that system (F. Tietze, L. H. Rome, J. DeB. Butler, G. S. Harper, W. A. Gahl, *Biochem. J.*, in press). Therefore, the demonstration of sialic acid countertransport may require a system other than normal fibroblast granular fractions loaded by exposure to ManNAc.

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Karyotype Analysis of *Leishmania* Species and Its Use in Classification and Clinical Diagnosis

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Chromosomes of four species of *Leishmania* represented by ten different geographic isolates were analyzed by pulsed field gradient gel electrophoresis (PFG) to assess chromosome stability in these parasitic protozoans. Among different geographic isolates of the same subspecies, more than two-thirds of chromosomes had similar sizes, ethidium bromide staining intensities, and locations of α,β-tubulin genes. However, among New World *Leishmania*, members of different species or subspecies have fewer than one-third of their chromosomes in common. Therefore, PFG karyotypes of *Leishmania* exhibit intraspecific variability similar to that reported for other parasitic protozoans. The greater similarities of the karyotypes of members of the same *Leishmania* subspecies may indicate that they represent valid taxa. These similarities also allowed the use of PFG in clinical diagnosis for rapid and accurate typing of patient isolates.

THE DNA SEQUENCE OF AN ORGANISM encodes species-specific information. In eukaryotes, this information is packaged into chromosomes in the nucleus. Chromosomes of parasitic protozoans cannot be resolved by light microscopy because they do not condense during the cell cycle. Thus, their genes cannot be mapped to specific chromosomes by cytogenetics, even in the rare instances when genetic markers are available.

Pulsed field gradient gel electrophoresis (PFG) has been used to separate protistan chromosome-sized DNA molecules up to about 2 megabase pairs or more, including those of some protozoan agents of human disease (*1–7*). The separated DNA molecules, which appear as discrete bands in agarose gels, have been shown to represent the DNA of the full-length chromosomes (*1, 8*).

We have applied the extraordinary resolving power of PFG to analyze the genomic organization of *Leishmania*. These obligate intracellular protozoan parasites of macrophages, monocytes, and histiocytes cause severe public health problems worldwide, including a recently discovered autochthonous focus of cutaneous leishmaniasis in Texas (*9*). *Leishmania* cause diseases ranging from skin ulcers (Oriental sore) to systemic visceral leishmaniasis, or kala-azar. The species and subspecies of the parasite (*10*) and the immunogenetic background of the host (*11*) affect disease development. Because only certain of its members may cause a given clinical syndrome, it is vital to elucidate the genetics of *Leishmania* and to classify the causative organism for epidemiological studies.

We first explored the variability of PFG karyotypes of the two developmental stages of *Leishmania*—the nonmotile amastigote, found within cells of vertebrate hosts, and the flagellated promastigote, found extracellularly in the gut of the sandfly vector. Both stages can be cultivated in vitro.

We examined the PFG karyotypes of *L. major*, an etiologic agent of cutaneous leishmaniasis from Africa (*12*). Several PFG gels were run at different pulse frequencies for all experiments described to separate optimally chromosomes of different molecular weights. With a 60-second pulse frequency, at least 15 bands were distinguished, with sizes between about 340 kb and 800 kb (Fig. 1). At this pulse frequency, large chromosomes (more than 800 kb) are not separated and some DNA remains in the sample well. The staining intensity of several bands is nonstoichiometric, indicating that multiple chromosomes are present in a band of one size class; this is also the case for yeast (*1*), *Trypanosoma brucei* (*5*), and *Plasmodium falciparum* (*7*). Nonstoichiometrically staining bands may represent more than one chromosome coincidentally of the same size or multiple copies of a single chromosome (aneuploidy). By using several pulse frequencies for optimal separation of DNA molecules of different lengths, at least 20 bands are revealed in different *Leishmania* species.

We determined the size of each chromosome to within ±20 kb with annealed lambda phage as size markers. Reproducibility of size determinations from replicate samples and for gels run at different pulse frequencies was good, with individual chromosomes being measured with a 5% coefficient of variation.

