FRACTIONATION AND COUNTERCURRENT DISTRIBUTION OF RIBONUCLEIC ACID

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SUMMARY

1. RNA has been prepared from rat and chicken liver by phenol–water and phenol–naphthalene-1,5-disulphonate (NDS) methods. The RNA prepared by the phenol–NDS method appears to be less degraded than RNA isolated by phenol–water.

2. RNA can be fractionated by addition of 2-butoxyethanol or 2-ethoxyethanol to solutions containing potassium acetate. The most insoluble fraction has the highest proportion of guanine and cytosine, while the fraction with the highest content of adenine and uracil is the most soluble. 2-Ethoxyethanol is better than 2-butoxyethanol for fractionation of RNA prepared by the phenol–NDS method.

3. C.C.D. of RNA and the fractions show that a complex mixture of interacting substances is present.

4. Fraction 1 (phenol–NDS preparation) appears to be the most undegraded RNA but action of ribonuclease can bring about a change in the C.C.D. pattern simulating that of the RNA prepared by the phenol–water method and without altering the base composition of the material.

INTRODUCTION

Evidence for the heterogeneity of ribonucleic acids (RNA) has been produced by several methods. Metabolic heterogeneity was demonstrated by SACKS, HURLEY AND YOUNG[1] by injection of radioactive phosphorus into rats and rabbits. MAGASANIK[2] has discussed results of the examination of base compositions of RNA present in different fractions obtained by differential centrifugation of tissue homogenates. Generally the base composition of the nuclear fraction differed from that of the cytoplasmic fractions and this conclusion has been supported by ELSON, TRENT AND CHARGAFF[3]. DE LAMIRANDE, ALLARD AND CANTERO[4], however, reported considerable variation in the base compositions of all their fractions from rat liver. Fractionation of RNA of ECTEOLA anion exchange resin by BRADLEY AND RICH[5] definitely demonstrated the large number of components in a given preparation and similar results were obtained by MUIRA AND SUZUKI[6] who used Dowex-2 and found fractions of differing molecular weights, but not of base composition. DAVIS AND ALLEN[7] fractionated yeast RNA by using salmine sulphate as a precipitant and releasing RNA by addition of sodium dodecyl sulphate solution, while BROWN, DAVIES,

COLTER, LOGAN AND KRITCHEVSKY[8] separated RNA of high molecular weight from Ehrlich ascites tumour cells by fractionation on a column of methylated bovine serum albumin. The present work demonstrates a relatively simple method of separating rat and chicken liver RNA into fractions of different base composition, and a study of the relationship of these fractions to each other and to the original RNA by application of the countercurrent distribution method of CRAIG AND CRAIG[9].

MATERIALS AND METHODS

Solvents

2-Methoxy-, 2-ethoxy- and 2-butoxy-ethanols are available as technical solvents and PORTER[10] reported a purification of these which yielded material having little absorption at 280 mμ if the original solvent had an absorbancy of less than 0.5 at 280 mμ. Frequently the original optical density was much higher than this and, as the method employed by PORTER did not give a sufficiently low O.D. at 260 mμ, a new method for the purification was devised. 4 l of the technical solvent were mixed with 1 g of palladised charcoal (5 %) and a stream of hydrogen was passed into this mixture through a sintered glass bubbler during 2 h. The solvent was filtered into a 3-necked flask for distillation and about 2 g of amidol (2,4-diaminophenol hydrochloride) were added. The solvent was distilled at the pressure of the water pump (approx. 15 mm) in a stream of N_2. A fractionating column 60 cm long was used and an arrangement made to collect fractions for measurements of the O.D. It is usually necessary to collect about 500 ml before the O.D. drops to 0.1 at 260 mμ, but this volume varies from one batch to another. When the O.D. was below 0.1 the solvent was collected until there remained about 100 ml of dark residue in the distillation flask. The solvent collected had an O.D. of 0.02–0.05 at 260 mμ and usually no absorption at 280 mμ. Under these conditions 2-methoxyethanol boils at about 31°, 2-ethoxyethanol at 42° and 2-butoxyethanol at 72°. These solvents may then be kept in dark bottles at room temperature for several weeks without an increase in the O.D. at 260 mμ.

