Kindt, T. J., Mandy, W. J., and Todd, C. W. (1970b), *Immunochemistry 7*, 467.
Kindt, T. J., Todd, C. W., Eichmann, K., and Krause, R. M. (1970c), *J. Exp. Med. 131*, 343.
Koshland, M. E., Davis, J. J., and Fujita, N. J. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1274.
Koshland, M. E., Reisfeld, R. A., and Dray, S. (1968), *Immunochemistry 5*, 471.
Krause, R. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 59.
O'Donnell, I. J., Frangione, B., and Porter, R. R. (1970), *Biochem. J. 116*, 261.
Offord, R. E. (1966), *Nature (London) 211*, 591.
Osterland, C. K., Miller, E. J., Karakawa, W. W., and Krause, R. M. (1966), *J. Exp. Med. 123*, 599.
Pan, S. C., and Dutcher, J. D. (1956), *Anal. Chem. 28*, 836.
Porter, R. R. (1970), *Harvey Lect.* (in press).
Prahl, J. W., and Porter, R. R. (1968), *Biochem. J. 107*, 753.
Press, E. M. (1967), *Biochem. J. 104*, 30c.
Press, E. M., and Hogg, N. M. (1970), *Biochem. J. 117*, 641.
Press, E. M., Piggott, P. J., and Porter, R. R. (1966), *Biochem. J. 99*, 356.
Wilkinson, J. M. (1969), *Biochem. J. 112*, 173.
Wilkinson, J. M., Press, E. M., and Porter, R. R. (1966), *Biochem. J. 100*, 303.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.

Retention of Common Nucleotide Sequences in the Ribosomal Deoxyribonucleic Acid of Eukaryotes and Some of Their Physical Characteristics*

John H. Sinclair† and Donald D. Brown†

ABSTRACT: The rRNA from *Xenopus laevis* has been hybridized with DNA from over 50 organisms, including prokaryotes and eukaryotes.

In all cases the DNA was prefractionated by preparatory centrifugation in CsCl. Analysis of hybridization profiles yielded the following information. (1) Without exception, the DNA of all eukaryotes analyzed had some molecular homology with *X. laevis* rRNA; no homology was found between *X. laevis* rRNA and the DNA of 8 prokaryotes. (2) There is a general tendency for rDNA to have a higher G + C content than bulk DNA from the same organism. The approximate buoyant density of rDNA from 50 eukaryotes has been tabulated and from this value the base composition of each rDNA has been estimated. (3) High levels of redundancy exist for ribosomal genes in all species examined. In plants and invertebrates a larger fraction of genome codes for rRNA than in vertebrates; in mammals this fraction is the smallest observed. (4) Clustering of ribosomal genes as evidenced by sharp banding of rDNA in CsCl occurs in most of the species examined.

The segment of the eukaryote chromosome termed the "nucleolar organizer" contains a unique set of genes which determine the structure of ribosomal RNA (rRNA).[1] In *Xenopus laevis* several hundred genes for the 18S and 28S rRNAs are clustered together on a single allele of the 18 haploid chromosomes (Wallace and Birnstiel, 1966). Individual 18S and 28S genes are adjacent and separated from the next pair by a "spacer" region of DNA (Brown and Weber, 1968b). The DNA which contains these three repeating nucleotide sequences (18S, 28S, and spacer DNAs) is termed rDNA, and it has a high G + C content relative to bulk *X. laevis* DNA and bands at a higher buoyant density in CsCl. This separation of rDNA from bulk DNA by CsCl centrifugation, first described by Wallace and Birnstiel (1966), adds considerable information and specificity to molecular hybridization studies. Since the rDNA is separated from bulk DNA, contamination of radioactive rRNA preparations with DNA-like RNA does not interfere with the measurement of hybridization to rDNA. From the buoyant density of rDNA its approximate base composition can be calculated. Finally, the distribution of rDNA in the gradient gives an indication of the extent to which these redundant genes are clustered within a genome.

These experiments were designed to analyze some physical properties of rDNA from a variety of eukaryotes. The combined techniques of equilibrium centrifugation of DNA followed by hybridization with radioactive rRNA yielded a variety of information from a single preparation of DNA. From examination of these gradients, it has been possible to unequivocally demonstrate homology between the rRNA of *X. laevis* and the rDNA of distantly related eukaryotes, and to estimate the buoyant density (and consequently the base composition) of these ribosomal genes. Results also indicate that the ribosomal genome is highly redundant

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[1] Abbreviations used are: rRNA, ribosomal RNA, including 18S RNA and 28S RNA; rDNA, ribosomal DNA, the portion of bulk DNA which contains gene sequences homologous to 18S and 28S rRNA; MAK, methylated albumin kieselguhr; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate).

in all species examined and that the genes tend to be clustered. A preliminary communication of this work has been published (Sinclair and Brown, 1968).

Materials and Methods

Culture of Cells and Preparation of Radioactive rRNA. High specific activity radioactive 28S and 18S rRNA of *X. laevis* was prepared from purified ribosomes of [^{3}H]uridine-labeled cells derived from adult kidney by techniques described earlier (Brown and Weber, 1968a). Its specific activity was 300,000 cpm/μg.

Source and Culture of Organisms and Cells for DNA. HeLa cells were grown in Waymouths MB752/1 tissue culture medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Livers of the following vertebrates were used as the source of DNA: monkey (*Rhesus*), rat, mouse, rabbit, guinea pig (all of these are common laboratory stocks maintained at the Carnegie Institution); chicken, turkey, goose (the latter three were domestic stocks obtained from local sources); *Bufo americanum*, *Bufo marinus*, *Rana sylvaticus*, *Rana palustris*, *Rana pipiens*, *Triturus viridescens*, *Ambystoma mexicanum*, *Ambystoma maculatum*, *Ambystoma tigrinum*, *Necturus maculosus*, *Xenopus laevis*, and *Fundulus* (Woods Hole Biological Laboratories). Erythrocytes were also used as a source of DNA for turkey, *A. mexicanum*, *R. pipiens*, *X. laevis*, *N. maculosus*, and *A. tigrinum*.

DNA was extracted from whole larvae of *Chironomous tentans* and *Drosophila melanogaster* while sperm was the source of DNA from *Spisula solidissima* and *Urechis caupo*. *Metritium* DNA was a gift of R. Britten.

