted into a beaker containing enough solid NaCl to make the final concentration of NaCl 1 *M*. When the salt is completely dissolved, the *p*H of the solution is raised to 7.0 by the careful addition of 0.1 *N* NaOH to the still cold solution. The nucleic acid is then precipitated as the sodium salt by the addition of two volumes of 95% ethanol. The white flocculent precipitate of Na-PNA is then centrifuged down and is washed with ethanol and acetone, and finally is dried in the air.

The final product is a white powder which dissolves readily in water to give a clear solution. The yield of this product is about 200 mg. (dry weight) when starting with 50 g. of rat liver (wet weight). This yield represents about 50% of the tissue ribonucleic acid on the basis of the Schneider[22] and Schmidt–Thannhauser[23] analyses of rat liver for PNA content. The material fails to give a biuret and Sakaguchi test for protein, and acid hydrolysis and paper partition chromatography for amino acids also have failed to show detectable contamination by protein. The product is also free of desoxy-sugar when made from normal tissues, since it gives no reaction with the diphenylamine reagent.

Analysis of the Sodium Ribonucleate.—Samples of the sodium salts of ribonucleic acids prepared as above were analyzed for both nitrogen and phosphorus. Nitrogen content was determined by the semi-micro Kjeldahl method, while phosphorus was determined by the method of Fiske and SubbaRow.[24] For these analyses, samples of the nucleates were dried *in vacuo* over P_2O_5. The digestion of the various samples was carried out with the sulfuric acid–copper sulfate–selenium mixture as recommended by Jones and co-workers.[25] The results of the analyses are reported in Table I. It can be seen that the nitrogen to phosphorus ratios are essentially the same for the three different mammalian sodium ribonucleates studied. However, the N/P ratio is considerably lower for wheat germ sodium ribonucleate and higher for the tumor ribonucleates. In the latter case the samples were always contaminated with protein, which accounted for the difference.

Viscosity.—The relative viscosity of a sample of sodium ribonucleate was studied over a considerable range of concentration. The NaPNA was dissolved in water and the relative viscosity was determined by means of an Ostwald type viscometer in a water-bath maintained at 30 ± 0.1°. The relative viscosity increased linearly with increasing concentration, reaching a value of 2.25 at a concentration of 3%.

Sedimentation in the Ultracentrifuge.—A sample of rabbit liver sodium ribonucleate was studied in the analytical ultracentrifuge to determine the degree of homogeneity of material prepared by this method. Seventeen mg. of the sodium ribonucleate was dissolved in 1 ml. of a mixture containing 0.2 *M* sodium chloride and 0.05 *M* phosphate buffer *p*H 6.8, as used by Volkin and Carter.[19] The sedimentation constant ($S_{20,w}$) for this sample is reported in Table I. The sedimentation constant for a sample of tumor ribonucleate is also reported in the same table. The latter material was contaminated with protein, and hence the value obtained for the rabbit liver nucleate is more indicative of the true sedimentation behavior of sodium ribonucleate prepared by the method described in this paper. For the determinations of the sedimentation constants, photographs were taken at 16-minute intervals after the maximum centrifugal speed was reached. The mean force was 255,000 *g* and the rotor temperature was between 25 and 27° during the runs.

Ultraviolet Absorption Spectra of Ribonucleates.—The extinction coefficients ($E^{1\%}_{1\ cm.}$) of a few samples of sodium ribonucleate prepared according to the method described in this paper are reported in Table I. All extinction coefficients were similar, and were slightly lower than those reported in the literature for ribonucleates prepared by other procedures. Lower extinction coefficients can mean less degradation of product rather than more contamination with foreign material, since it is known that chemical degradation can very materially increase the extinction coefficients or ribonucleates at 260 mμ.[17,18,26]

TABLE I

ANALYTICAL COMPOSITION OF SAMPLES OF SODIUM RIBONUCLEATE

Source	Nitrogen, %	Phosphorus, %	N/P	$E^{1\%}_{1\ cm.}$	$S_{20,w}$
Rat liver	12.1	7.5	3.58	191	
Calf liver	12.7	7.9	3.57		
Calf pancreas	12.6	7.8	3.58	195	
Rabbit liver					3.20
Wheat germ	12.0	8.8	3.02	190	
Human tumor[a]	13.4	6.0	4.85		
Walker carcinoma[a] (Rat)	13.1	6.1	4.92		2.77

[a] Both samples of tumor ribonucleate were contaminated by protein.