Phosphate solution

K_2HPO_4 was used for making the phosphate solution required for counter current distribution (C.C.D.). No difference in O.D. at 260 mμ was observed when Analar KH_2PO_4 and KOH were used instead. Since K_2HPO_4 contains a variable amount of moisture, a solution based upon specific gravity was used as a standard. K_2HPO_4, sp. gr. 1.320 at 20° was made up by weighing 1320 g K_2HPO_4 and adding 2.5 l distilled water. The solution was filtered, cooled to 20° and adjusted to sp. gr. 1.320 by addition of water (usually 100–200 ml). 33.3 % (v/v) H_3PO_4 was prepared by adding 350 g H_3PO_4 (Analar, sp. gr. 1.75) to 400 g of water. The phosphate (pH 7.5) solution was made by addition of 6 g of 33.3 % H_3PO_4 to every 100 g K_2HPO_4 (sp. gr. 1.320).

Solvent systems

They were made up by weighing and were allowed to remain at 20° overnight before being used.

Solvent system 1: 2-methoxyethanol 740 g, 2-butoxyethanol 360 g, phosphate,

pH 7.5, 1600 g, water 1420 g. This mixture produced approximately 1.5 volumes top phase per 1 volume lower phase.

Solvent system 2: 2-ethoxyethanol 3000 g, water 5600 g, ammonium sulphate 2000 g. This system gave nearly equal volumes of each phase.

Counter current distributions

The C.C.D.s were performed with a Quickfit and Quartz fully automatic machine (10 ml upper phase, 11 ml lower phase). RNA (15–30 mg) used for each distribution was first dissolved in water, and the phosphate and organic solvents then added in the correct proportions. After the distribution water was added to each tube to bring the contents into one phase and the O.D. was measured at 260 mμ.

Preparation of RNA

The phenol–water preparations were made essentially as described previously[11] and in addition the following modifications were made; naphthalene-disulphonate (NDS) preparations were made by homogenising the tissue in a volume equal to 8 × weight of the tissue of a solution of 0.5 % naphthalene-1,5-disulphonate disodium salt to every 100 ml of which 0.1 ml 2.5 *M* K_2HPO_4 had been added to bring the pH to 6.5. The mixture was then stirred and extracted with an equal volume of 90 % (w/w) phenol at 20° and the procedure described before was followed exactly. The yields were the same.

One preparation was made from rat liver which was homogenised in 0.18 % NaCl (equivalent molar concentration of 0.5 % NDS), extracted with phenol and isolated as described previously, and a further preparation was made by homogenising the rat liver with the phenol–water mixture in the Waring blendor and subsequent procedures were the same as in previous preparations.

Fractionation of RNA

Three fractions were obtained by the following method. RNA (3 g) was dissolved by stirring in water (450 ml) with KOAc (9 g). Alterations of the volumes mentioned for a given weight of material will produce a different result. The mixture was centrifuged in a Servall centrifuge at 5000 × g for 30 min at 0° to remove traces of insoluble material. The supernatant liquid was poured off, 2-butoxyethanol (300 ml distilled) was added and the mixture was set aside in the refrigerator overnight. A gelatinous precipitate had separated and was centrifuged off at 700 × *g*. This precipitate constituted fraction 1, and was washed twice with ethanol–water (3:1, v/v) when it separated as a white powder which was sometimes slightly grey on drying. 2-butoxyethanol (150 ml) was added to the clear supernatant fraction from fraction 1 and the mixture was again set aside overnight at 2°. This separation was facilitated if carried out in centrifuge bottles since the precipitate was not apparent although the solution appeared opalescent when shaken. This solution was centrifuged at 700 × *g* for 20 min and the material which remained at the bottom of the tube, after carefully pouring off the supernatant layer, constituted fraction 2. This residue separated as an oil, but when washed with ethanol–water (3:1, v/v) immediately separated as a white powder, which was washed once more with ethanol–water before drying in a vacuum desiccator. To the supernatant solution from fraction 2 ethanol (625 ml) was added and a flocculent precipitate appeared quickly but was

allowed to remain overnight before being collected by centrifuging. The final precipitate was washed twice with ethanol–water (3:1) before being dried in a vacuum desiccator and constituted fraction 3. The total recovery of material was about 90 %.

Fractionation of fraction 1 with ethoxyethanol

Fraction 1 from rat liver RNA (NDS preparation) (3.0 g) was dissolved in water (300 ml), KOAc (6 g) was added and then 300 ml 2-ethoxyethanol. Precipitation was allowed to proceed overnight at 2°, and the separated material was difficult to centrifuge but was deposited by centrifugation at 5000 × *g* for 45 min. After washing and drying this material weighed 2.2 *g*. The supernatant solution from this precipitate was mixed with 450 ml ethanol and the precipitate which constituted fraction 1/3 was separated by centrifugation, washed and dried (780 mg). The material which separated first was dissolved in water (200 ml), KOAc (4 g) added and 200 ml 2-ethoxyethanol; after standing overnight at 2° the precipitate was separated by centrifugation (5000 × *g* for 40 min), washed and dried and constituted fraction 1/1. Fraction 1/2 was obtained from the supernatant solution by adding of 300 ml ethanol and separation of the insoluble RNA by centrifuging. Rat liver RNA (phenol–water preparation) and chicken liver RNA were separated into 2 fractions only, the first precipitated by 2-ethoxyethanol and the second by ethanol as described for rat liver RNA.