DNA was extracted from nuclear pellets of *Nicotiana tabacum*, *Pisum sativum*, and *Cucurbita pepo*. The source of tissue was large green leaves, young sprouted stems plus roots, and fruit, respectively. In each case the tissue was suspended in 0.1 M Tris-HCl (pH 8.0)–0.01 M EDTA and ground in a mortar and pestle with glass beads. The homogenate was filtered through gauze and centrifuged at 500*g* for 10 min to sediment the nuclei. Nuclei were removed with a spatula so as to eliminate as much of the chloroplast and/or starch as possible. The nuclei were washed once again at low speed, suspended in 2.2 M sucrose, and centrifuged at 20,000 rpm in a Spinco Model L centrifuge for 30 min. The nuclear pellet at the bottom was removed, washed once again at low speed, and stored for extraction. Brine shrimp (*Artemia salina*) eggs were prepared for phenol extraction by grinding with glass beads. *Tetrahymena pyriformis*, strain W. (obtained from R. Ballantine), and *T. pyriformis*, GL-C (obtained from N. E. Williams), were grown on 2% proteose-peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% sequestrine (Geigy Agricultural Chemical Corp.) in 3-l. jars with aeration. Cells were broken with a Logeman homogenizer. *Euglena gracilis* (ATCC 10616) was grown in 0.1% sodium acetate, 0.1% beef extract, 0.2% tryptone, 0.2% yeast extract, 0.001% $CaCl_2$, and 0.5% glucose under fluorescent lights in diphtheria toxin bottles. Cells were treated with Pronase followed by freezing and thawing in sodium lauryl sulfate and then broken by homogenization. *Chlamydomonas reinhardi* (I.C.C. No. 89) was grown in Sueoka's three-tenths HSM medium (Sueoka, 1960) in large flasks with aeration. Cells were broken by the method used for *E. gracilis*. The blue-green algae *Plectonema boryanum* (a gift from R. Haselkorn) were grown in modified Chu No. 10 medium (Gerloff *et al.*, 1950) with aeration under fluorescent lights. Cells were broken by freezing, thawing, and grinding with glass beads in the DNA extraction buffer containing Pronase and sodium lauryl sulfate. Protoplasts of yeast (*Hansenula anomola*, NRRL Y-365; *Dabaromyces kloeckeri*, NRRL Y-833; *Saccharomyces carlsbergensis*, NCYC 74; and *Saccharomyces cerevisiae*, D-261, obtained from J. Mattoon) were prepared before DNA extractions. Cultures for DNA extraction were grown in 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose with aeration in large flasks. Both *Escherichia coli* and *Micrococcus luteus* were grown on the enriched *E. coli* medium. This consisted of a defined medium described by Stern *et al.* (1964) but contained an additional 10 g of glucose, 10 g of Casamino Acids, and 10 μg/ml of thymine per l.

Purification of DNA. Most of the DNA preparations used in these experiments were extracted from nuclei or whole cells with phenol. The tissue or nuclear pellet was suspended in SSC containing 1 mg/ml of Pronase and 0.5% sodium lauryl sulfate.

Pronase digestion was allowed to proceed for 1–4 hr at room temperature at which time an equal volume of a 1% solution of sodium lauryl sulfate in 0.1 M Tris (pH 9.0) and 0.1 M NaCl was added. Following Pronase digestion an equal volume of water-saturated phenol was added and the mixture gently shaken overnight. The aqueous layer was extracted once or twice more with phenol, then once or twice with chloroform–isoamyl alcohol (24:1, v/v). The DNA was precipitated with two volumes of 95% ethanol (4°) and resuspended in SSC. The DNA solutions were then treated with pancreatic RNase and α-amylase, followed by Pronase digestion and phenol extraction. In some cases in which DNA could not be wound out, it was further purified by absorption on a 2 × 2 cm MAK column (over Celite). The column was washed with 0.2 M NaCl–0.05 M Tris (pH 7.2) until no more ultraviolet-absorbing material was eluted. The DNA was eluted with 0.8 M NaCl–0.05 M Tris (pH 7.2) and then dialyzed against 0.1× SSC, concentrated *in vacuo* or against polyethylene glycol, and stored in SSC.

Cesium Chloride Centrifugation and DNA Immobilization. The utility of angle preparative rotors for separation of DNAs in accordance with buoyant density differences has been well illustrated (Fisher *et al.*, 1964; Flamm *et al.*, 1966). The principle advantage of a fixed-angle rotor is that it produces a shallower CsCl gradient than a horizontal rotor resulting in a greater separation of two DNAs of different buoyant density. However, the gradient produced by the fixed-angle rotor is nonlinear in the heavy end of the gradient. In order to predict density positions accurately in such a gradient, it is necessary consistently to use the same volume and initial density of CsCl in each tube and to include DNA of known density as a standard. In this study, DNAs from over 50 different organisms, with densities varying from 1.690 to 1.723 g per cm^3 determined by analytical CsCl, were examined in angle gradients containing *Micrococcus luteus* DNA as a density marker. All samples of DNA banded close to a line relating fraction number and CsCl density as determined by refractive index measurement or that predicted from measurements of tube diameter and angle. The predictable banding of these DNAs of established buoyant density confirms the accuracy of the line relating density and fraction number. The buoyant density of an unknown sample of DNA such as the genes for rRNA can simply and accurately be determined from this line. This was the method used to calculate all of the rDNA densities discussed in this paper.

In the course of these experiments, it was noted that when large amounts of high molecular weight DNA were applied

to preparative CsCl gradients, almost invariably the ribosomal satellite banded nearer the bulk DNA peak than was the case when low concentrations of sheared DNA were used (see also Brown and Weber, 1968a). Such an artifact is a serious problem if large amounts of DNA must be centrifuged. However, the DNA which has an altered position is apparently the bulk peak and not the ribosomal satellite. In gradients containing high molecular weight DNA, the position of the satellite was unchanged relative to the marker (*M. luteus*) under conditions which resulted in an apparent shift in the position of bulk DNA by as much as 0.01 g/cm^3. This effect appears to be related to the high viscosity of the concentrated band of DNA in these cases.

Such considerations led to these final conditions for preparatory CsCl centrifugation. All DNA preparations were sheared five times through a 26-gauge needle. This produces DNA with a weight average molecular weight of 5–6 $\times$ 10^6 (Brown and Weber, 1968a). About 100 μg of the tested DNA and 10–20 μg of *M. luteus* DNA in a final volume of 4.5 ml were centrifuged in a Spinco type 65 fixed-angle rotor at 33,000 rpm for 2 days at room temperature. The contents of each tube were collected and assayed for optical density. The DNA in each fraction was immobilized on an HA Millipore filter and the filters prepared for hybridization by the method of Brown and Weber (1968a).

