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Gene Products of Amphiuma: An Amphibian with an Excessive Amount of DNA

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The nuclei of Amphiuma contain 168 pg of DNA, 28 times that contained in human nuclei. Although many higher organisms appear to possess an excessive amount of DNA, in Amphiuma this has been carried to the extreme. Studies of this organism thus may provide some insight into how this excess DNA is used. This organism presumably evolved by numerous polyploidy and gene duplication events. Do its gene products present multiple electrophoretic forms? Are they quantitatively increased to the same degree as the DNA? Electrophoresis of Amphiuma glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, acid phosphatase, lactic dehydrogenase, and hemoglobin failed to show any evidence for multiple electrophoretic forms of these respective gene products. The amount of hemoglobin, G6PD, and 6PGD per red cell was increased to a comparable or even greater degree than the DNA. Analytical ultracentrifugation demonstrated a satellite band of DNA with a density of 1.720 corresponding to a GC content of 61%. This probably represents DNA coding for ribosomal RNA. Electron microscopy of liver nuclei showed a significant amount of condensed chromatin. The implications of these observations are discussed.

INTRODUCTION

The nuclear content of DNA varies considerably among higher organisms. Many of the chordates possess only a fraction of the amount of DNA present in mammals (Mirsky and Ris, 1951; Vendrely, 1955; Atkin *et al.*, 1965; Atkin and Ohno, 1967). This suggests that during evolution there occurred a number of episodes of gene duplication and polyploidy (Ohno *et al.*, 1968). This has occasionally been carried to the extreme and some organisms appear to possess more DNA than they would logically seem to need. This is especially true of the African lung fish and the Congo eel (Amphiuma). In man the nuclear content of DNA is approximately 6 pg. However, in

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the lung fish it is 100, and in Amphiuma 168 (Mirsky and Ris, 1951). In the latter organism there is thus a 28-fold increase in the amount of nuclear DNA compared to man. There is a proportionate increase in cell size as illustrated in Fig. 1, which shows a mixture of human and Amphiuma red blood cells. The question "Why so much DNA?" can reasonably be asked about mammals and most certainly it can be asked about Amphiuma. Gene loci in Amphiuma have presumably been duplicated many times. Have the gene products increased proportionately? Have the duplicated genes remained active? If they have, do the gene products show electrophoretic heterogeneity as expected from the accumulation of different mutations? To answer some of these questions we have investigated the quantitative and electrophoretic properties of some of the gene products of Amphiuma.

MATERIALS AND METHODS

The Amphiuma were obtained from the Carolina Biological Supply Company, Burlington, North Carolina. Two females and three males were studied.

Preparation of Erythrocyte Hemolysates

Heparinized whole blood was washed three times in normal saline and the red blood cells resuspended in 20 volumes of 0.007 M tris and 0.001 M EDTA. The samples were frozen in a methanol–dry ice bath and allowed to thaw. They were then centrifuged at 11,500 $\times g$ and the supernatant removed and frozen. When the sample was ready for use, it was thawed and extracted twice with toluene.

Preparation of Liver Hemolysates

Portions of liver were placed in cold normal saline and cut into small pieces. These were then placed in a Teflon homogenizer with an equal volume of 0.02 M tris and 0.12 M KCl, *p*H 8.6. The homogenate was frozen, thawed, and centrifuged at 10,000 $\times g$ for 5 min. The supernatant was removed and frozen until needed. At the time of use, the samples were thawed and extracted twice with toluene. A similar procedure was used for heart and muscle tissues.

Electrophoretic Techniques

All electrophoreses were performed with starch gel by the technique of Smithies (1955). The stain for glucose 6-phosphate dehydrogenase consisted of 10 mg glucose 6-phosphate (G6P) dipotassium salt (Sigma); 2 mg 3(4,5-dimethyl thiazolyl 1-2) 2,5-diphenyltetrazolium bromide (MTT); 2 mg phenazine methosulfate (PMS); and 2 mg NADP (TPN) dissolved in 10 ml of 1.1 M tris HCl buffer, *p*H 8.0. This was poured over the sliced gel which was then kept covered at room temperature for 30 min (Mathai *et al.*, 1966). The stain for 6-phosphogluconic dehydrogenase consisted of 20 mg of 6-phosphogluconic acid (PGA), 2 mg MTT, 0.2 mg of PMS, 2 mg TPN, and 15 mg of