Commercial yeast RNA

"Purification" was carried out by dissolving commercial yeast RNA (2 g) in water (50 ml) adding an equal volume of 2.5 *M* K_2HPO_4 mixed with 2.5 ml 33 % H_3PO_4 (1 volume H_3PO_4 sp. gr. 1.75 + 2 volumes H_2O) and 50 ml 2-methoxyethanol. The upper phase, which was dark brown, was separated and dialysed against 4 changes of distilled water when most of the colour disappeared. The contents of the dialysis bag were made up to 2 % with respect to KOAc and the RNA precipitated by 2 volumes of ethanol. After centrifuging, washing and drying the yield was 770 mg.

Action of ribonuclease on RNA fraction

Each fraction (50 mg) was dissolved in 4 % KOAc solution (25 ml) (fraction 1 dissolved somewhat slowly) and to each solution was added pancreatic ribonuclease (1 mg) in water (25 ml). The mixture was allowed to remain for 2 h at 20° and then shaken vigorously with 50 ml phenol (90 % v/v) for 30 min. The mixtures were poured into separating funnels and after 1 h the phenol layer was run off. Ethanol (80 ml) was added to the aqueous layer to precipitate degraded RNA, which was centrifuged off, washed with ethanol–water (3:1) and dried in a vacuum desiccator.

Action of ribonuclease on fraction 1/1 at different times

Fraction 1/1 from a NDS–phenol preparation from rat liver was used and 164 mg were dissolved in 74 ml of a solution of 2 % KOAc. Pancreatic ribonuclease (1 mg) in 2 ml water was added, and the mixture was shaken thoroughly and allowed to stand at 20°. 25 ml of solution were removed after 15, 30 and 60 min and in each case added immediately to 25 ml phenol (90 %). Each was shaken for 30 min before separating the phenol as described before and precipitating the degraded RNA with ethanol.

Action of ribonuclease and NDS on rat liver RNA

RNA (160 mg) was dissolved in water (75 ml) and KOAc (3 g) was added. This solution was divided into 3 portions of 25 ml each. To the first 25 ml was added 25 ml H_2O, to the second was added 1 mg ribonuclease in 25 ml water and to the third was added 1 mg ribonuclease in 1% NDS (adjusted to pH 7.0 with 2.5 *M* K_2HPO_4). The 3 solutions were allowed to stand for 20 min, at 20°, 90% phenol (50 ml) added to each and well shaken, the phenol layer was run off from a separating funnel and the RNA precipitated from the aqueous layer by addition of ethanol (80 ml). The insoluble material was centrifuged off, washed and dried as usual.

Analytical methods for phosphorus, ε(P) (CHARGAFF[12]) and base compositions have been described before[11].

Amino acids were estimated after hydrolysis with 5.8 *M* HCl for 24 h and separation of the amino acids by 2 way paper chromatography used ethyl methyl ketone–acetic acid–water (3:1:1, v/v/v) for the first way and ethyl methyl ketone–methanol–water–NH_3 solution (sp. gr. 0.88) (3:1:1:0.15, v/v/v/v) the second way. The colours were developed with ninhydrin and estimated as the cadmium complexes, (unpublished).

Ribonuclease was the crystallized commercial pancreatic enzyme (General Biochemicals Inc.).

RESULTS

Fractionation of RNA

The fractions produced by addition of 2-butoxyethanol to solutions of RNA in 2% potassium acetate varied slightly in amount and base composition from one preparation to another. However, the fraction with the greatest proportions of guanine and cytosine always separated first and a higher proportion of fraction 1 was always obtained from preparations made by the phenol–NDS method. The base compositions and yields of the fractions separated from rat-liver RNA prepared by the phenol–water and phenol–NDS methods are shown in Table I.

TABLE I

BASE COMPOSITIONS IN MOLAR PROPORTIONS AND ANALYTICAL FIGURES OF THE FRACTIONS OBTAINED FROM RAT-LIVER RNA BY PRECIPITATION WITH 2-BUTOXYETHANOL

Abbreviations: G, guanine; A, adenine; C, cytosine; U, uracil; P, phosphorus; ε(P) as defined by CHARGAFF[12].

