RIBONUCLEIC ACIDS FROM PANCREAS WHICH CONTAIN NEW COMPONENTS*

JOHN W. KEMP AND FRANK WORTHINGTON ALLEN

Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif. (U.S.A.)

Preparations of ribonucleic acids from pancreas vary greatly in composition, and with few exceptions are characterized by ratios of purine to pyrimidine which are greater than those of the ribonucleic acids from other sources[1]. In the case of one preparation of the ribonucleic acids from beef pancreas, BACHER AND ALLEN[2] have shown that the high ratios of purine to pyrimidine could be partly attributed to degradation by pancreatic ribonuclease which is incurred during the course of the isolation procedure. The yields in this procedure were low.

CRESTFIELD, SMITH AND ALLEN[3] have reported the isolation of the ribonucleic acids of baker's yeast by a procedure in which nuclease action is prevented and in which 60 to 70% of the ribonucleic acids are fractionated from the total by precipitation with 1 *M* sodium chloride at 0°. Certain of the remaining ribonucleic acids which are soluble in 1 *M* sodium chloride have recently been fractionated by DAVIS AND ALLEN[4] and shown to differ in the molar ratios of constituent nucleotides which include a new fifth nucleotide component.

In the present work the methods which were applied to the isolation and fractionation of the ribonucleic acids from yeast have been modified and applied to the isolation of the ribonucleic acids of pancreas. In order to assess the possibilities of enzymic degradation and fractionation, the nucleotide composition of the isolated ribonucleic acids was compared with that found for the ribonucleic acids prior to isolation from pancreatic tissue and found to be identical. The ratios of purine to pyrimidine are found to be similar to those for undegraded ribonucleic acids from other sources. The fifth nucleotide which has been reported by DAVIS AND ALLEN[4] to be present in the fractions of the ribonucleic acids from yeast has been found to be present in the ribonucleic acids isolated from the pancreas. In addition, certain unknown nucleotide components which occur with the guanylic acid fraction in paper chromatography are reported.

EXPERIMENTAL

Materials and analytical methods

Pancreatic tissue

Pancreas was obtained from four male mongrel dogs. Each animal was anesthetized with sodium pentothal by intraperitoneal injections of 30 mg/kg body wt. Twenty min after injection, the animals were ready for removal of the pancreas. The total time required to extirpate each pancreas

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was 45 sec. Upon removal of the gland, the excess blood was blotted off and the tissue dropped into acetone which had been cooled to —76° by means of an external bath of acetone and dry ice.

Piperidine

A practical grade was obtained from Eastman Kodak Company, Rochester, N.Y., and purified by distillation.

Salmine

Protamine sulfate was purchased from Krishell Laboratories, Inc. Portland, Ore. A 4% solution was adjusted to pH 6.5 with 1 *M* sodium hydroxide. Insoluble material was removed by centrifugation at 25,000 × *g* for 30 min.

Sodium dodecyl sulfate

Sodium dodecyl sulfate was recrystallized from Duponol "C" according to the procedure of CRESTFIELD, SMITH AND ALLEN[3].

Deoxyribonucleic acids

Deoxyribonucleic acids were estimated by the colorimetric diphenylamine procedure of DISCHE[5] as modified by BURTON[7].

Analysis of ribonucleic acids

Determination of the mononucleotide composition and mononucleotides released by the action of ribonuclease, as well as the estimations of phosphorus, protein and moisture content, were carried out as described by CRESTFIELD, SMITH AND ALLEN[3].

Chromatographic procedures

Chromatographic procedures were carried out according to the directions of CRESTFIELD AND ALLEN[8].

Electrophoresis

Paper electrophoresis as described by CRESTFIELD AND ALLEN[9] was employed: (a) to free small quantities of nucleotides from ultraviolet-absorbing contaminants which were eluted from the chromatographic paper (b) to resolve the new components which were found in the guanylic acid and (c) to resolve 5′ nucleotides from 2′ and 3′ nucleotides.

