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Characterization of Sea Urchin Ribosomal Satellite Deoxyribonucleic Acid*

James B. Patterson† and Darrel W. Stafford‡

ABSTRACT: Ribosomal satellite DNA from the sperm of the sea urchin *Lytechinus variegatus* has been examined by means of saturation hybridization with rRNA, denaturation analysis, and renaturation analysis. The saturation level of hybridization with homologous rRNA was about 0.37 μg of rRNA/μg of rDNA. Denaturation analysis indicated that the ribosomal satellite DNA was composed of two nearly equal components of 59.5% G + C and 65.2% G + C content. Renaturation kinetics yielded a $C_0t_{1/2}$ of 1.7×10^{-2} mole sec l.$^{-1}$ under conditions equivalent to 0.12 M phosphate buffer (pH 7.0). Renaturation studies further indicated that the satellite DNA behaved as a single family of ribosomal genes of identical or very similar repeating base sequences of about 5.2×10^6 amu.

The genes for ribosomal RNA in *Lytechinus variegatus* occur in a satellite DNA with a density in CsCl of 1.722 g/cm^3. This ribosomal satellite (rDNA)[1] comprises about 0.02–0.03% of the total DNA of the sea urchin sperm. In a previous paper (Patterson and Stafford, 1970) we showed that the rDNA could be isolated from the main band DNA by virtue of the differences in their G + C content, 63 and 35%, respectively. The rDNA isolation was achieved by selective denaturation of the main band DNA followed by separation of the native and denatured DNA in a polyethylene glycol–dextran two-phase system. It was further demonstrated by hybridization of sea urchin rRNA to DNA that the genes for rRNA were contained in the satellite DNA.

The isolation of a specific portion of the sea urchin genome should allow us to ask specific questions on the control of gene expression in development. Before the relevant questions could be asked, however, further physical and chemical characterization of the satellite were necessary. We report here the analysis of satellite DNA by means of denaturation, renaturation, and saturation hybridization experiments.

Materials and Methods

Reagents. All reagents used in these experiments were previously described (Patterson and Stafford, 1970), except for the following additional reagents. CTAB was purchased from Matheson Coleman & Bell. Cesium chloride was purchased from Varlacoid Chemical Co.

DNA Isolation. Sea urchin sperm DNA was isolated as previously described (Patterson and Stafford, 1970). Satellite DNA was isolated using the polyethelene glycol–dextran two-phase system followed by preparative CsCl density gradient centrifugation as previously described (Patterson and Stafford, 1970). Each preparation of satellite DNA was analyzed by CsCl analytical centrifugation at a concentration of 15 μg/cell. There was no detectable main band in any preparation, indicating that the satellite DNA used for these experiments was at least 99.8% satellite DNA.

Sea urchin ^{32}P-labeled rRNA was isolated from ribosomes of larvae at the pluteus stage of development. The larvae were grown in Millipore-filtered sea water made 50 μg/ml with respect to streptomycin and 300 units/ml with respect to penicillin (Glišin and Glišin, 1964). The larvae were labeled at the prism stage with 5 μCi/ml of [^{32}P]monosodium for 20 hr. Larvae from the eggs of one gravid female were washed once in Millipore-filtered sea water, once in Hultin's homogenization buffer (Hultin, 1961), resuspended in 15 ml of Hultin's homogenization buffer, and homogenized in a Dounce homogenizer with tight-fitting pestle. No whole cells were observed by phase microscopy. The postmitochondrial supernatant was made 1% with respect to Triton X-100, pelleted through 40% sucrose in Hultin's buffer, and resuspended in 5 ml of pH 5.1 buffer (1 M NaCl–0.01 M sodium acetate) and 1% SDS. The rRNA was prepared by extracting the ribosomes four times

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† Present address: Cell Biology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, N. C. 27709.
‡ To whom to address correspondence.