The ethidium bromide–stained PFG karyotypes of promastigotes, amastigotes from skin lesions, and amastigotes from culture (*13*) were the same at all pulse

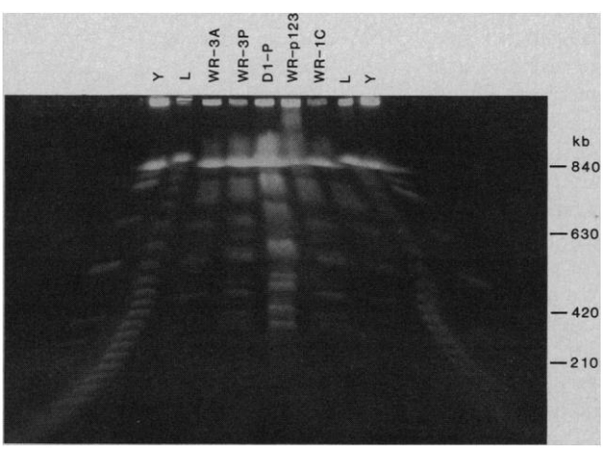

Fig. 1. Comparison of PFG karyotypes of *L. major*. See (*12*) for abbreviations. WR-3A, clone No. 3 amastigotes harvested from mouse skin lesions; WR-3P, clone No. 3 promastigotes; D1-P, promastigotes; WR-p123P, uncloned promastigotes from a 123rd subculture; WR-1C, clone No. 1 culture amastigotes. Annealed bacteriophage lambda DNA ladders (L) and yeast DNA (Y) are included as size standards. Samples were prepared (*2, 12, 13, 18*) and electrophoresed in a double inhomogeneous electrophoresis apparatus (20 by 20 cm) (15°C, 42 hours, 12 V/cm, pulse frequency of 60 seconds).

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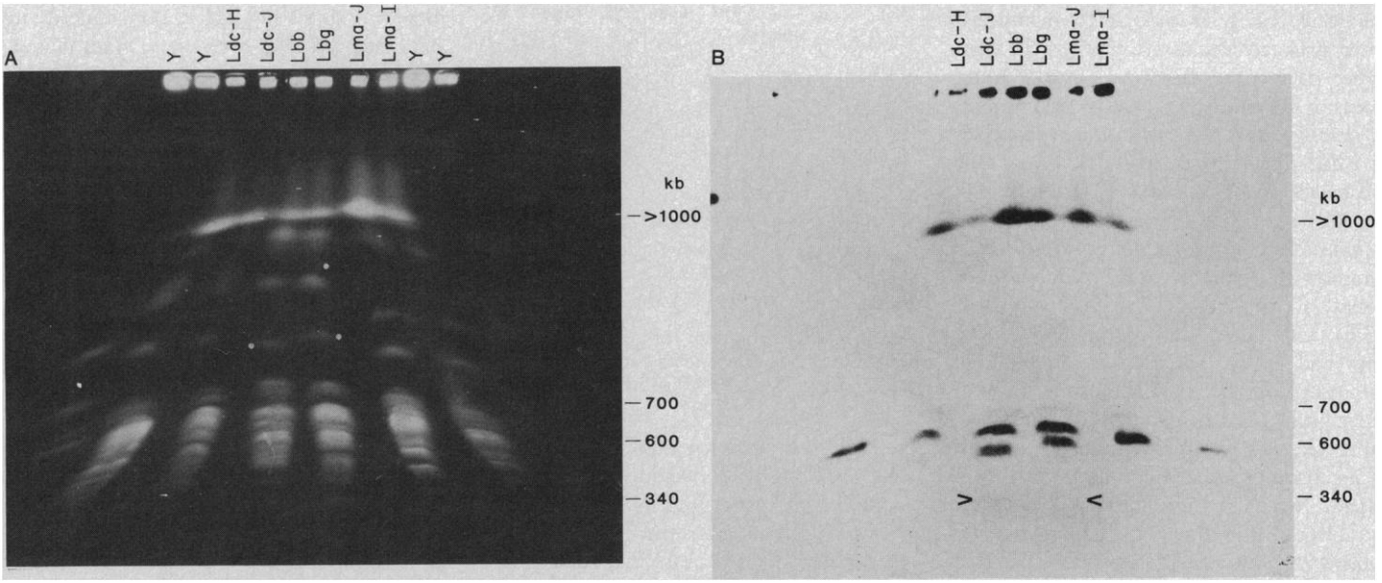

