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A New Exchanger for the Chromatographic Fractionation of Nucleic Acids[1]

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Fractionations of the nucleic acids have been accomplished by selective elution from various adsorbents and exchanger materials such as calcium phosphate (1), Dowex 2 (2), a modified cellulose exchanger Ecteola (3), histone-coated kieselguhr (4), methylated bovine serum albumin, and Celite (5, 6), and by the dissociation of denatured nucleoprotein complexes (7, 8). Of these materials the one which has seemingly proved to be the most useful is Ecteola, a triethanolamine derivative of cellulose (9). It has been used for the fractionation of both deoxyribonucleic acid (DNA) (3), and ribonucleic acid (RNA) (10, 11). It is reported, however, that as much as 3 weeks may be required to complete a fractionation (12). Attempts to shorten this time by increasing the elution rate resulted in a reduction of the resolving power of the exchanger and caused the peaks to overlap (12).

A survey of commercially available exchanger materials was made in an attempt to find a material which might offer advantages not exhibited by existing exchangers. One such product that has been investigated in this laboratory is a cationic starch called Cato-2 (formerly known as Cato-8, National Starch Products, Inc., 750 Third Ave., New York 17, N. Y.). This report describes the technique used for fractionating nucleic acids on columns of Cato-2 and presents results to substantiate the effectiveness of these fractionations.

METHODS

Cato-2 is manufactured under U. S. Patent No. 2,813,093 and is described therein as an ungelatinized tertiary amino alkyl ether of starch.

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It behaves as an anion exchanger. The sample of Cato-2 used in the experiments to be reported here (Lot EFR 239) has a nitrogen content of 0.23% and a capacity for DNA of about 3 mg/gm of large mesh exchanger (see below). The capacity for soluble RNA is about three times that found for DNA.

Cato-2 is supplied as a fine powder and as such is not suitable for use in columns because of impaired flow rates. To make it suitable for column use it must first be converted to a larger mesh size. One hundred grams of Cato-2 was suspended in 1 l water and allowed to settle for about 1 hr. The supernatant was decanted and the procedure repeated. The starch was then mixed with approximately an equal volume of water and filtered in a Büchner funnel (11 cm diameter). When the excess moisture disappeared from the top of the cake, it was lightly scored with a spatula. The vacuum was immediately released when the cake cracked in half on the scored line. The cake was then put bottom side down on an enamel pan and placed in a 165° oven for 3 hr, then removed and allowed to cool.

The pure white, nongelatinized top portion of the cake was crumbled off and sorted to remove traces of gelatinized material, lightly ground in a mortar, and then screened through perforated stainless steel screens. Good flow rates were obtained with material which passed a screen having $\frac{1}{32}$-in. holes, but most of the work reported here was done with material which passed through a screen having $\frac{1}{16}$-in. holes but not through a screen having $\frac{1}{32}$-in. holes.

For the fractionation of 1–2 mg nucleic acid, columns 0.8 cm in diameter have been used, containing about 10 cc of packed Cato-2 (about 2 gm of dry Cato-2). To assure equilibration with NaCl, 100 ml of 1 *M* NaCl was run through the columns at a flow rate of 0.4 ml/min. The excess 1 *M* NaCl was then washed out with about 40 ml of 0.01 *M* NaCl at the same flow rate. The excess Cato-2 was washed out to a predetermined volume and a glass wool plug was introduced to prevent resuspension of the Cato-2. Up to 2 mg nucleic acid in 5 ml of 0.01 *M* NaCl was allowed to percolate through the column at a flow rate of 0.2 ml/min. When the sample had passed into the column bed, washing was continued with 15 ml of 0.01 *M* NaCl. For the elution of the nucleic acids, the flow rate was increased to 0.4 ml/min and 2.0-ml samples were collected.

For linear gradient elution (13) four schedules have been used: (A) a linear gradient from 0.0 to 1.0 *M* NaCl; 50 fractions were collected; (B) a linear gradient from 0.0 to 1.0 *M* NH_4OH in 1.0 *M* NaCl; 50 fractions were collected; (C) 12 fractions were eluted with 1.0 *M* NaCl; (D) elution with 0.1 *M* NaOH in 1.0 *M* NaCl. Cato-2 swells in NaOH so

that the flow of liquid through a column stops. In order to elute with 0.1 *M* NaOH in 1.0 *M* NaCl, the Cato-2 was extruded from the columns into a centrifuge tube. The starch was gently shaken in about 50 ml of this reagent for 15 min, then centrifuged and the supernatant transferred to a 50-ml volumetric flask, diluted to volume with reagent, and assayed.