RNA–DNA Hybridization. As many as 80 DNA filters were hybridized together in 4$\times$ SSC at 70–72° for 15–20 hr (Brown and Weber, 1968a). About 1–3 μg of [^{3}H]rRNA/ml of solution was used, an amount which is less than the concentration of rRNA required to hybridize at saturation. All filters were digested with RNase, washed in 2$\times$ SSC, dried, and counted in a liquid scintillation spectrometer.

"Prehybridization" (*e.g.*, hybridization of DNA with unlabeled rRNA before CsCl centrifugation) was carried out as described by Brown and Weber (1968b).

Analytical Centrifugation of DNA. Buoyant density determinations were made according to methods described by Vinograd and Hearst (1962) and Sueoka (1961). DNA (1–5 μg) was suspended in CsCl and adjusted to an initial density of 1.700–1.710 g/cm^3. *M. luteus* DNA (ρ 1.731) (Schildkraut *et al.*, 1962a) was used as a density reference. Ultraviolet photographs were made after 20 hr at 44,000 rpm at 25°. Photographs were traced on a Joyce-Loebl microdensitometer. The G + C content of the DNA was estimated from its buoyant density by use of the equation given by Schildkraut *et al.* (1962a).

Results

Homology of X. laevis rRNA with rDNA of Eukaryotes. The DNA from a variety of eukaryotes was centrifuged to equilibrium in CsCl and the fractions hybridized with [^{3}H]rRNA of *X. laevis*. All results obtained are listed in Table I, including data for the organisms shown in the figures as well as that for others not shown.

Figure 1 illustrates some of the kinds of hybridization patterns which were found among amphibian DNAs. The level of hybridization with anurans (including *Bufo* and *Rana* species) was similar to that of *X. laevis* (Table I). One exception was DNA from *Rana sylvaticus* which showed a considerably larger amount of hybridization than other closely related *Rana* species (Figure 1). The level of hybridization with urodeles (including *Amphiuma*, *Necturus*, *Ambystoma*, and *Triturus*) was more variable, ranging from values nearly equal to homologous hybridization (the axolotl, for example) to

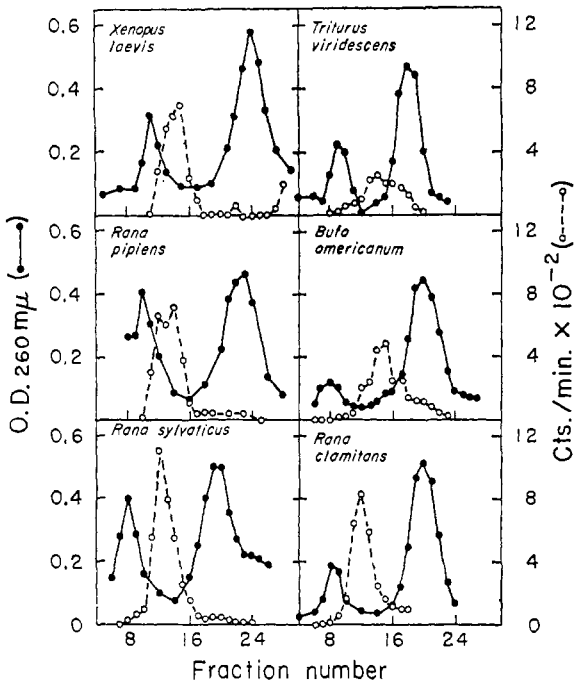

FIGURE 1: Cesium chloride centrifugation and rRNA hybridization profiles of DNAs from various amphibians. Filters were hybridized with ^{3}H-labeled *X. laevis* rRNA. The data were adjusted so that the amount of DNA in each gradient was 100 μg and the radioactivity is that amount which hybridized with 100 μg of DNA. (●) Optical density at 260 mμ; (○) counts per minute.

values of about 10–20% as high (*Necturus*). Although rDNAs of all amphibians which were analyzed had high buoyant densities compared to their main band DNA, the exact density varied considerably (Table I). Homogeneous banding of rDNA (*i.e.*, sharp peaks of hybridization) suggests a clustering of the redundant sequences in most species as is known to be the case for *X. laevis*.

X. laevis rRNA also hybridized well with DNA from other vertebrates, including carilagenous and bony fish, reptiles, birds, and mammals. The CsCl banding and hybridization levels with box tortoise, salmon, and turkey DNA (Figure 2) were similar to that with *X. laevis*. When *X. laevis* rRNA was hybridized with DNA from human, rabbit, and monkey (Figure 2) similar clustering and density profiles were obtained but the level of hybridization was lower. That the few counts found in the satellite region of higher vertebrate DNAs represent hybridization with ribosomal base sequences was confirmed by competition experiments (not shown here) in which unlabeled *X. laevis* rRNA eliminates all detectable counts. The low hybridization suggests that a smaller fraction of the genome in mammals is rDNA than in *X. laevis*. Others have also reported that mammals and chick have a smaller fraction of rDNA than values reported for *X. laevis* and many lower organisms (see references in footnote to column 5 in Table I).

Figure 3 illustrates that *X. laevis* rRNA is at least partially homologous with a variety of invertebrate rDNAs. In most organisms (starfish, *Limulus*, *Spisula*, and *Metridium*) the rDNA bands as a high-density satellite. The satellite for *Tetrahymena* is only slightly higher in density than bulk DNA and in *Drosophila* the hybridization band has the same density as bulk DNA. The rRNAs of the latter two eukaryotes are known to have a low G + C content. The same is true for the cellular slime mold *Dictyostelium discoides* (Table I). These DNAs hybridize with *X. laevis* rRNA over the main band.

TABLE I: Physical Properties of rDNA from Various Organisms.