Fig. 2. Comparison of PFG karyotypes of causative agents of leishmaniasis from Brazil, Surinam, and Peru. See (*12*) for abbreviations. (A) Ethidium bromide–stained gel. Electrophoresis at a pulse frequency of 90 seconds was as described for Fig. 1. As an example of how strains were compared, dots in lanes Lbb and Lbg indicate dissimilar chromosomes. (B) Southern blot of PFG karyotype after hybridization with α,β-tubulin. The smallest chromosomes (~230 to 330 kb) hybridize with tubulin only in the *L. braziliensis* strains Lbb and Lbg (arrowheads); these appear also in Fig. 3, where they are darker.

frequencies, indicating that gross chromosomal rearrangements do not occur during development. The PFG karyotype at one of the pulse frequencies is shown (Fig. 1). The slight differences in band sizes seen among lanes beginning with WR are not significant, and reflect the slight curvature of the double inhomogeneous electrical field. The karyotype did not change after extensive subculture (Fig. 1). Karyotypes of clones of *L. major* and of the parent stock cultures were the same. The identities in the karyotypes of amastigotes and promastigotes therefore indicate that the PFG analysis of a single stage may suffice for strain identification.

We then explored the difference between *Leishmania* species and subspecies by PFG karyotyping. The degree of similarity of two PFG karyotypes was evaluated by determining the number of chromosomes (i) falling within the same size ranges; (ii) having the same subjective degree of brightness when stained with ethidium bromide; and (iii) bearing the same information for several genetic markers.

Examples of how we evaluated chromosomal identity are shown in Figs. 1 to 3. Karyotypes in which resolved chromosomes are of the same size and staining intensity are shown in Fig. 1 (WR lanes), in Fig. 2A (chromosomes >700 kb in lanes Lma-J and Lma-I) and in Fig. 3A (lanes Lbb and PtB). Dissimilar chromosomes are indicated by dots in Fig. 2A and squares in Fig. 3A.