After elution with 0.1 *M* NaOH, the Cato-2 was discarded because of the large percentage of mechanical breakage of the Cato-2 particles during this extraction procedure. When the strongest eluting reagent used was 1 *M* NH_4OH, as when fractionating soluble RNA, the columns were reused after complete regeneration with NaCl.

When the nucleic acid sample contained as much as 80% DNA as in the samples of total tissue nucleic acids from mouse thymus (see below), the recoveries from the columns were better than 96% when uncorrected for column blank. There was some non-nucleic acid material that adsorbed at 260 mμ which was eluted from blank columns (mostly in area B). The average column blank per fraction was 0.006 optical density units. When a correction was made for this column blank, the recovery of nucleic acids (when mostly DNA) was about 85%. When the nucleic acid samples contained only RNA, however, the corrected recoveries averaged 99%.

RESULTS

Reproducibility

A sample of mouse thymus nucleic acids representing 87% of the total tissue nucleic acid content and consisting of a mixture of DNA and RNA in the ratio of 4:1 was prepared by a modification (14) of the detergent method of Crestfield, Smith, and Allen (15). Duplicate aliquots of the same sample of nucleic acid were submitted to linear gradient elution from two different columns of Cato-2 (Fig. 1). Although the samples were run on columns of different length so that some of the peaks in the shorter columns come off a tube or two sooner, the almost exact superimpossibility of the two diagrams is obvious. Furthermore, when duplicate aliquots of the same sample of mouse thymus RNA prepared by extraction with phenol (16) were submitted to separate columns and fractionated by the technique of discontinuous elution (see below), the amount of material eluted in the various peaks differed by less than 3% between the two columns (14).

Rechromatography

Although the method gives reproducible results for duplicate columns, it is also of importance to determine whether the emergence of material

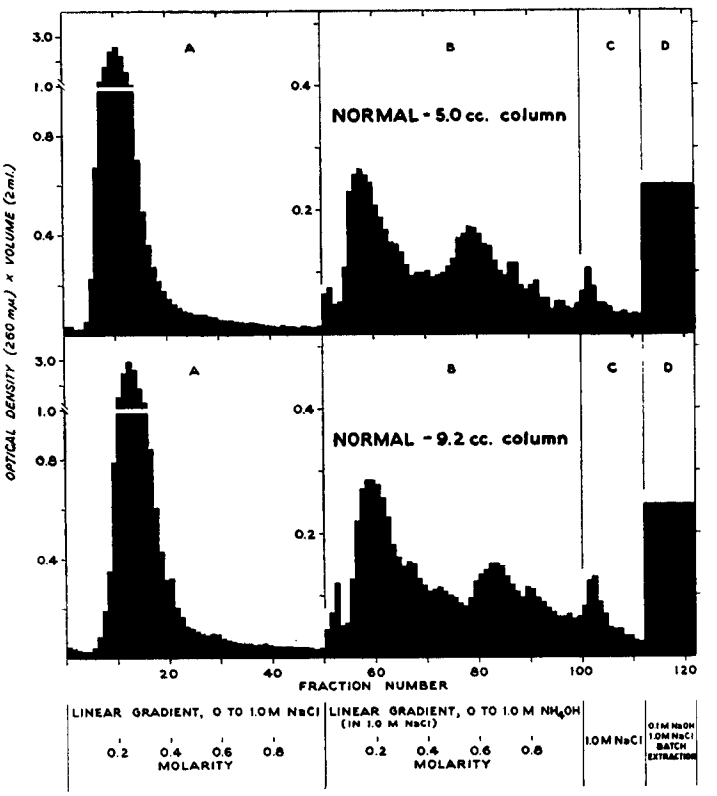

FIG. 1. Chromatography of duplicate samples of the same preparation of mouse thymus nucleic acids (DNA and RNA) representing 87% of the total tissue nucleic acids, on Cato-2 columns (0.8 cm diameter) of different length. Linear gradient elution was used at a flow rate of 0.4 ml; 2-ml samples were collected.