Class	Organism	Buoyant Density: Bulk DNA	Buoyant Density: rDNA	G + C Content: rDNA	G + C Content: rRNA	Amt of rDNA
		1	2	3	4	5
Mammalia						
	Homo sapiens (man, HeLa)	1.699	1.719	60	64[a]	
	Macaca mulatta (monkey, rhesus)	1.700	1.720	61		
	Rattus norwegicus (rat)	1.699	1.711	52	64.2[b]	-0.01–0.03
	Oryctolagus cuniculus (rabbit)	1.699	1.718	59	63[c]	
	Bos taurus (cow)	1.699	1.719	60	63[d]	0.04
Aves						
	Gallus domesticus (chicken)	1.700	1.723	64	64[e]	
	Meleargris gallopavo (turkey)	1.699	1.720	61		-0.03–0.06
	Anser anatidae (goose)	1.699	1.722	63		
Reptilia						
	Terrapene carolina (tortoise)	1.703	1.719	60		0.06
Amphibia						
	Amphiuma means	1.701	1.706	47		0.07
	Necturus maculosus	1.708	1.719	60		0.02
	Ambystoma trigrinum	1.704	1.717	58		0.04
	A. punctatum	1.704	1.718	59		0.02
	Trituris viridescens	1.702		55, 60	59[f]	0.04
	Bufo americanus	1.701	1.713	54		0.06
	B. marinus	1.704	1.717	58		0.06
	Rana sylvaticus	1.701	1.719	60		0.10
	R. pipiens	1.702	1.723	64		
	R. palustris	1.701				-0.05–0.07
	R. clamitans	0.701	1.721	62		
	Xenopus laevis	1.699	1.722	63	59[g]	0.06
Osteichthyes						
	Opsanus tau (toadfish)	1.687	1.719	60		
	Fundulus	1.699	1.711	52		
	Salmo (salmon)	1.700	1.705	46		
Chondrichthyes						
	Squalus sp. (shark)	1.704	1.714	55		0.08–0.1
Echinodermata						
	Asterias (starfish)	1.697	1.722	63		0.06–0.08
	Lytechinus (sea urchin)	1.696	1.722	63	57[h]	
Arthropoda						
	Cancer borealis (crab)	1.703	1.706	47		
	Artemia salina (brine shrimp)	1.695		46, 49		-0.12–0.15
	Limulus polyphemus (horshoe crab)	1.693	1.712	53		
	Drosophila melanogaster	1.701	1.701	39, 43	40[i]	
Echiuroida						
	Urechis caupo	1.700	1.710	51	52[j]	0.09
Mollusca						
	Loligo pealii (squid)	1.695	1.719	60		
	Spisula (clam)	1.696	1.713	54		0.02–0.05
Coelenterata						
	Metridium (sea anemone)	1.696	1.705	46		0.14
Protozoa						
	Tetrahymena pyriformis, W.	1.690	1.698	39	44[k]	-0.01–0.04
	Astasia longa	1.709				
Eumycophyta						
	Neurospora crassa	1.710	1.715	56	50[l]	0.3
	Saccharomyces cerevisiae	1.695			47[m]	0.4
	S. carlsbergensis	1.698	1.702	43		
	Hansenula anomala	1.695				
	Debaromyces kloeckeri	1.696				

TABLE I: (*Continued*)

Class	Organism	Buoyant Density: Bulk DNA	Buoyant Density: rDNA	G + C Content: rDNA	G + C Content: rDNA	Amt of rDNA
		1	2	3	4	5
Myxomycophyta						
	Dictyostelium discoides (cellular slime mold)	1.681[a]	1.681	23		
Chlorophyta						
	Chlamydomonas reinhardi	1.723	1.714	55		
Tracheophyta						
	Hordeum vulgare (barley)	1.702	1.705	46		0.3
	Triticum aestivum (wheat)	1.703	1.708	45	55[n]	0.12–0.14
	Pisum sativum (pea)	1.695	1.704	45	53[o]	0.12–0.14

1. "Bulk DNA" refers to main band DNA. (*a*) Ashworth (1966). 2. "rDNA bouyant densities" in this column were calculated from hybridization of preparative CsCl gradients with radioactive *X. laevis* rRNA as described in Methods. 3. G + C content of rDNA was determined by the relationship %G + C = (ρ − 1.660)/0.098 (Schildkraut *et al.*, 1962a). 4. rRNA values are from the literature and are an average of the 18S and 28S RNAs: (*a*) Amaldi and Attardi (1968); (b) Kirby (1965); (*c*) Wallace and Ts'o (1961); (*d*) Wang (1962); (*e*) Merits *et al.* (1966); (*f*) Gall (1966); (*g*) Dawid *et al.* (1970); (*h*) Glišin and Glišin (1964); (*i*) Ritossa *et al.* (1966); (*j*) Gould (1967); (*k*) Lyttleton (1963); (*l*) Henney and Storck (1963); (*m*) Kitazume *et al.* (1962); (*n*) Glitz and Dekkar (1963). 5. The "amount of rDNA" refers to the fraction of the DNA which hybridizes with radioactive rRNA of *X. laevis*. About 100 μg of DNA was run on CsCl, fractionated, applied to Millipore filters, and hybridized in stacks with 1–3 μg/ml of [^{3}H]rRNA. Hybridization was for 15–20 hr in 4× SSC at 70–72°. A filter with a known amount of *X. laevis* DNA was included in each vial as a control. The radioactivity under the satellite peak was totaled. The number of counts was then compared to the number of counts present on the filter having an equivalent amount of *X. laevis* whole cell DNA. If the count was identical, since 0.06% of the genome of *X. laevis* is rDNA (Brown and Weber, 1968a) then 0.06% of the DNA of this particular organism was homologous with *X. laevis* rRNA. When the count was different a direct ratio of the difference was used as the estimate of the "amount of rDNA." For organisms in which homology between *X. laevis* rRNA and the organism's DNA is less than 100% our "amount of rDNA" is naturally an underestimate. Literature reports for some of the organisms examined here are: HeLa, 0.003–0.005% for 28S (McConkey and Hopkins, 1964), 0.003–0.005% for 18 + 28S rRNA (Attardi *et al.*, 1965b); rat, 0.046% (Steele, 1968); chicken, 0.02% (Merits *et al.*, 1966); *B. marinus*, 0.025–0.056% (Miller and Brown, 1969); *X. laevis*, 0.057%; (Brown and Weber, 1968b); *D. melanogaster*, 0.27% (Ritossa and Spiegelman, 1965); *S. cerevisiae*, 2.4% (Schweizer *et al.*, 1969); *S. carlsbergensis*, 2.0% (DeKloet, 1970); wheat, 0.2% (Chen and Osbourne, 1970); pea, 0.3% (Chipchase and Birnstiel, 1963).

Ribosomes of *Dictyostelium* have physical properties which resemble bacterial ribosomes more closely than those from higher plants or animals (Ashworth, 1966). Nevertheless, some sequence homology between *D. discoides* and *X. laevis* rDNAs has been detected.

The fact that high levels of hybridization occur with DNA distinctly different in base composition from bulk DNA assures that homology really exists. In many invertebrates a larger fraction of DNA hybridizes with *X. laevis* rRNA than is the case for *X. laevis* DNA itself. Due to lack of knowledge as to the extent of homology between *X. laevis* rRNA and the heterologous DNAs the present results only define a minimum level of rDNA in these animals. Data on other invertebrates are presented in Table I.