[1] Abbreviations used are: SSC, standard saline citrate; MAK, methylated albumin kieselguhr; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; rRNA, ribosomal ribonucleic acid; rDNA, deoxyribonucleic acid containing the base sequences coding for rRNA; amu, atomic mass units.

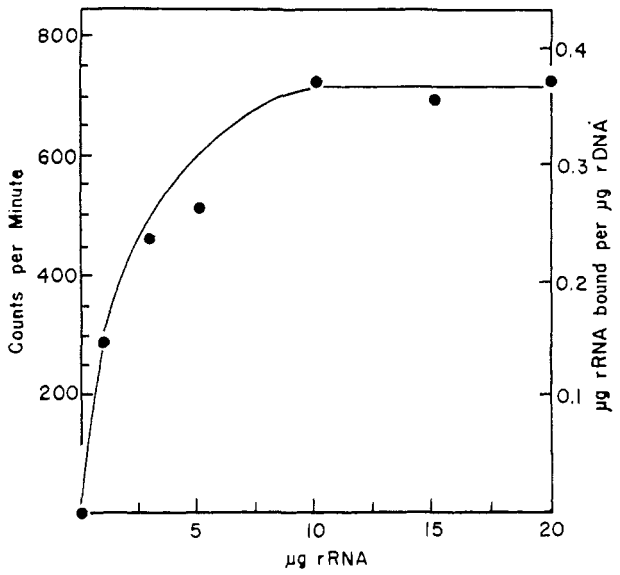

FIGURE 1: Saturation hybridization of homologous rRNA to sea urchin ribosomal satellite DNA. The rRNA and rDNA were prepared as described in the Materials and Methods section of the text. A background of 24 counts per minute has been subtracted from each point on the graph. rRNA (1 μg) was adsorbed to each filter. Incubation occurred at 67° for 4 hr in 0.6 ml of 2X SSC. The specific activity of the rRNA was 1950 cpm/μg. Counting efficiency was 91%.

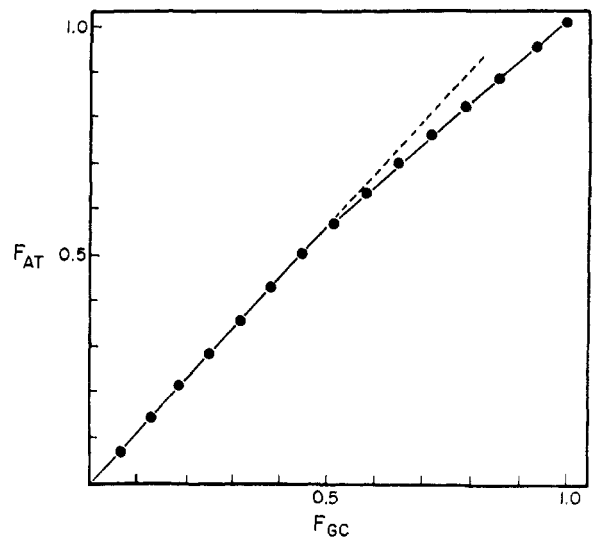

FIGURE 2: Spectral analysis of thermal denaturation of sea urchin ribosomal satellite DNA. F_{AT} is the fraction of all A·T base pairs and F_{GC} is the fraction of all G·C base pairs denatured at each temperature increment in the denaturation of DNA. F_{AT} and F_{GC} were calculated from hyperchromic spectral analysis (Hirschman and Felsenfeld, 1966). The G + C content of the first half of the graph of 59.5%, past the break point in the G + C content is 65.2%.