in any one peak along the elution diagram is a reproducible characteristic of that particular material on the column or is a secondary consequence of the over-all interaction between the exchanger and the various nucleic acid species present. If the former is true, then a peak should emerge from the column under the same conditions whether it has been adsorbed separately or as part of a mixture of nucleic acids. To test this, a sample of commercial DNA (Worthington Biochemical Corp., Freehold, N. J.) was fractionated on a column of Cato-2 (Fig. 2). The nucleic acid from the shaded area was recovered by alcohol precipitation and then resubmitted to a second column giving the results in the black areas. Ninety per cent of the resubmitted nucleic acid was again eluted from the column under the same conditions as before. Probability curves constructed for a theoretical resubmission experiment have the same

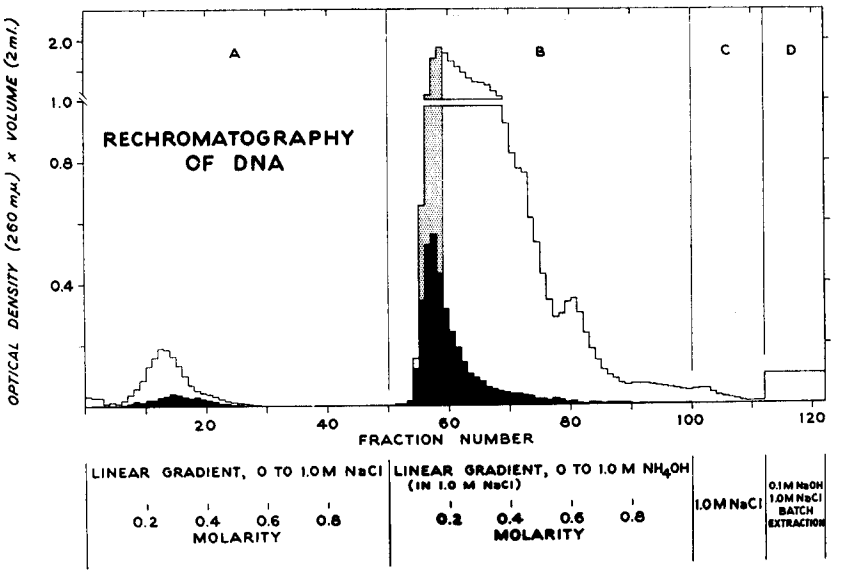

FIG. 2. Rechromatography of commercial calf thymus DNA on Cato-2 columns (0.8 × 18.3 cm): (*a*) original pattern given by solid line; (*b*) fraction to be resubmitted after recovery by alcohol precipitation indicated by shaded area; (*c*) results of resubmission experiment given in solid black areas. Linear gradient elution was used at a flow rate of 0.4 ml; 2-ml samples were collected.

shape as the curve obtained in area B. The degree to which a given peak will mimic its original behavior when resubmitted to columns of Cato-2 is therefore considered highly satisfactory.

Effect of Molecular Size

Figure 3 shows the results of one experiment to investigate the importance of molecular weight in determining the chromatographic behavior of the nucleic acids. The dark curve is the elution profile for a commercial sample of DNA (Worthington Biochemical Corp., Freehold, N. J.). The shaded area is the elution pattern for a deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) digest of a sample of this same DNA. It is apparent that molecular size does play a role in the fractionation obtained on columns of Cato-2. In other experiments it was found that adenine ribonucleotide was eluted in the same area as the digest of DNA, while uracil did not adhere to the column and was completely removed in the 0.01 *M* NaCl washing step. Since the largest polynucleotide in a deoxyribonuclease digest is probably an octanucleotide (17), either the column is not very selective for materials with molecular weights below about 3000 or else the conditions of elution do not make use of its selectivity (see below).