Effects of Acid and Alkaline Hydrolysis on the Extinction Coefficient of Ribonucleic Acid.—In order to verify the work of Chargaff, *et al.*, and of Tsuboi, experiments were performed to determine the effects on the ultraviolet absorption spectra of acid and alkaline hydrolysis of ribonucleic acid prepared from calf pancreas. A stock solution was prepared contalning approximately 2 mg. per ml. of the air-dried sodium ribonucleate. One-ml. aliquots were removed and were placed in 50-ml. volumetric flasks. To

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(26) K. K. Tsuboi, *Biochim. Biophys. Acta*, **6**, 202 (1950).

one flask was added sufficient $M/10$ phosphate buffer, pH 7.0, to dilute to the mark and this solution served as a non-hydrolyzed control sample. To a second flask was added 1 ml. of 10% trichloroacetic acid. The flask and contents were heated at 90° for 20 minutes. The solution was neutralized with NaOH and made up to volume with $M/10$ phosphate buffer of pH 7. To a third flask 1 ml. of 10% trichloroacetic acid was added and the flask and contents were incubated at 37° for 16 hours. The contents were then neutralized with NaOH and made up to volume with the $M/10$ phosphate buffer, pH 7.0. To a fourth flask was added 1 ml. of 1 N NaOH. The flask and contents were heated for 30 minutes at 95°. The contents were then neutralized with HCl and the volume was diluted to the mark with the $M/10$ phosphate buffer, pH 7.0. To the last flask, 1 ml. of 1 N NaOH was added and the flask was incubated at 37° for 16 hours. The contents were neutralized with HCl and made up to volume with $M/10$ phosphate buffer.

Blank solutions were made up by following the same procedures except that distilled water was used in place of the sodium ribonucleate.

The concentrations of the various samples with respect to sodium ribonucleate were determined from the control samples spectrophotometrically, using the extinction coefficient of the best available sample of calf pancreas sodium ribonucleate. The results can be seen in Table II, and Fig. 1. In each case hydrolysis caused an increase in the light absorption at 260 mμ over that of the non-hydrolyzed controls. The samples hydrolyzed with alkali showed greater increase in the absorption at 260 mμ than was observed for the samples hydrolyzed with trichloroacetic acid. In both the long and short periods of hydrolysis, an increase in absorption at 260 mμ of about 23% was obtained using alkali. In the case of hydrolysis with trichloroacetic acid, the increase in absorption obtained by hydrolyzing for 20 minutes at 90° was about 14%, and the increase by hydrolysis for 16 hours at 37° was about 16%. Similar results are obtained by the acid hydrolysis of desoxyribonucleic acid.

TABLE II

EFFECTS OF HYDROLYSIS ON CALF PANCREAS RIBONUCLEIC ACID

Values represent absorption coefficient ($E^{1\%}_{1\ cm.}$) of product obtained by hydrolysis under different conditions

Non-hydrolyzed control	5% TCA 90°, 20 min.	5% TCA 37°, 16 hr.	0.5 N NaOH 95°, 30 min.	0.5 N NaOH 37°, 16 hr.
195	223	227	238	241

Discussion

The procedure described in this paper for isolating sodium ribonucleate from normal animal tissues results in a yield of purified product which amounts to about 50% of the total ribonucleic acid. This material is quite free from protein as far as can be determined by the usual methods of detecting protein, such as color tests and assay of adequate amounts of acid hydrolysates for amino acids by paper chromatography. The method has been applied to yeast and wheat germ with satisfactory results, but it apparently does not work satisfactorily for the preparation of ribonucleic acid from tumor tissue. In the case of two human tumors which were investigated, there was considerable contamination of the final sodium ribonucleate by protein. It thus appears possible that the state of ribonucleic acid in a variety of normal tissues is different from the state of ribonucleic acid in tumor tissue, presumably because of a difference in the binding of the ribonucleic acid to protein. The tumor ribonucleoproteins appear to be more resistant to dissociation by the sodium dodecyl sulfate. This detergent appears to split normal tissue ribonucleoproteins quite satisfactorily on the other hand, as shown by the analysis of the isolated