Karyotypes of members of different spe-

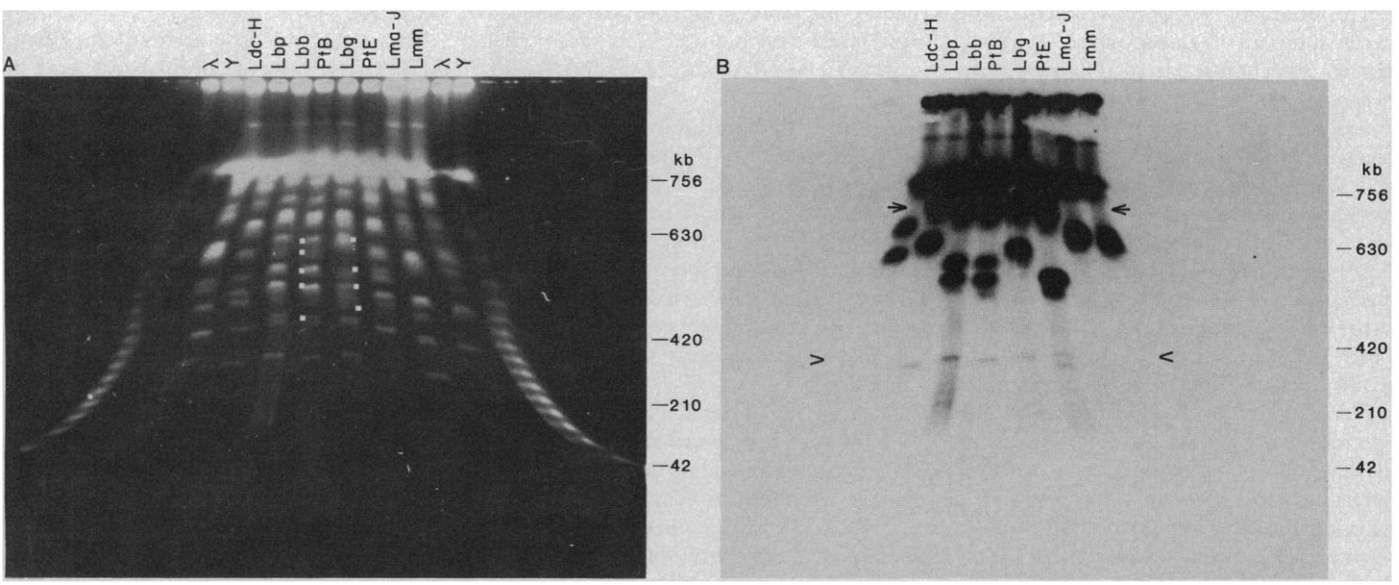

Fig. 3. Comparison of PFG karyotypes of South American species of *Leishmania*. See (*12*) for abbreviations. (A) Ethidium bromide–stained gel. Samples were electrophoresed as described in Fig. 1. As an example of how strains were compared, the dots in lanes Lbg and PtB indicate dissimilar chromosomes. (B) Southern blot of PFG karyotype after hybridization with α,β-tubulin. Note that the probe hybridizes with the smallest chromosomes (between ~230 and 330 kb) only in the *L. braziliensis* strains (between arrowheads). The location of the α,β-tubulin genes on chromosomes between ~670 to 715 kb is also seen only in *L. braziliensis* strains (between arrows). See Fig. 1 for methods.

cies were readily distinguished from each other, with fewer than one-tenth of chromosomes having the same sizes and staining intensities. Within the New World species *L. braziliensis* and *L. mexicana*, subspecies also had distinctive karyotypes. (Only one subspecies of *L. donovani* is recognized in the Western Hemisphere.) The subspecies *L. b. braziliensis* and *L. b. guyanensis* had in common the greatest number of chromosomes of all strains tested (almost one-third), and the two subspecies *L. m. mexicana* and *L. m. amazonensis* also had in common a number of chromosomes (about one-eighth). Surprisingly, the *L. braziliensis* subspecies *panamensis* had almost one-fifth of its chromosomes in common with *L. d. chagasi*, which it resembled more closely than it did the other two *L. braziliensis* subspecies. We therefore concluded that stocks of different etiologic agents of leishmaniasis had distinctive PFG karyotypes.

The great degree of diversity among karyotypes of South American stocks of different species and subspecies led us to ask whether each isolate might have an idiosyncratic karyotype, with no relationship to the species or subspecies to which it belonged. We therefore examined stocks that belonged to the same subspecies. Among New World isolates that we tested, the overall chromosome patterns were similar for different geographic isolates of the same subspecies (two stocks of *L. m. amazonensis* and two of *L. d. chagasi*), and distinctly different when compared with isolates of other species. (See resolved chromosomes >700 kb in Fig. 2A; other pulse frequencies resolved chromosomes <700 kb.)

This similarity between two stocks of the same subspecies (three-fourths to nine-tenths) was comparable to that seen when Senegalese (WR-300) and Egyptian (D-1) stocks of Old World species *L. major* were compared (Fig. 1). Karyotypes of the D-1 and WR-300 strains of *L. major* had only two uniquely different bands (which cannot be resolved at the pulse frequency shown in Fig. 1). Due to overloading, the D-1 stock's chromosomes migrate slightly more slowly than WR-300 stock's, but from this and other gels we conclude that about seven-tenths of chromosomes are of the same size and staining intensity. This degree of intraspecies variability for *L. major* (formerly classified as *L. tropica* subspecies *major*) is similar to the degree of variability within subspecies for the South American etiologic agents. We concluded that stocks of the same etiologic agents had similar PFG karyotypes.