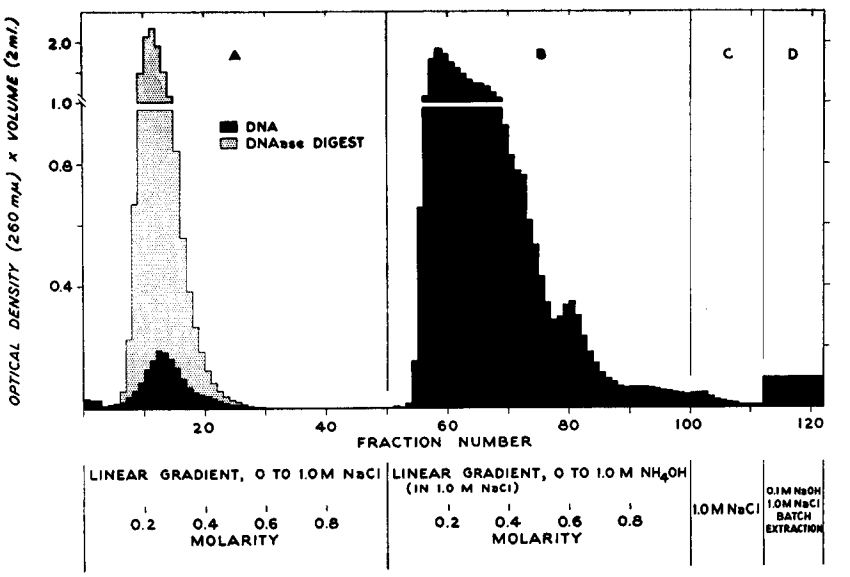

FIG. 3. Chromatography of commercial calf thymus DNA on Cato-2 columns (0.8 × 18.3 cm) before and after deoxyribonuclease digestion; using linear, gradient elution at a flow rate of 0.4 ml/min; 2-ml samples were collected.

Fractionation by Discontinuous Elution

The above result with the deoxyribonuclease digest was at first disappointing because the digest was eluted under the same conditions as a large part of the total mouse thymus nucleic acids (compare with Fig. 1). Quite different results were obtained, however, when the various samples were eluted by the technique of discontinuous elution instead of linear gradient elution. The method used for discontinuous elution does not differ from that already outlined for linear gradient elution as regards the preparation of the column, elution flow rates, etc., except that the eluting solutions were changed manually when the optical density at 260 mμ fell to a value below 0.040, and usually below a value of 0.020. To cover that portion of the diagram designated as area A in the linear gradient method, five discontinuous elutions have been used; followed then by the batch extraction of the Cato-2 as described in elution schedule D. The fractionations obtained by continuous elution and by discontinuous elution may be compared in Fig. 1 and Fig. 4, respectively.

With discontinuous elution adenylic acid could be eluted with 0.05 *M* salt, and deoxyribonuclease digests of DNA and ribonuclease digests of RNA could be eluted with 0.10 *M* salt. Less than 10% of the total tissue nucleic acids of mouse thymus was eluted with 0.10 *M* salt. These results with discontinuous elution indicate that the columns do distinguish be-

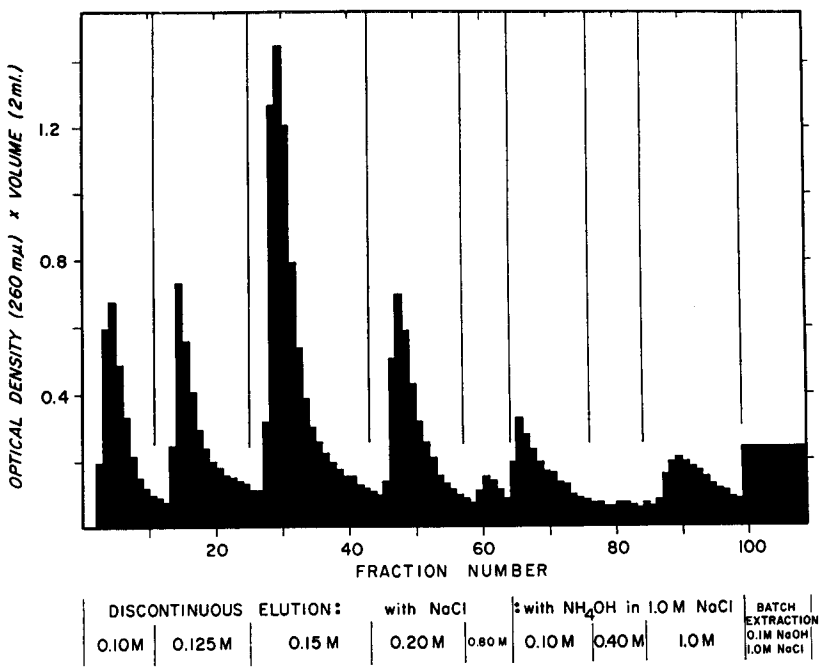

FIG. 4. Chromatography of mouse thymus nucleic acids (DNA and RNA), representing 92% of the total tissue nucleic acids, on a Cato-2 column (0.8 × 18.3 cm) using discontinuous elution at a flow rate of 0.4 ml/min; 2-ml samples were collected.

tween lower molecular weight polynucleotides. In an effort to obtain similar results with gradient elution, the rate of change of the gradient was decreased. Even a 20-fold decrease in the rate of change of the gradient still did not bring about fractionation in the neutral salt area.