Analysis of whole tissue

Pancreatic tissue was analyzed for total ribonucleic acids by a modification of the procedure of SCOTT, FRACCASTORO AND TAFT[10]. Determination of the molar ratios of the nucleotides of the ribonucleic acids in whole tissue was accomplished by digesting a sample of tissue with 1 *M* piperidine. 10 g of ground tissue were placed in a 2-l round bottom flask to which was added 500 ml of 1 *M* piperidine solution. The flask was stoppered with a ground glass stopper and the contents incubated at 55° for 48 h. The digest quickly thickened to a stringy, viscous mass which became completely fluid within 12 h. By 24 h all the tissue had been hydrolyzed and was in solution except for a very small amount of debris. Piperidine and water were removed from the hydrolysate by lyophylization. At this stage the ribonucleic acids were nearly completely hydrolyzed to nucleotides. The deoxyribonucleic acids were relatively unaffected. To remove the bulk of the proteins and deoxyribonucleic acids, the hydrolysate was dissolved in 100 ml of water and the pH decreased to 5 by the addition of 2 *M* formic acid. The copious precipitate of the proteins and deoxyribonucleic acids which formed was removed by centrifugation at 25,000 × *g* for 30 min. The supernatant liquid was decanted and the sediment was washed once with 25 ml of water. The supernatant fluid and washings were combined. The volume was reduced under vacuum and diluted to 10 ml. The solution contained tissue nucleotides as well as the nucleotides and oligonucleotides from ribonucleic acids. The residue retained 0.3% of the total D_{260} units.

The supernatant liquid contains the alkaline hydrolysis products of tissue ribonucleic acids, tissue nucleotides and coenzymes, together with certain non-nucleotide substances. Since the alkaline hydrolysis of tissue nucleotides and coenzymes yields 5′-mononucleotides and 2′,5′-and 3′,5′-diphosphonucleosides and the alkaline hydrolysis of ribonucleic acid yields 2′- and 3′-mononucleotides plus traces of 2′,3′-(3′,5′)-diphosphonucleosides, the analytical scheme must provide for the isolation of 2′- and 3′-mononucleotides from all other derivatives. Each analytical sequence therefore involved the following three steps. (1) Two dimensional chromatography, (2) purification of each of the four nucleotide areas by electrophoresis at pH 3.5, and (3) additional purification by electrophoresis in borate buffer at pH 9.2.

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Isolation of ribonucleic acids

The tissue which was freshly obtained from the animal and frozen at −76° was ground in a porcelain mortar which had previously been cooled in a dry ice–acetone bath. All the tissue was ground and blended together in order that a uniform sample could be obtained. The carbon dioxide trapped in the tissue was removed by evacuation. Pilot experiments indicated that the following extraction step was necessary in order to obtain all of the ribonucleic acids of the tissue.

Step 1. Extraction of nucleic acids. A small stainless steel Waring blendor of 150 ml capacity was placed in a tin can from which the ends had been removed. Clean copper shot was packed around the blendor and asbestos tape was wound around the can for insulation.

The blendor was heated at 105° for 1 h in a drying oven prior to use. 120 ml of boiling 2% solution of sodium dodecyl sulfate was poured into the hot blendor. 17 g of frozen ground tissue were added and blended for 3 min. The temperature fell from 100° to 87°, then gradually rose to 92° within 1 min. Foam was reduced by the addition of 1 ml of octyl alcohol. At the end of the 3-min interval the contents of the blendor were poured into an 800 ml beaker which was immersed in a dry ice–acetone freezing mixture. The contents of the beaker were stirred manually until the temperature dropped to 4°. The cold mixture was centrifuged at 25,000 × *g* for 30 min. The supernatant fluid was decanted. Three samples of tissue were treated in this manner so that, in all, 50 g of tissue were extracted. The supernatant fluids were combined to give a volume of 340 ml.