	G	*A*	*C*	*U*	*P*	*ε(P)*	*Yield (%)*
Phenol–water preparation							
Fraction 1	38.5	11.5	36.5	13.5	6.8	7450	28
2	35.1	17.6	31.4	15.9	6.5	8150	37
3	32.2	21.9	28.8	17.1	6.9	8500	35
Phenol–NDS preparation							
Fraction 1	36.0	17.5	31.4	15.1			50
2	33.4	19.8	30.8	16.0			30
3	30.9	24.2	27.6	17.7			20

TABLE II

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE FRACTIONS OBTAINED BY RE-PRECIPITATION OF FRACTION 1 FROM RAT-LIVER RNA WITH 2-ETHOXYETHANOL

Fraction	*G*	*A*	*C*	*U*	*Yield (%)*
Phenol–water preparation					
1/1	42.4	9.4	34.4	12.8	57
1/2	38.4	15.4	32.6	13.7	43
Phenol–NDS preparation					
1/1	38.0	15.3	32.2	14.5	60
1/2	35.2	18.1	30.6	16.1	15
1/3	30.8	20.2	30.0	19.0	26

TABLE III

PERCENTAGE BY WEIGHT OF RESIDUAL AMINO ACIDS ATTACHED TO FRACTIONS 1, 2 AND 3 FROM RAT-LIVER RNA (PHENOL–WATER PREPARATION)

The amino acids are expressed as a percentage of the whole air-dried RNA. Valine was also present. Abbreviations: Lys, lysine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Ser, serine; Threo, threonine; Leu, leucine + isoleucine.

Fraction	*Lys*	*Arg*	*Asp*	*Glu*	*Ala*	*Ser*	*Threo*	*Leu*
1	0.62	0.31	0.30	0.44	0.29	0.25	0.26	0.24
2	0.12	0.07	0.25	0.41	0.10	0.07	0.08	
3	0.12	0.02	0.21	0.40	0.12	0.09	0.09	

TABLE IV

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE DEGRADATION PRODUCTS OBTAINED BY ETHANOL PRECIPITATION AFTER REACTION OF FRACTIONS 1/1, 2 AND 3 FROM RAT-LIVER RNA WITH RIBONUCLEASE

Abbreviations as in Table I.

Degradation product from fraction	*G*	*A*	*C*	*U*	*Recovery (%)*
1/1	54.2	15.2	21.2	9.4	55
2	51.7	16.5	21.3	10.5	55
3	45.8	22.8	21.0	10.3	45

TABLE V

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE FRACTIONS RECOVERED BY ETHANOL PRECIPITATION AFTER REACTION OF FRACTION 1/1 FROM RAT-LIVER WITH RIBONUCLEASE FOR DIFFERENT TIMES

Time of reaction with ribonuclease	*G*	*A*	*C*	*U*	*Recovery (%)*
15 min	38.5	14.0	32.0	15.6	80
30 min	38.5	14.2	32.1	15.2	64
60 min	39.5	14.1	31.0	15.2	64

Chicken liver RNA prepared by the phenol–water method could be fractionated in a manner similar to rat-liver RNA prepared by the same method.

Chicken-liver RNA prepared by the phenol–NDS method yielded a much higher proportion of fraction 1 (75 %) and this was fractionated further by the use of 2-ethoxyethanol.

RNA from regenerating rat liver produced the same proportions of fractions 1, 2 and 3 whether this RNA was prepared in the presence or absence of NDS.

The fractionation of RNA could be extended by addition of 2-ethoxyethanol to solutions of fractions 1. The basis of the separation appeared the same as before and the base compositions of the fractions obtained from fraction 1 are shown in Table II.

Amino acids

RNA prepared by the phenol–water method usually contained 1–1.5 % residual amino acids, the greatest amount of which were attached to fraction 1. The distribution of the amino acids in the 3 fractions is shown in Table III. As these were determined after 2-way paper chromatography the amounts are unlikely to be absolute, but indicate the differences between the fractions. RNA prepared by the phenol–NDS method always contained less residual amino acids (0.5 %) but a larger proportion remained attached to fraction 1 than to fractions 2 and 3.

In addition to differences in residual amino acids and base compositions the 3 fractions also differed in their solubilities in 40 % (w/v) ammonium sulphate. Fraction 1 was almost insoluble, fraction 2 was partially soluble while fraction 3 was completely soluble. The fractions also showed different C.C.D. curves.