with equal volumes of phenol–cresol–8-hydroxyquinoline mixture (Kirby *et al.*, 1967) containing one-eighth total volume of 24:1 chloroform–isoamyl alcohol. The clear aqueous phase was pipetted off and combined with two volumes of cold 95% ethanol and the precipitate was pelleted and washed with 65% ethanol. The pellet was redissolved in a 2 ml of water, made 4 M with respect to sodium chloride, and refrigerated overnight. The precipitated RNA was pelleted through 6 M NaBr, washed with 4 M NaCl, and resuspended in 4 ml of 0.1 M sodium acetate. The rRNA was precipitated by adding 1% CTAB dropwise (Bellamy and Ralph, 1968), the pellet dissolved in H_2O, made 2X with respect to SSC, then precipitated with two volumes of 95% ethanol. The pellet was resuspended and reprecipitated two more times, then suspended in 2X SSC and passed through a Millipore filter. The rRNA was further purified by MAK column chromatography (Mandell and Hershey, 1960). The MAK column was first washed with 0.05 M sodium phosphate and 0.5 M sodium chloride until the eluate showed no $OD_{260\,nm}$. Only that fraction eluting between 0.5 M sodium chloride–0.05 M sodium phosphate and 0.9 M sodium chloride–0.05 M sodium phosphate was used in the experiments. The optical density spectra of such a rRNA preparation showed OD_{260}/OD_{280} = 2.13 and OD_{260}/OD_{230} = 2.50. The specific activity was 1950 cpm/μg.

Hybridization. Sea urchin satellite DNA was prepared as previously described (Patterson and Stafford, 1970).

Ribosomal satellite DNA from preparative CsCl density gradients was run on a Spinco Model E analytical ultracentrifuge to determine the purity of the sample. No main band DNA was detectable in sample loads of 4–15 μg of purified ribosomal satellite DNA in 12-mm Kel-F cells at 265 nm. Each DNA fraction of 1 μg was diluted to 4 ml with water, denatured at 97° for 10 min, quickly chilled, and made 6X SSC by adding 1 ml of ice-cold 30X SSC. Each fraction was collected on a nitrocellulose membrane filter which had been presoaked in 6X SSC for 4 hr. The filters were washed with 100 ml of 6X SSC, dried at room temperature for 2 hr, and then dried *in vacuo* for 12 hr at 60° (Gillespie and Spiegelman, 1965).

Filters upon which DNA had been adsorbed were placed flat, DNA up, on the bottom of silicon-coated scintillation vials. One filter was placed in each vial and a specific quantity of rRNA added to it. The final reaction volume was brought to 0.60 ml with 2X SSC. Replicate samples were run at each rRNA concentration. A small square of Parafilm was placed over the vial and the top was screwed on tightly to prevent evaporation. Incubation at 67° for 4 hr was terminated by incubation of each filter with 8 ml of heat-treated ribonuclease A at a concentration of 30 μg/ml for 5 min. After ribonuclease treatment, each filter was washed on both sides with 50 ml of 2X SSC at about 65°, dried, and counted by liquid scintillation spectrometry for 30 min. Counting efficiency was 91%.

Sedimentation Velocity. Sedimentation coefficients were determined by band sedimentation in a 12-mm Kel-F band forming centerpiece. The bulk solution for native DNA was 1.0 M NaCl; the bulk solution for denatured DNA was 0.9 M NaCl–0.1 M NaOH. Rotor speed was 25,980 rpm (Studier, 1965).

DNA Degradation. Ribosomal satellite DNA was exhaustively dialyzed against 0.875 M NaCl. Immediately before the renaturation experiment was run, the native rDNA was sheared by sonication. Air was evacuated from the rDNA until it boiled at room temperature, then the rDNA was saturated with nitrogen. The rDNA was next placed in ice–water bath and sonicated at a calibrated energy level for 20 sec using the microtip attachment on a Sonifer cell disruptor, Model W14OD. Alkaline sedimentation coefficients of rDNA sonicated in this manner were generally less than 7.5 S, they were unchanged after the renaturation experiments, and the bands were symmetrical.