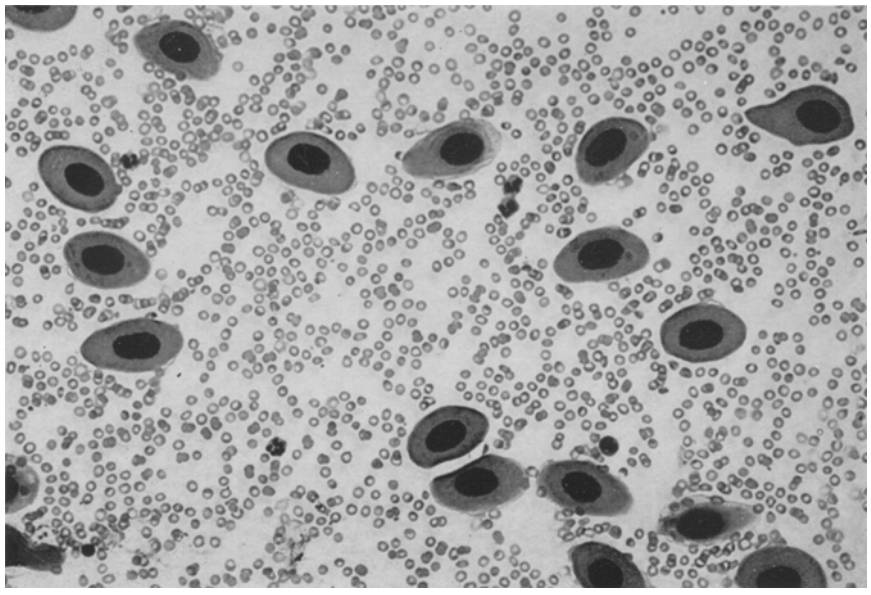

Fig. 1. A mixture of Congo eel and human red blood cells illustrates the large size of Amphiuma cells. 410×.

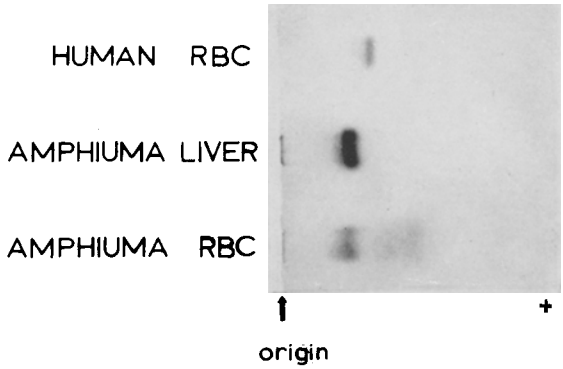

Fig. 2. Electrophoretic pattern of glucose 6-phosphate dehydrogenase of human red cells and Amphiuma liver and red cells. The band right of the Amphiuma red cell G6PD is hemoglobin (0.01 M tris, *p*H 9.0, 120 v for 15 hr).

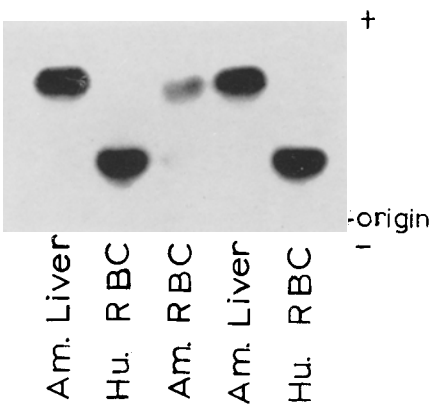

Fig. 3. Electrophoretic pattern of 6-phosphogluconate dehydrogenase of Amphiuma liver and red cell and of human red cell. The conditions are the same as those in Fig. 2.

catalase dissolved in 10 ml of 0.1 M tris HCl buffer, *p*H 8.0. The stain was poured over the sliced gel which was then incubated at 37 C for two or more hours. For glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase the procedure for preparation of homogenates was modified by the addition of TPN, 0.5 mg/ml, to all the homogenization steps.