To determine whether chromosomes of the same size might bear the same genetic information, we hybridized the PFG karyotypes with a probe for α,β-tubulin. In our New World isolates we identified tubulin genes on at least four DNA bands in *L. donovani*, on at least three bands in *L. mexicana*, and on at least five bands in *L. braziliensis* (Figs. 2B and 3B). The dispersed distribution of tubulin genes among chromosomes of New World isolates of *Leishmania* (Figs. 2B and 3B) was similar to that reported for *L. major* (cloned from LRC-L137), *L. donovani* (L52), and *L. mexicana* (L94), where tubulin genes were mapped to a total of four, four, and three bands, respectively (*4*).

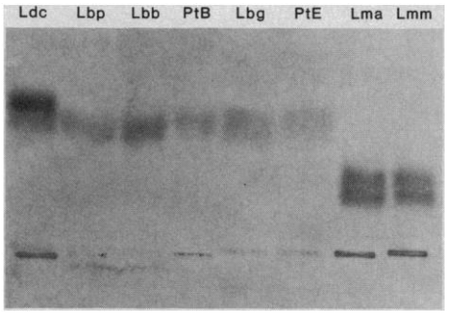

Fig. 4. Isoenzyme patterns of glucose phosphate isomerase (*11*) for *Leishmania* patient isolates and market stocks. See (*12*) for abbreviations.

All members of the *L. braziliensis* complex, and only these, carried sequences hybridizing with α,β-tubulin on a chromosome of ~690 kb (Fig. 3B, arrows), and also on their smallest chromosomes (between 230 to 330 kb) (Fig. 3B, arrowheads; Fig. 2B).

Based on the location of α,β-tubulin genes, *L. b. panamensis* was now clearly distinguishable from *L. d. chagasi* (Fig. 3B). The *L. mexicana* subspecies *mexicana* and *amazonensis* were more similar to each other than they were to members of other species (Fig. 3B).

Both by ethidium bromide staining and by the location of α,β-tubulin on individual chromosomes, the PFG karyotypes of the New World *Leishmania* show considerable intraspecies variation (Fig. 3). The extent of variation among members of the same subspecies seems to be less than the intraspecies variability; however, we will need to examine many more isolates to establish this point firmly.

We have also probed PFG chromosome patterns with a *T. brucei* Hsp 70 genomic clone (containing coding sequences for the 70-kD heat shock protein) and Pr8 (ribosomal RNA gene) (*14*). In different isolates, these probes hybridize with chromosomes of the same size, namely a chromosome more than 1 Mb in size. Data for three DNA sequences indicate that chromosomes of the same size carry the same genetic information in stocks of the same subspecies. It is likely, therefore, that a high degree of similarity in PFG karyotypes also reflects a high degree of genetic homology.

Because of the similarity in karyotypes of different geographic isolates of the same subspecies, PFG analysis can type clinical isolates. A *Leishmania* species was cultured from skin lesions of a patient infected in Brazil. The isolate was cloned in the second subculture, and clones and parent stock were identical by PFG, indicating that a single species was present. By PFG karyotyping, the isolate (PtB) resembled a marker stock of *L. b. braziliensis*, both by ethidium bromide staining of gels and by Southern blotting and hybridization with the α,β-tubulin gene probe (Fig. 3, lanes Lbb and PtB). Isoenzyme typing and radiorespirometry (*15*) confirmed that the isolate was *L. braziliensis*, although its subspecies was not determined by these techniques (Fig. 4). Because *L. b. braziliensis* can metastasize, to cause mutilating mucocutaneous leishmaniasis, chemotherapy and close follow-up were recommended.