Renaturation Analysis. The rDNA (0.8 ml) in 0.875 M NaCl was saturated with helium in a 1-ml quartz cuvet, stoppered, and set in the cuvet chamber of a Gilford 2000 spectropho-

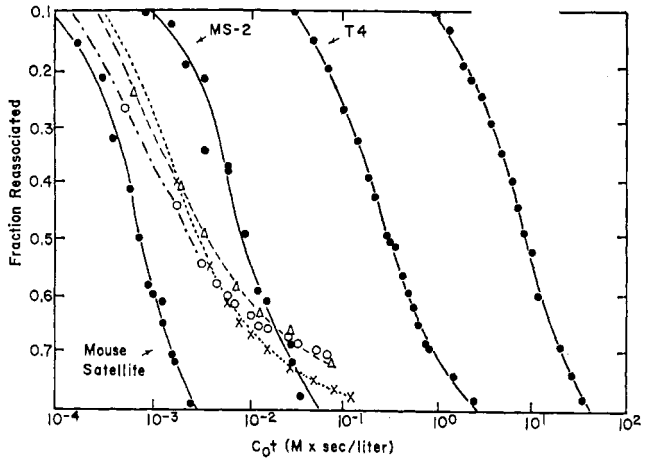

FIGURE 3: The reassociation curve for denatured sea urchin ribosomal satellite DNA. The renaturation occurred in 1.0 M sodium salt (Wetmur and Davidson, 1968) as described in the Materials and Methods section of the text. The $C_0t_{1/2}$ under conditions equivalent to 0.12 M phosphate buffer at neutral pH (Britten, 1970) was 0.017 mole sec $l.^{-1}$. The separate experiments were plotted on this graph. Single-strand molecular weights for the rDNA used were: (○) 0.54 × 10^6, (△) 0.20 × 10^6, and (×) 0.23 × 10^6. The curve for *E. coli* DNA, T 4 DNA, MS2 RNA, and mouse satellite DNA are from Britten and Kohne (1965) for reference.

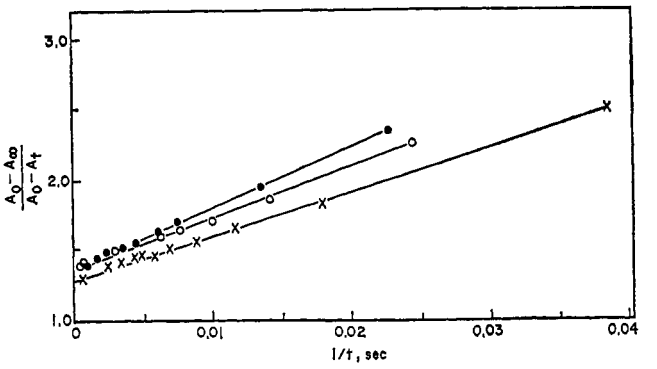

FIGURE 4: The renaturation of denatured DNA. Three separate experiments were plotted on this graph. Single-strand molecular weights for the rDNA used were (○) 0.54 × 10^6, (●) 0.20 × 10^6, and (×) 0.23 × 10^6. The straight lines enable one to precisely measure the slopes and thus determine the second-order rate constants.

tometer. Thermospacers attached to a thermostatically controlled glycerol–water bath kept the chamber temperature at 82.5°, 25° below the T_m of the rDNA. Each cuvet (blank and rDNA) was automatically monitored at 260 nm for 9 sec followed by a 5-sec temperature reading with a Gilford linear thermosensor. Total cycle time was 30 sec. The native rDNA was read at 260 nm after the temperature equilibration to establish the A_∞ value. The rDNA was denatured by adding 0.1 ml of 1.0 M NaOH, then read to establish the A_0 value. The average hyperchromicity value was 30%.

The mixing of 0.1 ml of preheated 2 M NaH_2PO_4 with the rDNA gave an immediate onset of the renaturation reaction which was monitored by the change in $OD_{260\,nm}$. Prior to each experiment, the linear thermosensor and wavelength were calibrated, and optical density standards were used to calibrate the recorder and absorbance meter.