Starch gel electrophoresis for acid phosphatase was by the technique of Hopkins *et al.* (1964). Thirty-microliter aliquots of homogenate were placed in the preformed slots. To prevent the gel from cracking, the starch concentration was increased to 75 g/500 ml and the gel was poured thicker than normal (9 mm). Lactic dehydrogenase was electrophoresed by the technique of Markert and Faulhaber (1965) and stained by the procedure of Ohno *et al.* (1967).

Hemoglobin starch gel electrophoresis (Smithies, 1955) was carried out with a 0.9 M tris, 0.02 M EDTA, and 0.5 M boric acid buffer at *p*H 8.6. Hemoglobin was stained with benzidine. To inhibit possible polymerization of hemoglobins the hemolysate of some samples was treated with iodoacetate by the technique of Riggs (1965).

Enzyme Assays

G6PD and 6PGD were assayed by a modification of the technique of Glock and McLean (1953). One cuvette contained a final concentration of the following: tris–HCl, 0.1 M, *p*H 8.0; glucose 6-phosphate, 0.6 mM; 6-phosphogluconate, 0.6 mM; NADP, 0.2 mM; and $MgCl_2$, 10 mM. A second cuvette was the same except that glucose 6-phosphate was left out. Both 6-phosphogluconate and G6P were left out of the third cuvette, which served as a blank. The difference in activity of the first and second cuvettes represented the G6PD activity, while the difference in activity of the second and third cuvettes represented the activity of 6PGD. The activity of both enzymes was expressed as micromoles of NADP reduced per minute per gram of hemoglobin at 37 C. The assays were kindly performed for us by Dr. E. Beutler.

Isolation of DNA

Liver or red blood cells were suspended in a solution of 2 × SSC (SCC—0.15 M sodium chloride, 0.015 M sodium citrate) containing 1% sodium lauryl sulfate and 3 mg/ml of pronase. The suspension was homogenized at high speed in a Virtis homogenizer for 3 min. It was then incubated at 37 C in a shaking water-bath incubator overnight. An equal volume of chloroform–isoamyl alcohol (24:1) was added and this was placed in a shaker at room temperature for 24 hr. This was then spun at 30,000 × *g* for 1 hr. The DNA was spun out from this supernatant following the addition of 1.5 volumes of 95% ethanol. The DNA was redissolved in 2 × SSC and incubated with 20 μg/ml of RNAase (heated to 60 C for 10 min to destroy DNAase) at 37 C for 1 hr. Pronase (also heat-treated), 1 mg/ml, was added and the solution incubated for an additional hour. An equal volume of water-saturated phenol containing 0.1% 8-hydroxyquindine was then added and heated to 60 C for 3 min. This was shaken well and cooled in an ice-bath. Following centrifugation at 5000 × *g* for 10 min, the supernatant was removed and dialyzed for 48 hr against several changes of 2 × SSC. An aliquot of this was then

placed on a Sephadex G-100 column and eluted with 2 × SCC to remove low-molecular-weight contaminants. This DNA was then dialyzed 48 hr against several changes of 0.01 tris, 0.001 EDTA.

Analytical Ultracentrifugation of DNA

One and one-fifth grams of dry CsCl were added to 1.0 ml of a solution of 8 μg/ml of Congo eel DNA plus 1.0 μg/ml of *Micrococcus lysodeikticus* DNA and with the use of an Abbe refractometer the density was adjusted to 1.71 g/cm^3. A 0.7-ml portion of this solution was placed in a standard centerpiece on an analytical D rotor and spun at 44,000 rpm for 24 hr. The UV photograph was scanned with a Joyce–Lobel microdensitometer. A density marker of *Micrococcus lysodeikticus* DNA (1.727 g/cm^3) was used and the density of the sample determined by the technique of Schildkraut *et al.* (1962).

Electron Microscopy

A small sample of fresh liver was minced into tiny pieces with a razor blade, fixed in 3% glutaraldehyde in phosphate buffer and then in 2% osmium tetraoxide, and stained in uranyl acetate. The specimens were then dehydrated in a graded series of ethanol washes and embedded in Epon. Sections of 600 Å were made with an LKG ultratome. These sections were stained in lead citrate (Reynolds, 1963) and examined in a Hitachi 11B electron microscope.