Separation of Biologically Active Nucleic Acids

Nucleic acids recovered from the peaks obtained by the method of discontinuous elution have demonstrated different biological activities. Soluble RNA from guinea-pig liver has been fractionated into samples which show a differential ability to incorporate specific amino acids (18). The RNA was fractionated by an elution schedule similar to that given in Fig. 4. It was found that the RNA which combines with tyrosine under the influence of an enzyme also found in the soluble fraction was eluted primarily in the 0.125 *M* NaCl peak, while the RNA which combines with leucine was found primarily in the 0.20 *M* NaCl peak. The ratio of the specific activities of leucine-RNA to tyrosine-RNA in the unfractionated material was 4:1. This ratio changed to 1:1 in the 0.125 *M* peak and was 21:1 in the 0.20 *M* NaCl peak. Assays for the incorpora-

tion of lysine, isoleucine, and threonine showed that some fractionation of RNA's specific for these amino acids was also achieved. We thus have an example of nucleic acids which differ not only chromatographically but also on the basis of a functional parameter.

Separation of Soluble and Microsomal RNA

It was reported that at least a partial separation of transfer RNA and microsomal RNA was obtained on columns of Cato-2 since 85–90% of the former was eluted with neutral 1.0 *M* NaCl while about 65% of the microsomal RNA remained adsorbed under these conditions and was eluted with alkaline salt solutions (18). We now find that when using washed mouse liver microsomes as the source of the RNA, better than 80% of the RNA is not eluted with neutral salt but can be eluted with alkaline salt solutions (19). The 20% of the RNA that is eluted by neutral salt does not come off the column until a concentration of 0.2 *M* is used, while about 50% of the soluble RNA is eluted from the columns with 0.15 *M* salt. It is remarkable that the washed microsomes of the type used for the isolation of the above RNA are still capable of incorporating amino acids into protein without the addition of soluble RNA. Furthermore the fraction of RNA isolated from washed microsomes that is eluted from the columns by neutral salt is capable of accepting amino acids in the presence of purified pH 5 enzymes. These results either point up the extreme heterogeneity of amino acid acceptor RNA's, or as preliminary experiments suggest only a portion of the amino acid acceptor RNA isolated from tissue supernatants obtained by spinning for 60 min at 105,000 $\times$ *g* is involved in microsomal protein synthesis; the remainder is probably involved in other pathways of amino acid metabolism.[2] Work to clarify this point is in progress.

Variation in Base Composition of Chromatographic Fractions

The various peaks obtained by discontinuous elution exhibit differences in chemical composition. Calf thymus RNA was prepared by extraction with 90% phenol (16). Forty-nine milligrams of RNA in 13 ml of 0.01 *M* NaCl were adsorbed on a column of $\frac{1}{32}$-in. mesh Cato-2 (2.1 cm $\times$ 32 cm) at a flow rate of about 1 ml/min. Twelve-milliliter samples were collected. Elution was performed at a flow rate of about 2 ml/min and was continued until the optical density at 260 mμ fell below a value of 0.020 before changing to the next eluting solution. The six fractions thus obtained were recovered by alcohol precipitation.

Aliquots of the recovered material were then subjected to alkaline hydrolysis (0.3 *M* KOH at 37° for 18 hr), and the nucleotides were

[2] See Bates, H. M., and Lipman, F., *J. Biol. Chem.* **235,** 22P (1960).

separated by the technique of paper electrophoresis in 0.4 *M* formate buffer at pH 3.2. Areas containing the nucleotides were located by ultraviolet photography (20). These areas were cut from the papers and the nucleotides were extracted with 0.01 *M* HCl. Concentrations were determined with a Beckman DU spectrophotometer using the following millimolar extinction coefficients at 260 mμ: adenylic acid, 14.2; guanylic acid, 11.8; cytidylic acid, 6.8; and uridylic acid, 10.0. The elution conditions and the nucleotide composition of the various fractions are given in Table 1.