Yeast RNA

Yeast RNA, purified as described in the experimental section, gave no precipitate upon addition of one portion of 2-butoxyethanol to a solution in 2 % potassium acetate, but a precipitate (78 %) was obtained upon adding the second portion of 2-butoxyethanol. A second precipitate (22 %) was isolated after addition of ethanol. The base compositions of the 2 fractions were nearly identical (G:A:C:U, 29:25:23:23).

Action of ribonuclease on fractions 1/1, 2 and 3 from rat liver

The base composition of the material insoluble in ethanol after treating fractions 1/1, 2 and 3 from rat liver with pancreatic ribonuclease during 2 h are shown in Table IV. These 3 materials were completely soluble in 40 % w/v ammonium sulphate and in solvent system 2 for C.C.D.

Action of ribonuclease on fraction 1/1 from rat liver at different time intervals

The base compositions of the materials precipitated by ethanol after reaction of fraction 1/1 from rat liver with pancreatic ribonuclease during 15, 30 and 60 min are shown in Table V.

Action of ribonuclease on rat liver RNA in the absence and presence of NDS

The base composition of the original RNA, and the materials recovered by ethanol precipitation after reaction with ribonuclease in the absence and presence of NDS are shown in Table VI.

TABLE VI

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE MATERIAL RECOVERED BY ETHANOL PRECIPITATION AFTER REACTION OF RAT-LIVER RNA WITH RIBONUCLEASE IN THE ABSENCE AND PRESENCE OF NDS

Material	*G*	*A*	*C*	*U*	*Recovery (%)*
RNA	34.5	18.2	31.1	16.2	88
RNA + RNAase	40.5	15.5	29.4	14.6	64
RNA + RNAase + NDS	39.4	16.3	29.7	14.6	82

Counter current distribution

C.C.D. has only recently been applied to RNA. WARNER AND VAINBERG[13] used a mixture of 2-propanol, formamide and phosphate buffer for separating synthetic polynucleotides, and HOLLEY AND MERRILL[14] have recently applied this system to the RNA from supernatant fraction of rat-liver homogenate.

The first problem was the choice of solvents, since nucleic acids are easily precipitated from salt solutions by organic solvents. The cellosolve-salt solution used by PORTER[10] was tried and some satisfactory systems consisting of diethyl carbitol, 2-ethoxyethanol and phosphate solutions were found for commercial yeast RNA. These systems were of no use for rat-liver RNA, which was largely insoluble in the mixture used for yeast RNA. It had been found that rat-liver RNA was completely extracted into the organic phase of a system of 2-methoxyethanol and phosphate buffer and this partition has been made the basis of the isolation of RNA (and deoxyribonucleic acid) free from glycogen. When 2-butoxyethanol and phosphate buffer were used in proportions approximating to those at the plait point, all the RNA was found in the aqueous phase. From these considerations empirical mixtures have been made from 2-methoxyethanol, 2-butoxyethanol and phosphate solutions, restrictions in choice being made by the solubility of the RNA, the partition of the RNA and the volume relationship of the 2 phases. Generally an increase in the proportion of 2-methoxyethanol or of phosphate ions increased the partition in favour of the upper phase, while an increase in the proportion of 2-butoxyethanol or of water resulted in a partition in favour of the lower phase.

It has not proved possible to dissolve whole RNA directly in the solvent systems for C.C.D., but the RNA remained soluble after being dissolved in the water first and subsequently adding the phosphate solutions and then the organic solvents. HAUSSMAN AND CRAIG[15] have recently employed a similar device for the dissolution of proteins for C.C.D.

The C.C.D. curves for rat-liver RNA prepared by the phenol–water and phenol–NDS methods are shown in Fig. 1 and 2 respectively. The position of the peaks was the same in preparations by the same method but the relative heights varied from one preparation to another, especially with NDS method. The curve obtained with chicken-liver RNA made by the phenol–NDS method was similar to Fig. 2 but very much less material travelled in the organic phase.

The C.C.D. curves for fractions 1, 2 and 3 from rat-liver RNA prepared by the phenol–water method are shown in Fig. 3. The whole RNA did not show the considerable peak in tube 1 that was present in fraction 1. Fraction 3 was the only one

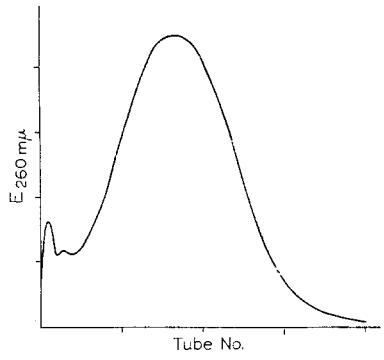

Fig. 1. C.C.D. curve for rat-liver RNA (phenol–water preparation) in solvent system 1. The maxima occur at tubes 2 and 34 after 96 transfers.