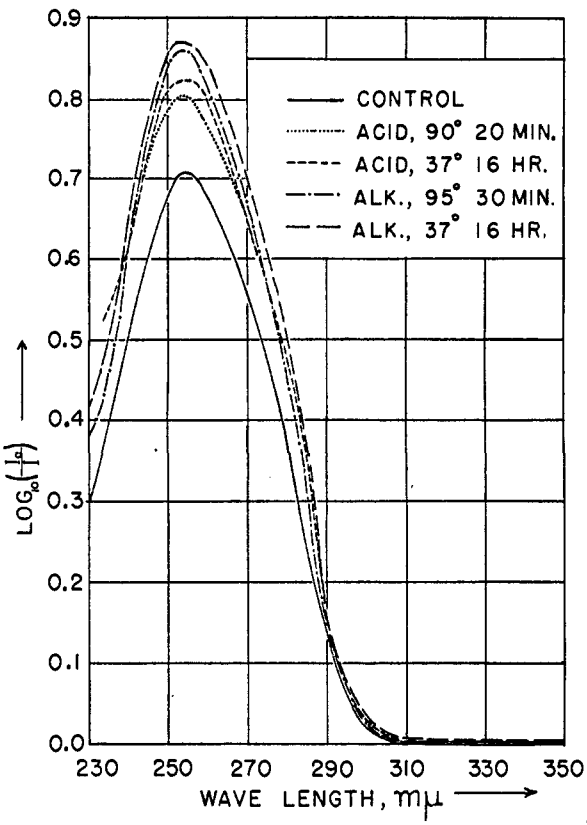

Fig. 1.—Absorption spectrum of calf pancreas PNA (*control*) together with spectra of the same material after various treatments with acid and alkali.

nucleic acids. The nature of this apparent difference in the binding of the ribonucleic acid to protein will require further study.

The analysis for phosphorus and nitrogen of the sodium ribonucleates isolated by the sodium dodecyl sulfate procedure showed that the N/P ratios were lower than ratios obtained by other methods of isolation. This constitutes additional evidence that this method is quite capable of providing a very satisfactory product. When used in conjunction with the method already described[2] for the preparation of sodium desoxyribonucleate, it is possible to extract both nucleic acids from the same tissue sample in quite pure state. The nucleic acids have been extracted in this fashion from as small an amount of tissue as one rat liver in sufficient yield for analytical purposes, although in this case the purity of the products may not be as high as when larger amounts of tissue are used.

The isolation of sodium ribonucleate from wheat germ was carried out by using the above procedure with defatted wheat germ. As shown in Table II, the percentage of phosphorus in the latter ribonucleate was found to be higher than for sodium ribonucleates prepared from animal sources, which might suggest the possibility of some contamination by polyphosphates in material isolated from plant sources. However, other interpretations are also possible.

The viscosity of sodium ribonucleate of different concentrations was found to vary linearly with

concentration. Ribonucleic acid isolated by our procedure was found to be similar in regard to viscosity to the high molecular weight ribonucleic acid obtained from tobacco mosaic virus.[9] The sedimentation constant for rabbit liver sodium ribonucleate, a typical example of our product, indicates that this material probably has a higher molecular weight than ribonucleic acid prepared with the use of guanidine hydrochloride according to Volkin and Carter.[17] It was found by the latter authors that the sedimentation constant of rabbit liver ribonucleate is more than doubled when the *p*H is lowered from 6.8 to 4.8. In view of the fact that the sedimentation data obtained by Cohen and Stanley[9] for the freshly prepared nucleic acid from tobacco mosaic virus were obtained from sedimentations in acid solution at *p*H 4.9, it is possible that their material if it had been studied at *p*H 6.8 would have shown a sedimentation constant comparable to that observed for our ribonucleic acid. If this assumption is correct, our ribonucleic acid appears to have an average molecular weight close to that of the ribonucleic acid of tobacco mosaic virus. Analysis of our ribonucleate by means of the ultracentrifuge also indicated that this material is probably rather polydisperse.