Step 2. Isolation of crude ribonucleic acids. The crude ribonucleic acids were precipitated from the combined extracts by pouring while stirring into 680 ml (2 volumes) of 95% ethyl alcohol which contained 10 ml of a saturated solution of sodium chloride. This was left to stand 12 h at 4°. The crude ribonucleic acids were collected by centrifugation at 3200 × *g* for 30 min. The precipitate was washed with two 100-ml portions of cold 67% ethyl alcohol. One ml of a saturated solution of sodium chloride was added to each washing to insure complete precipitation of the ribonucleic acids. The nucleic acids were collected by centrifugation, suspended in 80% ethyl alcohol and left to stand 48 h at 4°.

Step 3. Purification of ribonucleic acids. The crude ribonucleic acids were collected by centrifugation at 10,000 × *g* for 30 min. The precipitate at this point contained denatured proteins and 90% of the total ribonucleic acids in the tissue. The ribonucleic acids were extracted from the precipitate with 200 ml of 0.05 *M* phosphate buffer at pH 6.5 in order to prevent the alkaline pH which was noted to occur by CRESTFIELD, SMITH AND ALLEN[3] when water was used. The precipitate and the buffer were blended with a spatula to the consistency of a thin paste. The suspension was allowed to stand for 3 h at 20°. The insoluble proteins were removed by centrifugation at 25,000 × *g* for 1 h at 4°. The supernatant fluid was decanted and the residue was extracted a second time. The supernatant fluids from each extraction were combined and the pH found to be 6.7. Most of the suspended material was removed by filtration with pressure through a D-7 Steriflo asbestos pad. The filtrate was examined for ribonuclease activity by permitting a 1-ml aliquot to stand at room temperature for 48 h. Aliquots of 10 µl each were removed at 0, 24, and 48 h, and applied to a sheet of Whatman No. 1 filter paper. The chromatogram was developed in the first dimensional solvent. No ribonuclease activity could be detected.

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Step 4. Precipitation of ribonucleic acids with salmine. The ribonucleic acids were separated from the bulk of the soluble proteins by precipitation of the nucleic acids with salmine according to a procedure devised by DAVIS AND ALLEN[4]. 20 ml of the salmine solution at pH 6.5 were added with stirring to the supernatant fluid from step 3. The precipitate which formed was dense and flocculent. Precipitation was allowed to continue for 10 h at 4°. The salmine nucleate was collected by centrifugation at 10,000 × *g* for 10 min.

Step 5. Dissociation of salmine nucleate. The salmine nucleate was dissociated with saturated solution of sodium picrate at pH 5.5. The salmine nucleate was added to 60 ml of saturated sodium picrate at 25° and slowly stirred 4 h then cooled to 4° and left in the cold for 6 h. The picrate ion displaced the ribonucleic acids from the salmine and formed an insoluble salmine picrate. The ribonucleic acids were now in solution. The insoluble salmine picrate was removed by centrifugation at 25,000 × *g* for 1 h at 4°. The clear supernatant fluid was dialyzed for 24 h in distilled water to remove most of the sodium picrate. The dialyzed supernatant liquid contained ribonucleic acids and traces of sodium picrate which failed to dialyze. The volume at this point was 80 ml.

Step 6. Purified ribonucleic acids. Solid sodium chloride was added to the supernatant liquid of step 5 to bring the concentration to 1 *M* with respect to the salt. The solution was left at 4° with the expectation that ribonucleic acids would precipitate in a manner similar to that which CRESTFIELD, SMITH AND ALLEN[3] had observed for ribonucleic acids from yeast. After 12 h no precipitation occurred. The solution was then added with stirring to two volumes of 95% ethyl alcohol. A copious precipitate of ribonucleic acids formed. After 4 h the ribonucleic acids were collected by centrifugation, and washed three times with 67% ethyl alcohol to remove the traces of sodium picrate, then dissolved in 50 ml of water and dialyzed for 36 h. The slight turbidity which was present was removed by filtration with pressure through a D-7 Steriflo asbestos pad. The filtrate was lyophylized. The yield of ribonucleic acids amounted to 640 mg/50 g tissue and represented 48% of the total ribonucleic acids as determined by the method of SCOTT, FRACCASTORO AND TAFT[10].