RESULTS

Electrophoreses

Glucose 6-Phosphate Dehydrogenase

The Amphiuma liver and red blood cell glucose 6-phosphate dehydrogenase migrated as a single band slightly slower than human red cell G6PD (Fig. 2).

6-Phosphogluconic Dehydrogenase

Both Amphiuma liver and red blood cell 6-phosphogluconate dehydrogenase migrated as a single electrophoretic band. The rate of migration was the same and was approximately twice that of human red blood cell 6PGD (Fig. 3).

Acid Phosphatase

Amphiuma red blood cell acid phosphatase migrated as two electrophoretic bands (Fig. 4). The liver acid phosphatase also migrated as two bands, one at the same rate as the slower of the red blood cell bands, the other migrated cathodally. The human red blood cell and liver acid phosphatases both migrated cathodally. This same pattern was found on four different individual Amphiuma.

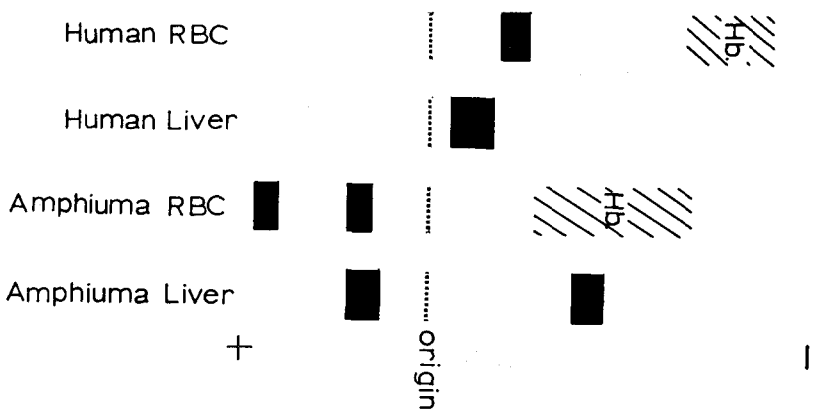

Fig. 4. A diagram of the electrophoretic pattern of human and Amphiuma red cell and liver acid phosphatase (2.5 mM succinic acid, 4.6 mM tris, *p*H 6.0, 120 v for 15 hr). The sliced gel was stained with phenothalene.

Lactic Dehydrogenase

Amphiuma skeletal muscle showed a single major M band and a very faint H band (Fig. 5). Amphiuma heart muscle showed a single H band. Both the liver and the red blood cells showed both M and H bands (the most rapidly migrating spot on the Amphiuma red blood cell represents hemoglobin). By comparison the human liver showed all five isozyme bands characteristic of this tetrameric molecule. This failure of A and B subunits to associate and form tetramers is similar to that of teleost fish (Markert and Faulhaber, 1965) and the hagfish (Ohno *et al.*, 1967).

Hemoglobin

Starch gel electrophoresis of Amphiuma hemoglobin showed a major, rapidly migrating band constituting 80% of the total hemoglobin and a slower band constituting approximately 18% of the total hemoglobin (Fig. 6). There was a suggestion of two faint bands within this region. There was a very faint region migrating more slowly. Addition of iodoacetate to the hemolysate (Riggs, 1965) to inhibit hemoglobin polymerization did not result in any marked change in the pattern. It did produce a moderate increase of the faint, slowly migrating hemoglobin band.

Quantitative Assays

The results of the G6PD and 6PGD assays are shown in Table I. Based on the values published by Mirsky and Ris (1951), the ratio of Amphiuma DNA per nucleus to that of man is 28 : 1. Since human red blood cells are not nucleated the Amphiuma red cell–human red cell ratios for cell volume, hemoglobin, and enzymes cannot be strictly compared to that of the DNA ratios. However, as seen in Table I, the gene products, hemoglobin, G6PD, and 6PGD show a proportionate if not greater increase per cell than that of DNA.