The mild hydrolysis by acid or alkali was found to result in rather marked changes in the ultraviolet absorption spectrum as shown in Table II. The observed increases in absorption at 260 mμ after acid and alkaline hydrolysis are both slightly less than the increases obtained by Tsuboi[26] with mouse liver ribonucleic acid, although the control value of the extinction coefficient obtained by this author for untreated ribonucleic acid was higher than that reported above for our ribonucleic acid prepared with the use of sodium dodecyl sulfate. It is likely that the nucleic acid obtained by Tsuboi had already suffered degradation during the isolation procedure. It is apparent that the extraction of ribonucleic acid from tissues by the Schmidt–Thannhauser or the Schneider procedures for the analysis of the nucleic acids will result in changes in the absorption coefficients of the nucleates.

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The Preparation of Nitro Compounds from Oximes. I[1]

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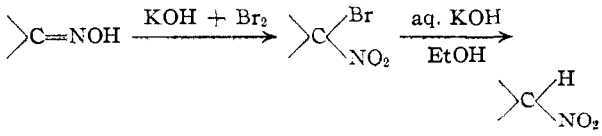
The conversion of oximes to nitro compounds by hypobromite oxidation to an α-bromonitro compound, followed by reductive debromination, has been examined. Only strictly alicyclic ketoximes give the corresponding nitro compounds and, even here, the yields are poor. With aldoximes and the oximes of aliphatic and aromatic ketones, the sequence fails completely.

In 1899 Forster[2] quantitatively prepared bromonitrocamphane from camphor oxime by treatment with potassium hydroxide and bromine. He later reduced the bromonitrocamphane with aqueous alcoholic potassium hydroxide and isolated nitrocamphane in 80% yield.[3] These reactions may be summarized as



Forster's preparation of bromonitrocamphane has been repeated by Ginnings and Noyes[4] and by Knapp and Lipp.[5] Cherkasova and Mel'nikov[6] have reported the preparation of chloronitro and bromonitro compounds from aliphatic aldoximes and ketoximes by the hypohalite oxidation[7] first described by Forster. However, their experimental procedure for the oxidation of aldoximes was not described in detail and has not been verified in this Laboratory.

This method of synthesizing aliphatic and alicyclic nitro compounds was undertaken in order to determine its generality, since it would be a very useful adjunct to the existing procedures for preparing nitro compounds.

Oximes of the 14 carbonyl compounds listed in Table I have been allowed to react with aqueous sodium hydroxide–bromine solution at 0–5° producing aliphatic and alicyclic bromonitro compounds in the indicated yields. The reaction failed with all aromatic oximes[8] and aldoximes. Pure bromonitro compounds readily purified by

(1) Presented in part before the Division of Organic Chemistry of the American Chemical Society, Atlantic City, N. J., September 17, 1952.

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(4) R. M. Ginnings and W. A. Noyes, THIS JOURNAL, **44**, 2567 (1923).

(5) H. Knapp and P. Lipp, *Ber.*, **73**, 915 (1940).

(6) M. Cherkasova and N. N. Mel'nikov, *J. Gen. Chem., (U.S.S.R.)*, **19**, 321 (1949).

(7) R. Robin, *Ann. chim.*, **16**, 77 (1921), investigated the oxidation of oximes with iodine and sodium carbonate and found that aromatic aldoximes yielded peroxides. With aliphatic aldoximes the aldehydes were reported to be reformed and ketoximes were unattacked by this reagent.

(8) The failure of this reaction with α-indanone, fluorenone and benzophenone oximes parallels the observation by I. D. DePaolini, *Gazz. chim. ital.*, **61**, 551 (1931), that ketoximes derived from phenyl ketones did not produce bromonitroso compounds when treated with bromine in pyridine solution.