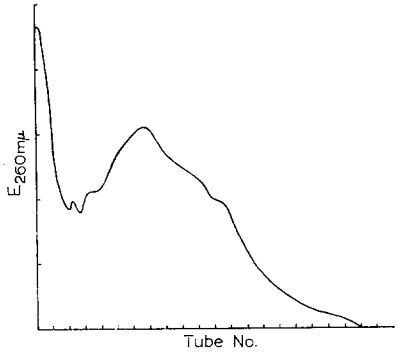

Fig. 2. C.C.D. curve for rat-liver RNA (phenol–NDS preparation) in solvent system 1. The maxima occur at tubes 1 and 26 after 96 transfers.

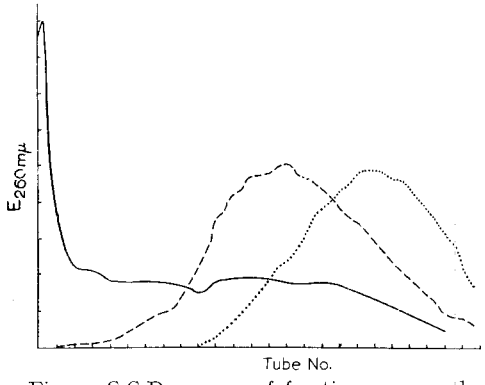

Fig. 3. C.C.D. curves of fractions 1, 2 and 3 obtained by fractionation of rat-liver RNA (phenol–water preparation) with 2-butoxyethanol. ——— fraction 1; – – – – fraction 2; · · · · · fraction 3. The main maxima occur at tubes 1, 56 and 76 respectively for each fraction after 200 transfers in solvent system 1.

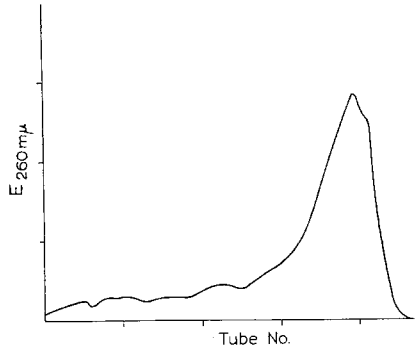

Fig. 4. C.C.D. curve of fraction 3 of rat-liver RNA (phenol–water preparation) in solvent system 2. The maximum occurs at tube 78 after 96 transfers.

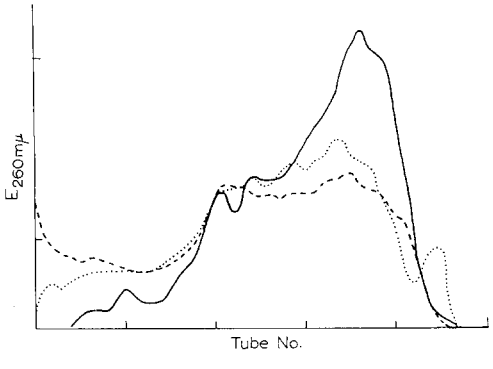

Fig. 5. C.C.D. curves of the products of the action of ribonuclease on fractions 1, 2 and 3 from rat-liver RNA (phenol–water preparation) in solvent system 2. – – – – – fraction 1 (maxima at tubes 0, 42 and 70); . . . . . , fraction 2 (main maximum at tube 65); ——— fraction 3 (main maximum at tube 72).

completely soluble in solvent system 2 and the curve obtained is shown in Fig. 4. The peak at tube 78 was also found when yeast RNA ("purified" as described) was distributed in the same system.

When the 3 fractions from rat-liver RNA prepared by the phenol–water method were separately treated with ribonuclease, the insoluble material which separated

upon addition of ethanol to each fraction was found to be completely soluble in solvent 2. The C.C.D. curves are shown in Fig. 5.

To examine the effect of ribonuclease on fraction 1, this fraction from a rat-liver phenol–NDS preparation was refractionated with 2-ethoxyethanol and the C.C.D. curve of this material in solvent system 1 (Fig. 6) showed that very little material was passing into the organic phase.

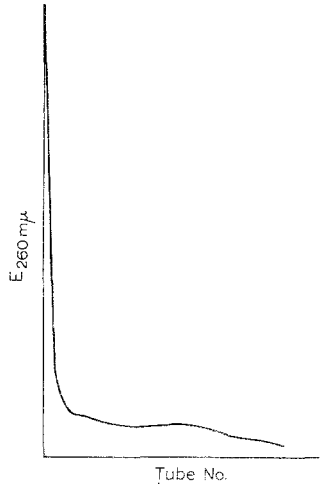

Fig. 6. C.C.D. curve of fraction 1/1 obtained by fractionation of rat-liver RNA (phenol–NDS preparation) fraction 1 with 2-ethoxyethanol. Solvent system 1, 96 transfers.