A second *Leishmania* species, PtE, was cultured from skin lesions of a patient infected in Ecuador. The location of α,β-tubulin genes on two of its chromosomes (at 690 and 330 kb, indicated by arrows) was characteristic of *L. braziliensis* subspecies (Fig. 3). It was not clearly identifiable as one of the marker subspecies tested; by ethidium bromide staining, PtE was most similar to *L. b. panamensis*, with which it had one-third of chromosomes in common. Isoenzyme typing confirmed its identity as *L. braziliensis* (Fig. 4), and the isolate was subsequently typed by monoclonal antibodies as *L. b. panamensis* (*16*).

These observations indicate that the *Leishmania* species as now defined may have a highly flexible chromosomal organization in that members of the same species may bear the same gene on different sized chromosomes. Such variability in size also occurs among different geographic isolates of *Trypanosoma brucei* and *Plasmodium falciparum* (*5–7*). Alternatively, the currently defined species might actually be complexes (*17*). If so, characteristics of any given isolate may not be shared by other members of the same complex, which may be biologically distinct organisms.

PFG karyotypes appear to be more similar among members of the same subspecies, in which chromosomes of similar size carry genes for α,β-tubulin, Hsp-70, and ribosomal RNA. Although more typed stocks must be analyzed by PFG to gauge the extent of variation within subspecies, the *Leishmania* subspecies may represent valid taxonomic entities deserving the status of species.

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12. The marker strains (abbreviations for figure legends), year of isolation, and geographic origin were: *L. major* WR300 (WR), also held as MHOM/SN/00/DK-106, year unreported; Senegal [described in M. Strobel, B. Ndiaye, C. Renaud-Steens, J. P. Dedet, J. P. Marchand, *Med. Mal. Infect.* **8**, 98 (1978)]; *L. major* D-1 (D1), 1984, from a dog in Egypt (a generous gift of Dr. T. A. Morsy, Ain Shams University, Cairo); *L. donovani chagasi* JBV (Ldc-J), 1984, Maranhaõ, Brazil; *L. donovani chagasi* HBI (Ldc-H), 1984, Bangu, Rio de Janeiro, Brazil; *L. mexicana amazonensis* IOC (Lma-I), 1984, Bahia, Brazil (all three strains were generous gifts of Dr. M. C. de A. Marzochi, Instituto Oswaldo Cruz, Rio de Janeiro); *L. mexicana amazonensis* Josepha (Lma-J), 1975, Brazil [P. D. Marsden, C. C. Cuba, A. C. Barreto, R. N. Sampaio, R. A. A. Rocha, *Trans. R. Soc. Trop. Med. Hyg.* **73**, 391 (1979)]; *L. mexicana mexicana* 1VLM (Lmm), also called WR183, 1958, Panama [M. de V. Coelho and E. Coutinho-Abath, *Rev. Inst. Med. Trop. Sao Paulo* **77**, 136 (1965)]; *L. braziliensis guyanensis* MHOM/SR/80/CUMC-1 (Lbg), 1980, Surinam; *L. braziliensis panamensis* ATCC30879 (Lbp), also held as WR209 and LV641, 1974, Panama [B. E. Beacham, R. Romito, H. D. Kay, *Am. J. Trop. Med. Hyg.* **31**, 252 (1982)]; *L. braziliensis braziliensis* MHOM/PE/83/CUMC-2 (Lbb), 1980, Peru. All strains except D-1 were isolated from humans. We confirmed the nominal identity of each marker strain by typing its isoenzymes of mannose phosphate isomerase (MPI), glyceraldehyde phosphate dehydrogenase (GAPDH1), phosphogluconate dehydrogenase (6PGDH), glucose phosphate isomerase (GPI), and acid phosphatase (ACP). Cultivation of promastigotes [S. H. Giannini, *Trans. R. Soc. Trop. Med. Hyg.* **79**, 458 (1985)] and isoenzyme analysis [R. D. Kreutzer, M. E. Semko, L. D. Hendricks, N. Wright, *Am. J. Trop. Med. Hyg.* **29**, 199 (1980)] have been described.
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15. We are grateful to Joan E. Decker-Jackson, Walter Reed Army Institute of Research, for performing the radiorespirometric identification. The patient isolate from Brazil (PtB) had the low profiles of substrate utilization characteristic of *L. braziliensis*. Further details of the technique are in M. L. Chance and B. C. Walton, Ed., *Biochemical Characterization of Leishmania* (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, 1982).
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The Mechanism of Binding of a Polynucleotide Chain to Pancreatic Ribonuclease