Denaturation Analysis. The rDNA was dialyzed exhaustively against 0.01 M phosphate (sodium, equimolar) then saturated with helium in a 1-ml quartz cuvet. The chamber was equilibrated at 2° increments for at least 15 min before each reading. Readings were made at 5-nm intervals from 230 to 290 nm. The linear thermosensor was checked against a thermocouple placed in the blanking cuvet and found to agree within ±0.1°. The temperature range was 62–92°. All absorbance measurements were corrected for water expansion and melting curves were constructed at each wavelength to check the accuracy of the observed absorbances. The data were analyzed by the methods described by Felsenfeld and his coworkers (Felsenfeld and Sandeen, 1962; Felsenfeld and Hirschman, 1965; Hirschman and Felsenfeld, 1966; Hirschman *et al.*, 1967) using their parameters for a quadradic analysis. The computer program was written by Mr. Nirmal K. Mishra in PL-1 language for an IBM 360 computer.

Results

Saturation hybridization can be used as a measure of the amount of DNA in a given sample which contains the bases complementary to a sample of RNA (Nygaard and Hall, 1963; Gillespie and Spiegelman, 1965). We hybridized sea urchin rRNA to ribosomal satellite DNA as described in Materials and Methods and the results are shown in Figure 1. The level of hybridization was about 0.35–0.38 μg of rRNA bound per μg of rDNA. Control experiments were performed by incubating nitrocellulose filters containing main band DNA and nitrocellulose filters alone with ^{32}P-labeled sea urchin rRNA. The counts per minute for main band DNA and no DNA saturation levels of rRNA were 57 and 25 cpm above background, respectively. In Figure 1 the counts per minute shown represent the total counts per minute minus 24 cpm.

We analyzed rDNA by the elegant methods of Felsenfeld and his coworkers. The melting curve for rDNA (not shown) was biphasic but not to the extent exhibited by *Xenopus* (Dawid *et al.*, 1970). The rDNA melted from 67 to 87° in 0.01 M phosphate (equal molar amounts of monobasic and dibasic sodium phosphate). The T_m was 77°, indicating a G + C content of about 63% (Schildkraut and Lifson, 1965). A plot of the fraction of G·C base pairs *vs.* the fraction of A·T base pairs denatured at temperatures from 62 to 90° is shown in Figure 2. The curve is divided into two straight lines of approximately equal length. The G + C content of that DNA melting in the first part of curve is 59.5%; after the break the G + C content is 65.2%. The mean G + C content is about 63%.

rDNA was sheared by sonication to single-strand molecular weights ranging from 0.20 × 10^6 to 0.54 × 10^6 amu as determined by alkaline sedimentation velocity (Studier, 1965) for renaturation studies. Figure 3 shows the renaturation curves of three samples of rDNA as determined by loss of hyperchromicity. All samples are 50% renatured at about 2.4 × 10^{-3} mole sec^{-1} $l.^{-1}$ in 1.0 M Na^+. This corresponds to a $C_0t_{1/2}$ of about 1.7 × 10^{-2} mole sec $l.^{-1}$ when adjusted to conditions equivalent to 0.12 M phosphate buffer (pH 7.0) (Britten, 1970). The curves for *Escherichia coli* DNA, T4 DNA, MS2 RNA, and mouse satellite DNA are taken from Britten and Kohne (1965) for reference.

A different second-order plot of the kinetic data is shown in Figure 4. This plot is analogous to the one used by Subirana and Doty (1966). Three rDNA samples of differing molecular weights and concentrations are shown. Each gives a straight line allowing the calculation of k_2, the experimental second-order rate constant (Wetmur and Davidson, 1968).

Table I summarizes the results of the kinetic analysis of sea

TABLE I: Renaturation Analysis of rDNA.