In Figure 4 the hybridization of *X. laevis* rRNA with the DNA of two higher plants is examined. The rDNA of these plants is of higher density than bulk DNA and a very high level of hybridization is observed. It is obvious from these figures as well as from hybridization levels with yeast (Table I) that *X. laevis* rRNA shows extensive homology with eukaryotes as distantly related as plants and fungi.

Several attempts have been made to detect homology between *X. laevis* rRNA and the DNA of prokaryotes. No measurable homology was detected with any of the six bacterial DNAs examined, with T_2 or T_7 bacteriophage, with *X. laevis* mitochondrial DNA (Dawid and Brown, 1970), or with DNA of another prokaryote, the blue-green alga *Plectonema boryanum* (Brown *et al.*, 1967). The results of hybridization with a bacterium (*Clostridium perfringens*) and *P. boryanum* are shown in Figure 5. Not only is the level of hybridization very low when compared to that with *R. pipiens*, but very little of the radioactivity which binds to these filters is competed for by unlabeled *X. laevis* rRNA. This is in distinct contrast to the situation in higher vertebrates, where a similar small number of counts is entirely eliminated by unlabeled *X. laevis* rRNA. Furthermore, saturation competition studies have shown that under conditions in which mouse rRNA competes with *X. laevis* rRNA for over 90% of the *X. laevis* rDNA sequences, bacterial rRNA competes for no sequences at all (Sinclair and Brown, 1968). Failure to detect hybridization with DNA from prokaryotes cannot be attributed to the small number of ribosomal genes in the bacterial genome, since the 9–10 rRNA genes of a bacterium such as *B. subtilis* comprise about 0.39% of the genome (Smith *et al.*, 1968) compared to less than 0.1% in *X. laevis* and even less in higher vertebrates.

Buoyant Density of rDNA from Different Organisms as Determined by CsCl Centrifugation and RNA–DNA Hybridization. As discussed above, the technique of CsCl centrifugation and molecular hybridization permits a determination of the buoyant density of ribosomal genes. The presence of two

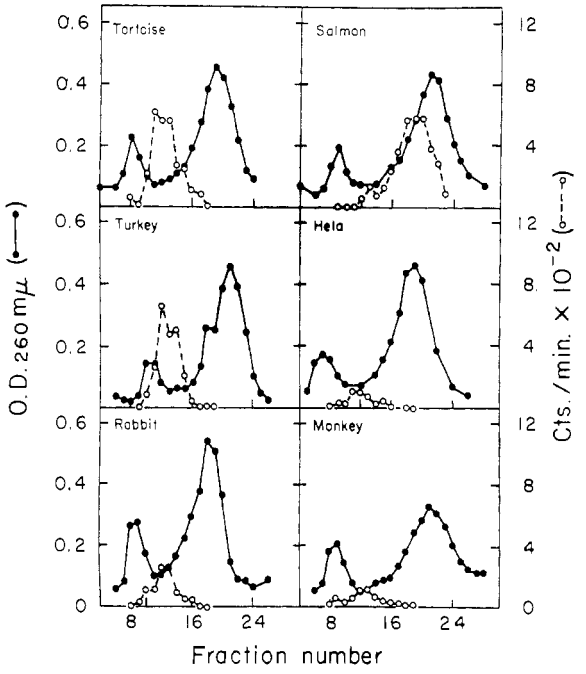

FIGURE 2: Cesium chloride centrifugation and rRNA hybridization profiles for DNAs from several vertebrates. Same conditions as in Figure 1. (●) Optical density at 260 mμ; (○) counts per minute.

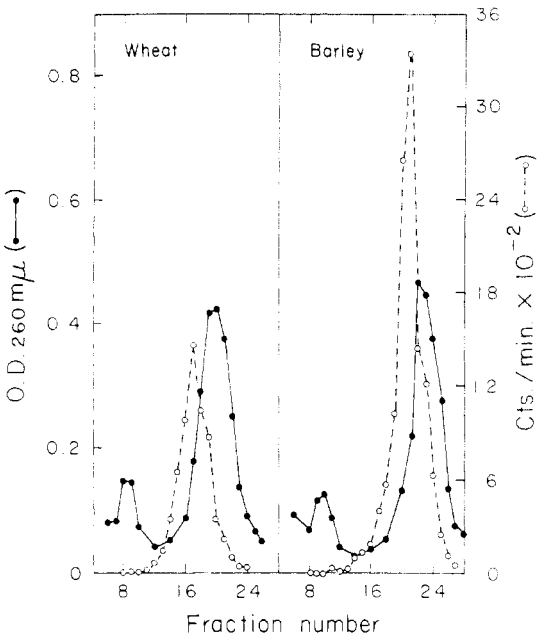

FIGURE 4: Cesium chloride centrifugation and rRNA hybridization profiles for DNAs from higher plants. (●) Optical density at 260 mμ; (○) counts per minute.

density markers in each gradient (*M. luteus* and the bulk DNA of each organism) adds confidence to the method. From this buoyant density the G + C content of the rDNA genes can be estimated. The ribosomal genes of the majority of the organisms examined banded as a reasonably discrete peak (Figures 1–4). Column 2 of Table I lists the buoyant density of rDNA for a variety of organisms. Generally the rDNA has a higher buoyant density than the bulk DNA. In some organisms the densities are the same and in one instance rDNA had a lower buoyant density than bulk DNA (*Chlamydomonas*).

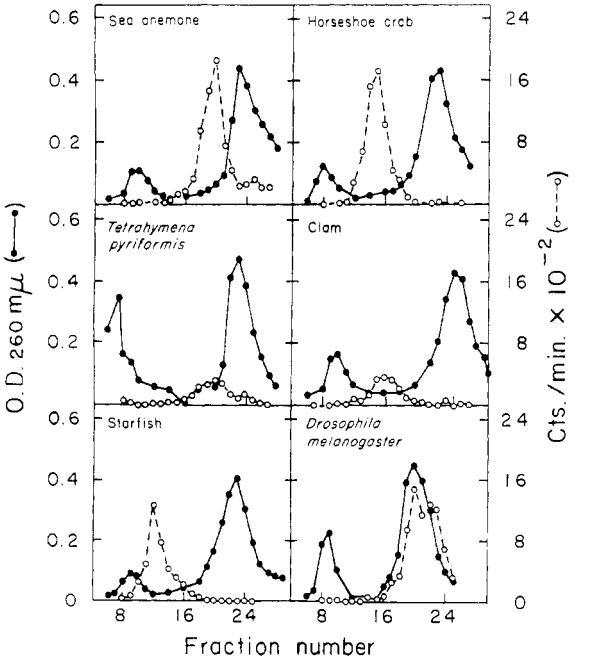

FIGURE 3: Cesium chloride centrifugation and rRNA hybridization profiles for DNAs from several invertebrates. (●) Optical density at 260 mμ; (○) counts per minute.