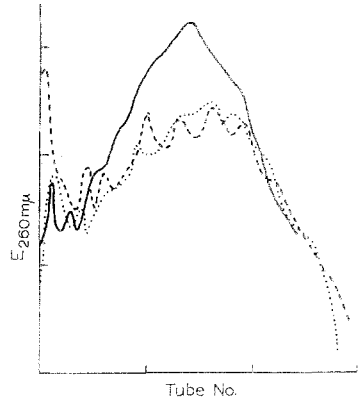

Fig. 7. C.C.D. curves of the products of the action of ribonuclease on fraction 1/1. – – – –, 15 min after addition of enzyme; · · · · · ·, 30 min after addition of enzyme; ————, 60 min after addition of enzyme; (peaks at tubes 2, 6 and 24). Solvent system 1, 96 transfers.

Treatment of this fraction with ribonuclease quickly altered the C.C.D. curve and after 15 min the high peak in tube 0 disappeared and a complex pattern appeared (Fig. 7). A peak at tube 24 appeared after 30 min while after 1 h the curve was, in shape, very similar to that of rat-liver RNA extracted by the phenol–water method (Fig. 1). During this alteration in the C.C.D. curve there was no alteration in the base composition of each product.

DISCUSSION

The addition of naphthalene-1,5-disulphonic acid to the water in which the tissue was homogenised produced RNA which differed in its mode of fractionation with 2-butoxyethanol and its C.C.D. pattern compared with RNA prepared in the absence of NDS. The differences found between the RNAs from rat liver were more marked when the preparations were made from chicken liver and less marked in RNA from regenerating rat-liver.

Fractionation

The basis of the fractionation of RNA by 2-ethoxy- and 2-butoxyethanol is not clear but possibly depends upon the cleavage of aggregates formed through hydrogen bonds of the A-U and G-C types as in the synthetic polynucleotides[17]. It is obvious from the base compositions of the fractions that in all cases a mixture rich in G-C

is being separated from one rich in A-U. The differences between the fractionations of the RNAs prepared by phenol–water and phenol–NDS methods became more clear when these compounds were studied by C.C.D.

Counter current distribution

A comparison of the C.C.D. curves of rat-liver RNA (phenol–water preparation) (Fig. 1) with each of the curves of the three fractions shows immediately that the curves of the fractions cannot be summated to give the original curve of whole RNA. However, when the 3 fractions were mixed in the proportions in which they were present in whole RNA and examined by C.C.D. the curve obtained was similar to that of whole RNA again, and the peak in tube 1 of fraction 1 was not apparent. These curves indicate that RNA, as isolated by this method, is a complex mixture of components which interact with one another and are not easily separated by solvent system 1. Examination of the C.C.D. curves of the RNA prepared in the presence of NDS shows that a higher proportion of the material with a partition preponderantly in the aqueous phase is present in these preparations, and a greater proportion of this is present in the chicken-liver RNA than in the rat-liver RNA. The amount of fraction 1 obtained by precipitation with 2-butoxyethanol from RNA is related to the height of the peak in tubes 0 or 1.

It seemed possible that the reason for the difference between the RNA isolated by the phenol–water and phenol–NDS methods was that the phenol–water RNA was in fact more degraded and NDS was inhibiting some nuclease present in the cell. No inhibition was noted if the cells were extracted directly with a mixture of phenol and water and the RNA isolated, since the same C.C.D. pattern was produced as that by RNA isolated after homogenising the liver in water and then extracting with phenol. The action of NDS may be partly due to the Na^+ as addition of NaCl to the water yielded RNA with a slightly increased peak in tube 0.

Pancreatic ribonuclease was not inhibited by NDS (Table VI) but since 2 different ribonucleases have been reported to be present in rat liver[18] this failure to inhibit may not be relevant.

Effects of ribonuclease

Pancreatic ribonuclease is known to cleave phospho-diester bonds between pyrimidine nucleotides and in accordance with this uridylic and cytidylic acids were lost from fractions 1/1, 2 and 3 upon treatment with ribonuclease. The material insoluble in ethanol after treatment with ribonuclease had a high content of guanine, and since the ratio of guanine to adenine was greater after enzyme treatment, particularly in fractions 2 and 3, it must be assumed that more adenine than guanine is associated with the disrupted pyrimidine nucleotides.