Isolation of the fifth nucleotide

The fifth nucleotide found by DAVIS AND ALLEN[4] in ribonucleic acids from yeast was isolated from an alkaline digest of ribonucleic acids from the pancreas by means of the improved two-dimensional chromatography system described by CRESTFIELD AND ALLEN[8]. In the first dimension, the fifth nucleotide appeared on the trailing edge of the guanylic acid–uridylic acid zone. In the second dimension the fifth nucleotide advanced slightly ahead of the guanylic acid zone. The chromatogram was resubmitted to the ascending flow of the second dimensional solvent in order to achieve greater resolution.

Larger quantities of the fifth nucleotide were obtained by subjecting an aliquot of the alkaline digest of the ribonucleic acids to paper electrophoresis in ammonium formate buffer at pH 2.5 and an ionic strength of 0.4 and an applied potential of 16 V/cm. Uridylic acid which was contaminated with the fifth nucleotide is resolved from the other nucleotides under these conditions. The uridylic acid zone from the electrophoretic separation was subjected to two-dimensional chromatography to separate the fifth nucleotide from the uridylic acid.

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New unknown components from the guanylic acid fraction

In the analysis of the alkaline hydrolysis of whole tissue, it was hoped to resolve 5'-mononucleotides from the corresponding 2'- and 3'-mononucleotides in order to permit a valid partition between ribonucleic acids and the tissue nucleotides, since no known tissue nucleotide is a 2'- or 3'-mononucleotide or would be converted to a 2'- or 3'-mononucleotide by alkaline hydrolysis under the present conditions. The procedure was to chromatograph to separate the respective 2'- and 3'-mononucleotides, (the 5'-mononucleotides are in unknown positions) and then to submit each zone to electrophoresis in borate buffer at pH 9.2. Under these conditions the 5'-mononucleotides will separate from the other nucleotides. Comparative relative mobilities and spectral ratios permit identification of the 5'-mononucleotide. The major characteristic is a faster movement in borate buffer than in ammonium acetate buffer at pH 9.2. It was expected that from isolated ribonucleic acids such a sequence of separations would not yield 5'-mononucleotides and, therefore, no extra areas. In the instance of guanylic acid unknown areas were encountered.

In the attempt to separate with electrophoresis the fifth nucleotide from the guanylic acid fraction, two new components were found. The guanylic acid fraction separated by two dimensional chromatography was eluted from the zone on the chromatogram. The eluate was concentrated in a vacuum and subjected to electrophoresis in ammonium formate buffer at pH 2.5 and an ionic strength of 0.4 and an applied voltage of 16 V/cm. In this buffer two zones moved faster as anions than did guanylic acid. By eluting the guanylic acid zone from which the two components had been separated and subjecting the eluate to electrophoresis in ammonium acetate buffer at pH 9.2 and an ionic strength of 0.1 with an applied voltage of 27 V/cm, it was possible to separate a third component which moved faster than guanylic acid.

RESULTS AND DISCUSSION

Two preparations of ribonucleic acids were made; one sample was prepared from tissue which had been ground to fineness in solid carbon dioxide prior to extracting with the hot detergent. The sample of ribonucleic acids which was extracted precipitated at 4° from a 1% solution made 1 *M* with respect to sodium chloride. The phosphorus content was 8.3%, and the deoxyribonucleic acids were present to the extent of 0.6%. The yield of this preparation was 40% of the total. The second sample was prepared from ground tissue which was homogenized in a Waring blendor with hot detergent. This sample did not precipitate with 1 *M* sodium chloride as did the first sample. The phosphorus content was lower (7.8%), and the deoxyribonucleic acids content was 0.9%. The protein content of both samples was less than 1% as determined by the procedure of KUNKEL AND TISELIUS[11]. The yield of ribonucleic acids was 62% of the total. Both samples exhibit the same nucleotide content as the ribonucleic acids in whole tissue.

In Table I a comparison of the moles of nucleotide released by alkaline hydrolysis from the isolated ribonucleic acids is made with those released by an alkaline hydrolysis of whole tissue. It is observed that the moles of nucleotide released are the same in each case. By this criterion it is concluded that ribonuclease activity had been blocked during the isolation procedure and that no fractionation occurred.