Organisms in Table I are listed in a phylogenetic sequence from vertebrates down through protozoa and fungi then back up to higher plants. Although the rDNA of some classes of organisms has a similar buoyant density, this similarity does not hold for all classes. All of the mammals, birds, and reptiles examined have a consistently high buoyant density. Other classes of vertebrates such as bony fishes and amphibians, on the other hand, show appreciable variation in rDNA buoyant densities. The rDNAs of yeast and higher plants are only slightly higher in buoyant density than their respective bulk DNAs.

Hybrids of X. laevis rDNA and Heterologous rRNA. Saturating levels of homologous rRNA hybridize with rDNA to produce an RNA–DNA hybrid which has a much higher buoyant density in CsCl gradients than denatured DNA (Wallace and Birnstiel, 1966; Brown and Weber, 1968b). It might be expected that any rRNA which is homologous with a significant fraction of *X. laevis* rDNA would hybridize to form a particularly high buoyant density RNA–DNA hybrid. Detection of such hybrids by this method would provide additional evidence for homology between the organism from which the DNA was taken and the one from which the RNA was obtained.

rRNA from a number of organisms has been examined with respect to its ability to form high buoyant density hybrids with *X. laevis* rDNA. Evidence for hybridization was obtained with rRNA of all of the eukaryotes examined. Banding profiles of *X. laevis*, rat, *U. caupo*, *T. pyriformis*, *N. crassa*, *and* spinach rRNA hybrids with *X. laevis* rDNA are shown in Figure 6. Hybrids of *X. laevis* DNA with starfish, chick, and *S. cerevisiae* rRNA have also been examined with similar results. The increase in density of rDNA following hybridization with these heterologous rRNAs is probably not due to

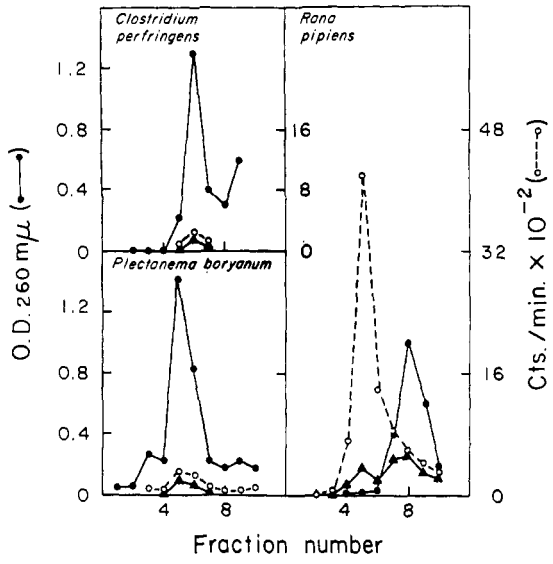

FIGURE 5: Cesium chloride centrifugation and rRNA hybridization profiles for DNAs from prokaryotes compared to *R. pipiens*. Same conditions as in Figure 1, except: 35 drop fractions were taken, to which 0.5 ml of water was added before reading the optical density. After adsorption on filters, each filter was split into two; one-half was hybridized in the usual manner while the other half was hybridized in the same way with the addition of 15 μg/ml of unlabeled *X. laevis* rRNA to the hybridization solution, which corresponds to about a 20-fold excess of unlabeled rRNA. (●) Optical density at 260 mμ; (○) counts per minute with only radioactive rRNA; (▲) counts per minute with both labeled and unlabeled rRNA.

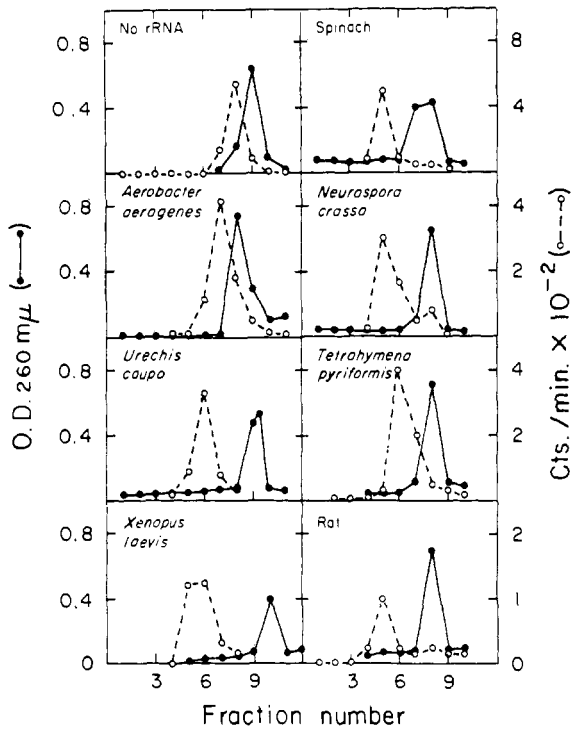

FIGURE 6: Comparative banding behavior of heterologous rRNA–*X. laevis* rDNA hybrids in CsCl. In each case about 50 μg of *X. laevis* DNA was sheared by passing it through a 26-gauge hypodermic needle five times, then denatured by making the solution 0.1 N with KOH. Traces of rRNA in the DNA preparation were hydrolyzed by incubating at 18° overnight (Brown and Weber, 1968b). Prehybridization of rDNA was carried out with 20 μg of various heterologous rRNAs at 70° for 45 min. The reaction mixture was diluted to 2× SSC and treated with 10 μg/ml of pancreatic RNase for 20 min at room temperature. Then CsCl was added, followed by centrifugation and fractionation. RNA hybridized to DNA was removed by alkali prior to immobilization of the DNA on filters. The filters containing DNA were then hybridized with ^{3}H *X. laevis* rRNA. (●) Optical density (○) counts per minute.

unhybridized ends of rRNA or to loose aggregates since each hybrid mixture was treated with RNase prior to CsCl centrifugation. This indicates that a considerable length of rRNA is involved in the hybrid and supports other observations which suggest that the degree of homology among distantly related eucaryotic organisms is quite high. When bacterial rRNA was prehybridized with *X. laevis* rDNA, there was no shift in the banding position of rDNA (Figure 6).