TABLE 1
COMPOSITION OF CALF THYMUS RIBONUCLEIC ACID FRACTIONS

Chromatographic fraction	Per cent of total RNA	Nucleotide composition (moles/100 moles)				$\frac{G}{A}$	$\frac{C}{U}$	$\frac{A+G}{C+U}$
		C[a]	A	G	U			
Unfractionated RNA		32.1[b]	18.1	31.6	18.2	1.75	1.76	0.99
0.125 *M* NaCl	4.1	32.9	16.8	32.1	18.2	1.91	1.81	0.96
0.150 *M* NaCl	6.4	31.9	18.1	31.4	18.5	1.74	1.72	0.98
0.200 *M* NaCl	24.7	31.5	18.5	31.4	18.6	1.70	1.70	1.00
0.800 *M* NaCl	12.5	31.3	18.7	31.6	18.5	1.69	1.69	1.01
1.0 *M* NaCl + 1.0 *M* NH_4OH	51.5	31.7	18.7	30.3	19.2	1.62	1.65	0.96
1.0 *M* NaCl + 0.1 *M* NaOH	0.8	—	—	—	—	—	—	—
	Recovery[c]	98%	102%	97%	103%			

[a] C, A, G, and U represent the nucleotides: cytidylic acid, adenylic acid, guanylic acid, and uridylic acid, respectively.

[b] Average deviation is 2%.

[c] Nucleotide composition of calf thymus RNA calculated as the sum of the increments contributed by each fraction and expressed as the per cent of the values found for the unfractionated sample.

The various RNA fractions show a progressive increase in adenine content relative to the guanine content as the eluting solutions necessary for their removal from the column become more concentrated. A similar progressive change has been obtained in the base composition of DNA fractions (4, 7). A chemical explanation for this phenomenon has been published (4, 10).

The various RNA fractions show a purine to pyrimidine ratio near unity. This is of special significance in the fractions eluted by neutral salt since the major portion of these fractions is composed of RNA from the soluble fraction of the cells (18, 19). The relatively smaller size of this class of RNA has implied the lack of a requirement for base pairing as is found in DNA and more recently in microsomal RNA (21). It

would now appear that there may be base pairing even in these smaller ribonucleic acids.

DISCUSSION

It is unreasonable to assume that one exchanger material would meet the requirements for the complete fractionation of anything so complex as the total nucleic acids of a tissue. An exchanger exhibiting weak basic properties is required for the efficient release of the larger molecular weight nucleic acids, and somewhat more basic exchangers are to be preferred for the fractionation of lower molecular weight polynucleotides; nevertheless, the selection of an exchanger material for a particular separation is largely empirical. Discussions of some of the problems involved in the fractionation of the nucleic acids with special emphasis on the influence of the chemical and physical heterogeneity of the nucleic acids (4, 10) and the heterogeneity of the exchanger materials themselves have been published (10).

Although Cato-2 was not developed commercially for chromatographic use, it has proved to be very useful in this capacity. A simple pretreatment of Cato-2 converts it to a mesh size more useful for column work. It exhibits characteristics that are to be desired in any exchanger material: (*a*) duplicate columns yield duplicate results; (*b*) isolated peaks rechromatograph as they did originally; (*c*) it distinguishes between samples which differ chemically and/or by molecular weight; and (*d*) it apparently does not alter the sample during its passage through the column since fractionated soluble RNA retains its biological activity as a binding agent for specific amino acids. Cato-2 offers an important technical advantage in that fractionation remains sensitive at much faster flow rates than those employed with other exchangers. This allows the completion of an experiment in days instead of weeks.

Preliminary accounts of the application of this method to the study of the nucleic acid changes in isologous tumor and x-irradiated tissues have appeared (22, 23). This work will be reported in more detail elsewhere.

SUMMARY

1. A new exchanger material, Cato-2 (a cationic starch), has been found to be suitable for the chromatographic fractionation of the nucleic acids. Duplicate columns yield duplicate results and isolated peaks rechromatograph as they did originally.

2. Much faster flow rates than those usually employed with other exchangers can be used with Cato-2 without loss of resolution. This allows the completion of an experiment in days instead of weeks.

3. That the fractions obtained under these conditions represent real and significant differences in the nucleic acids present is documented by the fact that the fractions differ not only in chemical composition but also in biological activity.

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