$s^{pH\ 13}_{20,w}$	Single-Strand Mol Wt $\times 10^{-6}$	k_2 (l. mole^{-1} sec^{-1})	Kinetic Complexity[a] ($\times 10^{-6}$)	Analytical Complexity ($\times 10^{-6}$)	Kinetic: Analytical Ratio
7.0	0.20	1108	4.45	5.2	0.86
7.4	0.23	1005	5.26	5.2	1.01
10.4	0.54	1375	5.85	5.2	1.12

[a] The kinetic complexity is corrected to 64% G + C by a factor of 1.27 (Wetmur and Davidson, 1968).

urchin rDNA. The k_2 values were taken from the lines in Figure 4. The kinetic complexity was calculated using the empirical formula given by Wetmur and Davidson (1968) for

$$N_I \quad \frac{5.5 \times 10^8 (s^{pH\ 13}_{20,w})^{1.25}}{1.27 k_2} \tag{1}$$

a DNA of 64% G + C content. The mean kinetic complexity was about 5.2×10^6 amu.

Discussion

Eukaryotic organisms which have been studied transcribe their ribosomal precursor RNA from a large transcriptional unit. The precursor RNA is then modified and degraded to its characteristic rRNAs. There is no reason to believe that sea urchins are an exception. Perry and coworkers (1970) studied ribosomal transcriptional units from a number of organisms. They found a correlation between presumed evolutionary progression and the size of the transcriptional unit. By fitting sea urchins to this scale one may estimate that its ribosomal precursor RNA would be about 2.7×10^6 amu. Doubling this value to account for both strands gives an estimate of 5.4×10^6 amu for the size of the repeating unit of sea urchin rDNA. Our hybridization saturation data yield an estimate of about 5.2×10^6 amu for the size of the gene for rRNA in sea urchins.

The reasoning used in arriving at this estimate for the analytical complexity of rDNA is as follows. Hybridization of rRNA–rDNA yields a figure of about 37% at saturation. Doubling this figure gives about 75% as the amount of the DNA that acts as template and complement. rRNA (26 S + 18 S) in the sea urchin *Arbacia* (Sy and McCarty, 1968) should give a total molecular weight value of about 2.08×10^6 amu (Loening, 1970). Double this value, 4.16×10^6, plus the 25% that does not hybridize yields a total ribosomal gene size of about 5.2×10^6 amu.

Renaturation kinetic data indicates that the ribosomal genes react as multiple copies of size about 5.2×10^6 amu and that there is little or no heterogeneity of the rDNA. Because the kinetic complexity of the DNA is the same as the estimated analytical complexity it seems safe to conclude that there is little heterogeneity in the rDNA.

Several methods for determining the second-order rate constant of DNA renaturation and its relationship to genome size have been published. Most commonly cited is the work of Britten and Kohne (1965) and Wetmur and Davidson (1968). We believe that the method presented here is in several aspects superior. Wetmur and Davidson's excellent paper provides an empirical relationship between the experimental second-order rate constant, k_2, and the kinetic complexity, N_D. The equation to determine k_2 is

$$\frac{A_0 - A_\infty}{A_t - A_\infty} = \frac{k_2 P_T t}{2} + 1 \tag{2}$$

where A_0 = the $OD_{260\,nm}$ at zero reaction time or maximum hyperchromicity, A_∞ = the $OD_{260\,nm}$ at infinite reaction time or minimum hyperchromicity, and P_T = total DNA phosphate concentration, $1.47 \times 10^{-4} A_\infty$ mole l.$^{-1}$.

With less sophisticated experimental apparatus or more complex DNA this form of the equation does not always yield straight line plots. However, a form analogous to one used by Subirana and Doty (1966) which yields straight-line plots can be derived directly from eq 2. First subtract one from both sides of the equation to obtain

$$\frac{A_0 - A_t}{A_t - A_\infty} = \frac{k_2 P_T t}{2} \tag{3}$$

By inverting eq 3 and adding one to each side of the equation, we obtain

$$\frac{A_0 - A_\infty}{A_0 - A_t} = \frac{2}{k_2 P_T t} + 1 \tag{4}$$

The equation is now in slope intercept form. Because the k_2's in eq 2 and 4 are the same, the empirical formulation of Wetmur and Davidson, eq 1, can be used to determine the kinetic complexity of the rDNA.