Discussion

Qualitative Homology Between rRNA of X. laevis and Heterologous rDNAs. This analysis of rDNA is essentially a qualitative one which was designed to detect homology between a single labeled ribosomal RNA (from *X. laevis*) and DNAs from a variety of organisms. Extensive sequence homology was found with DNAs of organisms as distantly related as protozoa, higher plants, and fungi. Hybridization conditions in these experiments (4× SSC at 70°, followed by RNase treatment) are quite stringent. This does not imply that the heterologous RNA–DNA hybrids are perfectly matched. In fact, experiments designed to detect mismatching indicate that heterologous hybrids melt out with T_m values from 2 to 10° lower than the homologous rRNA–rDNA hybrids (D. D. Brown and J. H. Sinclair, unpublished).

The specificity of hybridization has been enhanced by prefractionating the DNAs in CsCl before hybridization. In most cases gradient centrifugation results in a partial or complete separation of ribosomal genes from the main band of DNA due to their high G + C content. Buoyant density estimates of these genes made from the preparative CsCl gradients agree approximately with published base composition values for rRNAs of the organisms used (Table I). Exact agreement of these two values is not necessarily expected. In rDNA of *X. laevis*, additional sequences (spacer DNA) of a base composition different from the ribosomal genes are interspersed between the genes for 18S and 28S rRNA (Brown and Weber, 1968a; Birnstiel *et al.*, 1968; Dawid *et al.*, 1970). Furthermore, the rDNA of *X. laevis* has a high content of 5-methyldeoxycytidylic acid residues which reduces the buoyant density of the DNA (Dawid *et al.*, 1970). Despite these potential influences on the buoyant densities of rDNA, the G + C contents derived from the densities are in approximate agreement with the G + C content of the rRNA. This is especially evident in those species whose rRNA is similar in base composition to the bulk DNA. *Tetrahymena*, *Drosophila*, and slime mold are such examples and their rDNAs are approximately the same buoyant density as main band DNAs. The only eukaryote examined whose rDNA predicts a lower G + C content than for the bulk DNA is *Chlamydomonas reinhardi*. In this organism main band DNA has an unusually high density (63% G + C) while the buoyant density of rDNA predicts that its base composition is 54% G + C.

There is considerable precedence for our observations of high levels of RNA–DNA hybridization between a vertebrate rRNA and distantly related species. High homology levels have been reported for *Paramecium* rRNA and DNAs from several other protozoa (Gibson, 1967), for HeLa cell rRNA and several eukaryote DNAs (Attardi *et al.*, 1965b), for to-

bacco and Chinese cabbage rRNA with DNA from several other plants (Matsuda and Siegel, 1967), for pea rRNA with DNA from a spectrum of eukaryotes (Bendich and McCarthy, 1970), and for rRNA of *S. cerevisiae* and *S. lactis* with DNA from several other species of yeast (Bicknell and Douglas, 1970). Several of these authors combined direct hybridization studies with competition hybridization to confirm homology. Laird and McCarthy (1968) have also demonstrated that the RNAs of several organisms compete with *D. melanogaster* rRNA for *D. melanogaster* DNA. Both the direct hybridization and competition experiments of these workers are in general agreement with the results of this paper. We have also carried out competition studies with a variety of organisms and the results indicate that homology is high (Sinclair and Brown, 1968).

Although the above reports are generally in good agreement with our own, there are two or three apparent discrepancies which might be mentioned. Laird and McCarthy (1968) for instance, observed essentially no competition of rRNA from yeast, bee, mouse, frog, and *E. coli* for rDNA sites of *D. melanogaster*. This relatively low level of conservation of base sequence homology is surprising when compared to the reports previously discussed, as well as when compared to our own work. Certainly if significant homology exists among vertebrates, plants, and fungi, etc., it is surprising to find none between *Drosophila* and bees. Bendich and McCarthy (1970) reported the surprising result that *E. coli* rRNA binds to pea DNA at about 25% of the level *E. coli* binds to *E. coli* DNA. If both pea (Chipchase and Birnstiel, 1965) and *E. coli* (Kennell, 1968; Avery *et al.*, 1969) have approximately the same amounts of their genome as rDNA (0.3–0.4%) then this represents 25% homology. They found similar high levels of hybridization when pea rRNA was hybridized with *E. coli* DNA. In fact, the levels of hybridization between pea rRNA and bacterial DNA was higher than the level of pea rRNA and the DNA of some plants and animals. These results are contrary to others who have compared pea and *E. coli* (Chipchase and Birnstiel, 1963; Attardi *et al.*, 1965a) and contrast with our own findings and those of others which fail to detect homology between eukaryotes rRNA and the DNA of prokaryotes. Part of the discrepancy between Bendich and McCarthy's and our own results may be attributable to the less stringent conditions they used (*e.g.*, omitting the RNase step after hybridization). Our experiences with RNase digestion does not suggest that this difference in technique is an adequate explanation for the different results they obtained, however.

The extent of rRNA–rDNA homology which we find is somewhat surprising in view of the divergence of nucleotide sequences in the total nDNA among eukaryotes (Hoyer *et al.*, 1964; Dutta *et al.*, 1967). Another reason for not having expected such a large amount of homology among rDNAs of different species is the great variation in overall base composition of rRNAs among eukaryotes. The base compositions in terms of G + C content of rRNA from several organisms are shown in column 4 of Table I. There is obviously a wider range in base composition of rRNAs than is the case for bulk DNA.

Quantitation of rDNA by Heterologous Hybridization. In column 5 of Table I we have presented figures estimating the fraction of the genome of different organisms which will hybridize with *X. laevis* rRNA. For organisms with rDNA having base sequences very similar to rDNA base sequences in *X. laevis*, this is a useful estimate of the size of the ribosomal genome. In organisms in which base sequence homology with *X. laevis* is less similar this figure is naturally an underestimate of the size of the ribosomal genome. We include this information for two reasons. First, it makes available in one place, a comparison of the fraction of DNA from a wide range of organisms which will hybridize with a single preparation of rRNA under the same set of conditions. The opportunity to directly compare the values obtained at least partly overcomes the inherent disadvantages due to the known breakdown in homology. Second, we have reason to believe that the homology breakdown is not so extensive as might at first be expected. Estimates of regions which are totally nonhomologous have been made by competition experiments. Values ranging from 30 to 60% homology have been found between the rRNAs of *Tetrahymena*, tobacco, and yeast with *X. laevis* DNA (Sinclair and Brown, 1968). *Urechis* rRNA competes the hybridization of *X. laevis* rRNA with its homologous rDNA by about 60% (Dawid and Brown, 1970). Nevertheless, the breakdown in homology between *X. laevis* rDNA and that of the organism being examined must be kept in mind in using these data. It is also important to keep in mind that these hybridizations were carried out at concentrations of rRNA below saturation levels. Although it has been demonstrated that varying amounts of rRNA in the reaction mixture give quantitatively accurate values with *X. laevis* rDNA (Brown and Weber, 1968a), this work referred only to the homologous hybridization reaction and has not been clearly documented for heterologous systems. Despite the uncertainties of using a single rRNA for assaying a variety of DNAs and of carrying out hybridizations at subsaturating conditions, it is interesting that our results are consistent with values obtained by others using homologous saturating conditions. (Some literature values are given as a footnote to column 5 of Table I.) Our estimates for mammals are somewhat lower than those reported for rat. This is not surprising considering the differences in techniques used and considering the uncertainties in quantitating this very small genome. In the case of birds we obtain values about twice the value reported by Merits *et al.* (1966) for chicken. For *Drosophila*, our value is about half that reported in the literature. This might be expected on the basis of homology breakdown. Though base sequence divergence is so great as to make quantitative comparison impossible, our results for fungi and plants also support literature reports of a large ribosomal genome in these organisms. In spite of the underestimation of the rDNA content inherent in this method, it is still possible to conclude that the fraction of the genome which is homologous with rRNA increases with decreasing phylogenetic position among animals. Further, plant genomes tend to have high rDNA contents.