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TABLE I

A COMPARISON OF THE CHEMICAL PROPERTIES OF RIBONUCLEIC ACIDS ISOLATED FROM DOG PANCREAS WITH THOSE FOR RIBONUCLEIC ACIDS IN WHOLE TISSUE

	Isolated ribonucleic acids	*Ribonucleic acids in whole tissue*
Adenylic acid, moles per 100 moles	18	18
Cytidylic acid, moles per 100 moles	32	32
Guanylic acid, moles per 100 moles	30	30
Uridylic acid, moles per 100 moles	15	15
5th nucleotide, moles per 100 moles	1	1
Component 1A + 1B, moles per 100 moles	3	3
Component 2, moles per 100 moles	2	2
Oligonucleotides, moles per 100 moles	3	3
Uridylic acid liberated by ribonuclease, per cent of total	60	—
Cytidylic acid liberated by ribonuclease, per cent total	51	—
5th nucleotide liberated by ribonuclease, per cent total	64	—
Guanylic acid liberated by ribonuclease, per cent of total	1	—
Phosphorus, per cent dry weight	7.8	—
$E^{1\%}_{1cm}$ 260 mμ, 0.01 *M* HCl	200	—
$E^{1\%}_{1cm}$ 260 mμ, 0.01 *M* HCl after alkaline hydrolysis	284	—
$E^{1\%}_{1cm}$ 260 mμ, 0.01 *M* HCl after ribonuclease hydrolysis	268	—

All data are calculated on a basis of dry weight.

The precipitation of one sample of ribonucleic acids in 1 *M* sodium chloride in contrast to the other remains unexplained.

The total ribonucleic acids in the tissue, as determined by a modified procedure of SCOTT *et al.*[10], amounted to 2.2% of the tissue. The figure so obtained is approximately 18% higher than that for the ribonucleic acids in whole tissue which was obtained by chromatography of the alkaline digest and estimation of the ribonucleotides by spectrophotometry. Such a determination showed that ribonucleic acids more nearly approach a figure of 1.8% of the tissue. It would appear that the figure for the content of ribonucleic acids in pancreas, as determined by the method of SCOTT, FRACCASTORO AND TAFT[10], is too high and that a better estimation is found from the direct determination of the individual nucleotides. The yield in the present procedure then increases from 48% to 62%.

The fifth nucleotide which was found in three successive fractions of ribonucleic acids from yeast to the extent of 3, 7, and 9% respectively by DAVIS AND ALLEN[4] was found in the amount of 1% in ribonucleic acids from the pancreas. The spectral ratios of the compound are similar to those of the compound found by DAVIS AND ALLEN[4]. The electrophoretic mobilities and chromatographic movements also compare favorably.

In addition to the fifth nucleotide mentioned in the foregoing paragraph, three additional unknown components were isolated from the guanylic acid fraction. The spectral ratios of these components which are termed 1A, 1B and 2 are compared with those of guanylic acid in Table II. Components 1A and 2 have spectral ratios similar to those of guanylic acid, whereas component 1B is considerably different.

The electrophoretic mobilities of these unknown components, as determined by the procedure of CRESTFIELD AND ALLEN[3], are given in Table III. None of these compounds are the 5′ isomer of guanylic acid which has a mobility equal to or lower

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TABLE II

SPECTRAL RATIOS OF THE COMPONENTS SEPARATED FROM THE GUANYLIC ACID FRACTION BY PAPER ELECTROPHORESIS

Sample	*pH*	*250/260*	*280/260*	*290/260*
Guanylic acid	2	1.04	0.68	0.38
	12	1.02	0.65	0.15
Component 1A	2	1.09	0.68	0.45
	12	0.92	0.61	0.01
Component 1B	2	0.74	0.64	0.38
	12	0.76	0.69	0.06
Component 2	2	1.03	0.62	0.38
	12	1.01	0.66	0.37