In the sea urchin *Lytechinus variegatus*, the ribosomal satellite comprises 0.2% of 0.3% of the total DNA. The percentage has not been precisely determined but if it is taken as 0.25% and the size of each repeating unit containing the ribosomal gene is determined, then the number of copies of ribosomal genes in the haploid sea urchin genome can be estimated.

Each *Lytechinus* sperm contains 0.90×10^{-12} g of DNA (Mirsky and Ris, 1951) or 2.25×10^{-15} g of DNA in the ribosomal satellite. For 2.25×10^{-15} g of rDNA cell^{-1} $\times$ 6.0×10^{23} amu g^{-1} there is 1.35×10^9 amu rDNA cell^{-1}. If each ribosomal gene has 5.2×10^6 amu copy^{-1}, then *Lytechinus* sperm contains about 260 copies of the ribosomal gene per haploid cell.

The denaturation analysis indicates that the satellite contains two regions of DNA which are distinctly different in their average G + C content. The two values were 59.5 and 65.2% G + C. The G + C content of these two regions does not relate precisely to the G + C content of the ribosomal subunit RNAs of two other genera of sea urchins. *Arbacia punctulata* rRNA was examined by Sy and McCarty (1968). The 18S rRNA was 56.1% G + C and 26 S was 60.7% G + C. Emerson and Humphreys (1970) found 18S rRNA to be 54.4% G + C and 26S rRNA to be 61.0% G + C in *Strongylocentrotus purpuratus*. The small differences could be explained if the nonconserved portion of the rDNA transcriptional unit were higher in G + C. Although this has not been examined in sea urchins, it has been found to be the case in other eukaryotic organisms that possess rRNA of the high G + C content type.

An examination of partially denatured rDNA by electron microscopy should provide important information about the topological distribution of the two regions of different G + C content (Inman, 1967; Inman and Bertani, 1969).

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Isolation and Characterization of the Bovine Parathyroid Isohormones*

Henry T. Keutmann,† Gerald D. Aurbach, Bess F. Dawson, Hugh D. Niall, Leonard J. Deftos, and John T. Potts, Jr.

ABSTRACT: Several different molecular forms of bovine parathyroid hormone (BPTH) have been isolated in high yield from extracts of pooled bovine parathyroid glands. Difficulties were previously encountered in the final stages of purification due to the persistence in the purest hormone preparations of several nonhormonal peptides similar in size and charge to the parathyroid polypeptides. These problems have been overcome by the introduction of 8 M urea into the buffers used in the final chromatography step on carboxymethylcellulose. In addition to the predominant form of the hormone (BPTH-I), two other biologically active hormonal peptides (BPTH-II and -III) were also isolated. These three active polypeptides, each containing 84 amino acid residues, all possess amino-terminal alanine and are devoid of cystine. The amino acid compositions of BPTH-I and -II differ only in the presence in BPTH-II of a single threonine residue (BPTH-I lacks threonine) and one less valine residue. BPTH-III also contains threonine; this form of the hormone is the most basic, eluting last from carboxymethylcellulose.

The purification procedure has proven satisfactory for preparation of sufficient BPTH-I to permit the complete structural analysis of the molecule.

The isolation of parathyroid hormone in completely pure, homogeneous form has been the object of extensive investigations over the 40-year period since the first active extracts from bovine parathyroid glands were reported by Collip (1925). These early procedures involved use of hot hydrochloric acid; subsequently it was appreciated that this caused considerable hydrolysis of the polypeptide during the extrac-

* From the Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Metabolic Diseases Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received February 9, 1971.* This work was supported in part by Grants AM-04501 and AM 11794 of the National Institute of Arthritis and Metabolic Diseases.

† To whom to address correspondence.