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References

Amaldi, F., and Attardi, G. (1968), *J. Mol. Biol. 33*, 737.

Ashworth, J. M. (1966), *Biochim. Biophys. Acta 129*, 211.

Attardi, G., Huang, P., and Kabat, S. (1965a), *Proc. Nat. Acad. Sci. U. S. 53*, 1490.

Attardi, G., Huang, P., and Kabat, S. (1965b), *Proc. Nat. Acad. U. S. 54*, 185.

Avery, J. R., Midgley, J. E. M., and Pigott, H. G. (1969), *Biochem. J. 115*, 395.
Bendich, A. J., and McCarthy, B. J. (1970), *Proc. Nat. Acad. Sci. U. S. 69*, 349.
Bicknell, J. N., and Douglas, H. C. (1970), *J. Bacteriol. 101*, 505.
Birnstiel, M., Speirs, J., Purdom, I., and Jones, K. (1968), *Nature (London) 219*, 454.
Brown, D. D., and Weber, C. S. (1968a), *J. Mol. Biol. 34*, 661.
Brown, D. D., and Weber, C. S. (1968b), *J. Mol. Biol. 34*, 681.
Brown, D. D., Weber, C. S., and Sinclair, J. H. (1967), *Carnegie Inst. Washington Yearb. 66*, 580.
Chen, D., and Osbourne, D. J. (1970), *Nature (London) 225*, 336.
Chipchase, M. J. H., and Birnstiel, M. L. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 1101.
Dawid, I. B., and Brown, D. D. (1970), *Develop. Biol. 22*, 1.
Dawid, I. B., and Brown, D. D., and Reeder, R. H. (1970), *J. Mol. Biol. 51*, 341.
DeKloet, S. R. (1970), *Arch. Biochem. Biophys. 136*, 402.
Dutta, S. K., Richman, N., Woodward, V. W., and Mandel, M. (1967), *Genetics 57*, 719.
Fisher, W. D., Cline, G. B., and Anderson, N. G. (1964), *Anal. Biochem. 9*, 477.
Flamm, W. G., Bond, E., and Burr, H. E. (1966), *Biochim. Biophys. Acta 129*, 310.
Gall, J. G. (1966), *Nat. Cancer Inst. Monogr. 23*, 475.
Gerloff, G. C., Fitzgerald, G. P., and Skoog, F. (1950), *in* Symposium on the Culture of Algae, Brunel, J., Prescott, G. W., and Tiffany, L. H., Ed., Dayton, Ohio, Charles F. Kettering Foundation, p 27.
Gibson, I. (1967), *J. Protozool. 14*, 687.
Glišin, V. R., and Glišin, M. V. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1548.
Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.
Gould, M. (1967), Ph.D. Thesis, University of California, p 33.
Henney, H. R., and Storck, R. (1963), *Science 142*, 1675.
Hoyer, B. H., McCarthy, B. J., and Bolton, E. T. (1964), *Science 114*, 959.
Kennell, D. (1968), *J. Mol. Biol. 34*, 85.
Kirby, K. S. (1965), *Biochem. J. 96*, 266.
Kitazume, Y., Yčas, M., and Vincent, W. S. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 265.
Laird, C. D., and McCarthy, G. J. (1968), *Genetics 60*, 303.
Lyttleton, J. W. (1963), *Exp. Cell. Res. 31*, 385.
Matsuda, K., and Siegel, A. (1967), *Proc. Nat. Acad. Sci. U. S. 74*, 673.
McConkey, E. H., and Hopkins, J. W. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 1197.
Merits, I., Schulze, W., and Overby, L. R. (1966), *Arch. Biochem. Biophys. 115*, 197.
Miller, L., and Brown, D. D. (1969), *Chromosoma 28*, 430.
Ritossa, F. M., Atwood, K. C., Lindsley, D. L., and Spiegelman, S. (1966), *Nat. Cancer Inst. Monogr. 23*, 449.
Ritossa, F. M., and Spiegelman, S. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 737.
Schildkraut, C. L., Mandel, M., Levisohn, S., Smith-Sonneborn, J. E., and Marmur, J. (1962b), *Nature (London) 196*, 795.
Schildkraut, C. L., Marmur, J., and Doty, P. (1962a), *J. Mol. Biol. 4*, 430.
Schweizer, E., McKechnie, C., and Halvorson, H. W. (1969), *J. Mol. Biol. 40*, 261.
Sinclair, J. H., and Brown, D. D. (1968), *Carnegie Inst. Washington Yearb. 67*, 404.
Smith, I., Dubnau, D., Morell, P., and Marmur, J. (1968), *J. Mol. Biol. 33*, 123.
Steele, W. J. (1968), *J. Biol. Chem. 243*, 3333.
Stern, J. L., Sekiguchi, M., Barner, H. D., and Cohen, S. S. (1964), *J. Mol. Biol. 8*, 629.
Sueoka, N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 83.
Sueoka, N. (1961), *J. Mol. Biol. 3*, 31.
Vinograd, J., and Hearst, J. E. (1962), *Fortschr. Chem. Org. Naturst. 20*, 372.
Wallace, H., and Birnstiel, M. L. (1966), *Biochim. Biophys. Acta 114*, 296.
Wallace, J. M., and Ts'o, P. O. P. (1961), *Biochem. Biophys. Res. Commun. 5*, 125.
Wang, T. Y. (1962), *Arch. Biochem. Biophys. 97*, 387.