The C.C.D. patterns of the products of the action of ribonuclease on the 3 reactions were very similar in solvent systems 1 and a second solvent system, based on 2-ethoxyethanol and ammonium sulphate solution, has also been used. Fraction 1/1 was completely insoluble in this system, while fraction 2 was only partly soluble. In contrast to this the products of the action of ribonuclease on the fractions were all completely soluble in this system. The C.C.D. curves again showed many similarities in a complex pattern which showed better resolution than solvent system 1. The effect of ribonuclease on fraction 1/1 was more marked than on fraction 2 and 3. Examination of

the action of smaller amounts of ribonuclease on fraction 1/1 during shorter periods showed that while the base composition remained unaltered the C.C.D. pattern changed considerably. The peak at tube 0 decreased after 15 min, a second peak at tube 24 became evident and was more pronounced after 1 h. This peak is not in the same position as that of the C.C.D. pattern for RNA extracted with phenol–water, but then fractions 2 and 3 having partitions more in favour of the upper phase are not present. However, the position of this peak is in reasonable agreement with that found for the second peak of rat-liver RNA (phenol–NDS preparation) in solvent system 1 (Fig. 2). The curve, after 1 h, is quite similar in shape to that of whole RNA extracted by the phenol–water method and it is particularly noticeable that during this period of activity there was almost no change in the base composition.

These changes in the C.C.D. pattern of fraction 1/1 subsequent to the action of ribonuclease and culminating in a C.C.D. pattern similar to whole RNA extracted by the phenol–water method, indicate that some similar enzyme reaction may be taking place during the isolation of the RNA from the tissue. Moreover, it might be expected that the enzyme was less active, or more easily inhibited, in chicken than in rat liver, and perhaps more active in regenerating rat-liver.

DICKMAN AND RING[19] have shown that, prior to hydrolysis of phosphodiester linkages, pancreatic ribonuclease caused a non-hydrolytic alteration of the structure of yeast RNA and dilatometric experiments by VANDENDRIESSCHE[20] showed that addition of ribonuclease to RNA solutions produced a temporary increase in the volume of the solution, whereas hydrolysis of the RNA would have been expected to decrease the volume.

The changes observed in the C.C.D. pattern of fraction 1/1 after reaction with ribonuclease could be explained by an alteration of the structure of the RNA molecules or by a very limited hydrolysis which would not affect the base compositions. The evidence suggests that RNA, isolated by the phenol–water method is, to some extent, broken down more than RNA isolated by the phenol–NDS method. NDS may not be the most suitable salt for the purpose of inhibiting the enzyme since the C.C.D. curves of material extracted in this way show some variation and NDS does not inhibit pancreatic ribonuclease.

The suggestion that RNA extracted by the phenol–water method is somewhat degraded finds support in the low content of uridylic and cytidylic acids found in pancreatic RNA extracted by this method[11]. Since commercial pancreatic ribonuclease was inhibited *in vitro* by extraction with phenol some other nuclease may be responsible for the degradation. It is of interest that KEMP AND ALLEN[21] have prepared RNA from the pancreas of the dog by extraction with a hot solution of sodium dodecylsulphate and this RNA was not deficient in pyrimidines.

Very recently LASKOV, MARGOLIASH, LITTAUER AND EISENBERG[22] have also reported that RNA isolated from rat-liver by the phenol–water method was degraded. They attributed the degradation to impurities in the phenol and to the action of heavy metals, since they found that RNA with a greater sedimentation coefficient could be isolated if the phenol was distilled first and ethylenediamine tetraacetate was present during the isolation. It is possible that some of the degradation of the RNA which they observed may also be enzymic in nature if, in fact, the crude phenol contained some enzymic "activator" (*cf.* the degradation of RNA by Ce^{+++} and La^{+++} reported by BAMANN, TRAPMANN AND FISCHLER[23]).

LASKOV *et al.*[21] starved their rats for 48 h before the preparation of RNA. It is of course possible that RNA after this period may not be the same as RNA in the non-fasting rat. The present preparations were made on animals which were not fasted and glycogen was removed by extraction with 2-methoxyethanol from phosphate buffer. LASKOV *et al.*[22] stated that 2-methoxyethanol caused some degradation of RNA. This degradation was probably due to the peroxide content of the technical solvent since the material which represents fraction 1/1 in the present work remained in the aqueous phase and showed no signs of degradation during repeated extraction with 2-methoxy- and 2-butoxyethanol mixtures in the presence of phosphate ions. It was essential to purify the solvents as described in order to obtain consistent results with C.C.D.

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