TABLE III

COMPARISON OF THE RELATIVE ELECTROPHORETIC MOBILITIES OF THE COMPONENTS SEPARATED FROM THE GUANYLIC ACID FRACTION BY PAPER ELECTROPHORESIS WITH THE 2′, 3′, AND 5′ ISOMERS OF GUANYLIC ACID, AND WITH 2′,5′-(3′,5′)-DIPHOSPHOGUANOSINE

	pH 2	*Ammonium formate buffer*			*Sodium borate buffer*
		pH 2.5	*pH 3.6*	*pH 9.2*	*pH 9.2*
Component 1A	—	0.65	—	1.10	1.40
Component 1B	—	0.75	—	1.30	1.40
Component 2	—	0.48	—	1.32	1.40
2′-Guanylic acid	—	0.48	0.63*	1.10*	1.14*
3′-Guanylic acid	—	0.48	0.57*	1.10*	1.14*
5′-Guanylic acid	—	< 0.48	< 0.57	< 1.10	~ 1.3*
2′, 5′-(3′, 5′)-Diphosphoguanosine**	between 0.75–1.17	> 0.75	1.17	between 1.5–1.7	between 1.5–1.7

* Data from CRESTFIELD[12].
** Data from CRESTFIELD AND ALLEN[13].

than the 2′ or 3′ isomers at pH 2.5 in ammonium formate buffer, and also at pH 9.2 in ammonium acetate buffer but a greater mobility at pH 9.2 in sodium tetraborate buffer. Component 2 has the same mobility as guanylic acid at pH 2 but a greater mobility at pH 9.2 in the other two buffers. The fact that it has nearly the same mobility in ammonia buffers as in borate buffers rules out the possibility of the 5′ isomer. Components 1A and 1B cannot be the 5′ isomer because they each have a greater mobility at pH 2.5 than does guanosine-5′-phosphate under the same conditions. Mobility studies also rule out the possibility of the new components being guanosine diphosphate.

It was first suspected that the unknown components might be dinucleotides of guanylic acid when each was observed to be partially degraded by acid to yield a compound with the same electrophoretic mobility as guanylic acid in ammonium acetate buffer at pH 9.2. By subjecting each compound to the action of snake venom (*Crotalus adamanteus*) at pH 9.2 in ammonium acetate buffer, no detectable degradation could be observed by means of electrophoresis.

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By excluding the new components from the purine nucleotides the ratio of purine to pyrimidine nucleotides is 0.92. Two of the unknown components are very likely to be derivatives of guanine due to the similarity of their spectral ratios with those of guanylic acid. If this is the case, the ratios of purine to pyrimidine will be close to unity.

SUMMARY

The method of CRESTFIELD, SMITH AND ALLEN[3] for the isolation of the ribonucleic acids from yeast has been modified and applied to the isolation of the ribonucleic acids of pancreas. In order to assess the possibilities of enzymic degradation and fractionation, the nucleotide composition of the isolated ribonucleic acids has been compared with that found for the ribonucleic acids prior to isolation from pancreatic tissue and found to be identical. The ratios of purine to pyrimidine are unity which are similar to those for undegraded ribonucleic acids from other sources.

The fifth nucleotide which has been reported by DAVIS AND ALLEN[4] to be present in the fractions of the ribonucleic acids from yeast has been found to be present in the ribonucleic acids isolated from the pancreas. In addition certain unknown nucleotide components which occur with the guanylic acid fraction in paper chromatography are reported.

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THE EFFECT OF DEUTERIUM OXIDE ON SURVIVAL OF MICE WITH ASCITES TUMOR*

ANN M. HUGHES, BERT M. TOLBERT**,
KARL LONBERG-HOLM*** AND MELVIN CALVIN

Radiation Laboratory and Department of Chemistry University of California, Berkeley, Calif. (U.S.A.)

The improved availability of mass-produced deuterium at reasonable prices ($ 28 per pound for D_2O) has made it of interest to renew the study of metabolic effects of deuterium in plants and animals. The older literature on this subject has been ably

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** Present address: Department of Chemistry, University of Colorado, Boulder, Colorado.
*** U.S. Public Health Service predoctoral fellow, Department of Biochemistry, University of California, Berkeley 4, Calif.

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