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The crystalline complex of pancreatic ribonuclease (RNase) with oligomers of $d(pA)_4$ has been solved by x-ray diffraction methods and refined by standard procedures to a conventional crystallographic R factor of 0.22 at 2.5 angstrom resolution. The asymmetric unit is a complex of one RNase molecule associated with four $d(pA)_4$ oligomers. Although the DNA in this complex is segmented, and therefore shows some discontinuities, it nevertheless traces a continuous path 12 nucleotides in length that passes through the active site cleft of the enzyme and over the surface of the protein. The DNA makes a series of eight to nine electrostatic bonds between its phosphate groups and lysine and arginine residues on the protein, as well as specific chemical interactions at the active site. The path described by the sequence of nucleotides is likely to be that taken by an extended polynucleotide chain when it is bound by the enzyme.

RIBONUCLEASE (RNASE) FROM BOVINE pancreas is among the most thoroughly studied of all protein molecules in terms of structure, chemistry, and enzymatic mechanism (*1–3*). Two functional roles have been ascribed to the protein. Its major function is to cleave RNA at points 3′ to pyrimidine residues to yield fragments having 3′ phosphate termini. A second property of the protein derives from its preferential affinity for, and ability to form complexes with, single-stranded DNA. It is a helix destabilizing or DNA unwinding protein (*4, 5*).

The structure of the protein's active site in the immediate vicinity of enzymatic catalysis has been well established by x-ray and neutron diffraction (*6, 7*), and by chemical

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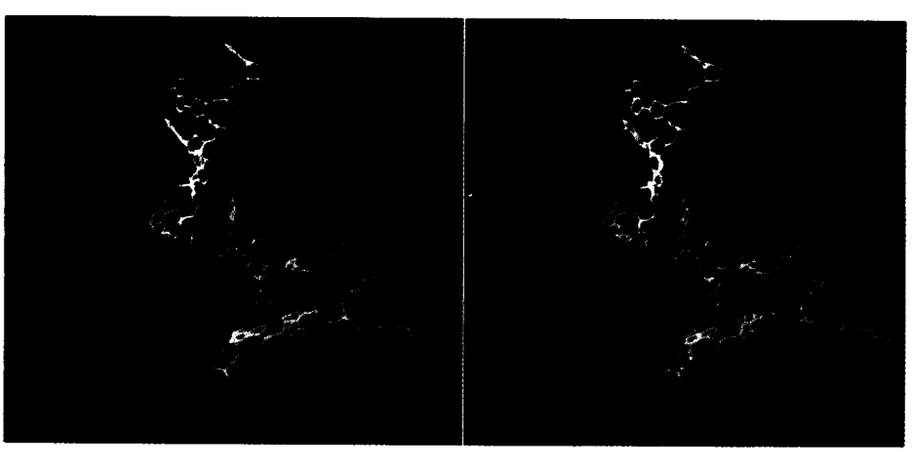

Fig. 1. The structure of the complex formed between bovine RNase A and four tetramers of $d(pA)_4$ as determined by x-ray crystallographic techniques. The four deoxyoligomers I–IV are shown in green, violet, yellow, and red, respectively, and extend in a consistent 3′ to 5′ direction from the top of the figure to the bottom. The conventional crystallographic R factor for the structure at 2.5 Å resolution is 0.22.