Table I. DNA, Hemoglobin, Glucose 6-Phospgate Dehydrogenase, and 6-Phosphogluconic Dehydrogenase in Amphiuma and Human Red Blood Cells

	Units	Amphiuma	Human	Ratio Amphiuma/human
DNA/nucleus	10^{-12} g	168	6.0	28
Hemoglobin/RBC	10^{-12} g	2700	28	97
RBC volume	μ^3	10,700	85	126
G6PD	a. Micromoles/min/g Hb	9.6	7.9	
	b. Micromoles/min/RBC	26,000	220	118
6PGD	a. Micromoles/min/g Hb	2.7	4.3	
	b. Micromoles/min/RBC	7300	120	61

Centrifugation of DNA

The microdensitometry tracing of the UV photograph of an analytical centrifugation of Amphiuma red blood cell DNA with a *Micrococcus lysodeikticus* marker is shown in Fig. 7. The density of the main peak was 1.705 g/cm^3 and that of the satellite peak was 1.720 g/cm^3. These correspond to GC contents of 46 and 61%, respectively (Schildkraut *et al.*, 1962). These values are analogous to those in *Xenopus laevis* of 1.698 g/cc^3 for the main band and 1.723 g/cc^3 for the satellite bands (Wallace and Birnstiel, 1966; Birnstiel *et al.*, 1965). The GC content of the satellite band (61%) corresponds closely to the GC content of 63% found in amphibian ribosomal RNA (Wallace and Birnstiel, 1966). This strongly suggests that the Amphiuma satellite DNA represents the DNA coding for ribosomal RNA, as has been shown for the satellite in *Xenopus laevis* by the hybridization studies of Wallace and Birnstiel (1966).

Electron Microscopy

Sixty-five Amphiuma liver nuclei were viewed. Figure 8(a) illustrates a low-power view of one of these. There is relatively more condensed chromatin than is usually seen in liver nuclei of organisms with less nuclear DNA, as can be seen by comparison to Fig. 9 which illustrates a liver cell nucleus from a field vole, *Microtus agresti.* A higher-power view of the Amphiuma nuclei [Fig. 8(b)] shows the large intranuclear granules in the euchromatin similar to the 400-Å granules in amphibian erythrocytes (Davies and Tooze, 1966). Linear structures were seen within the condensed chromatin parallel to the nuclear membrane, as described by Davies (1967).

DISCUSSION

The excessive amount of DNA present in each cell of Amphiuma may have arisen by one of three general mechanisms: (1) polyteny, (2) polyploidy, or (3) multiple localized gene duplications resulting from unequal crossing-over or related mechanisms. The appearance of the 28 Amphiuma chromosomes (Donnelly and Sparrow, 1965; Donnelly and Sparrow, 1963) is illustrated in Fig. 10. The relative size of the largest human

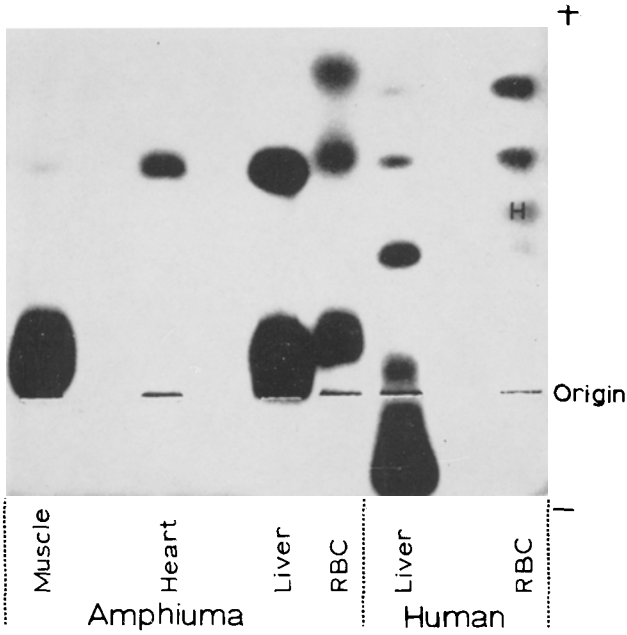

Fig. 5. Amphiuma and human lactic dehydrogenase (0.7 M tris, 0.5 M boric acid, 0.02 M EDTA, *p*H 8.6). Electrophoresis at 120 v for 15 hr. H denotes hemoglobin.

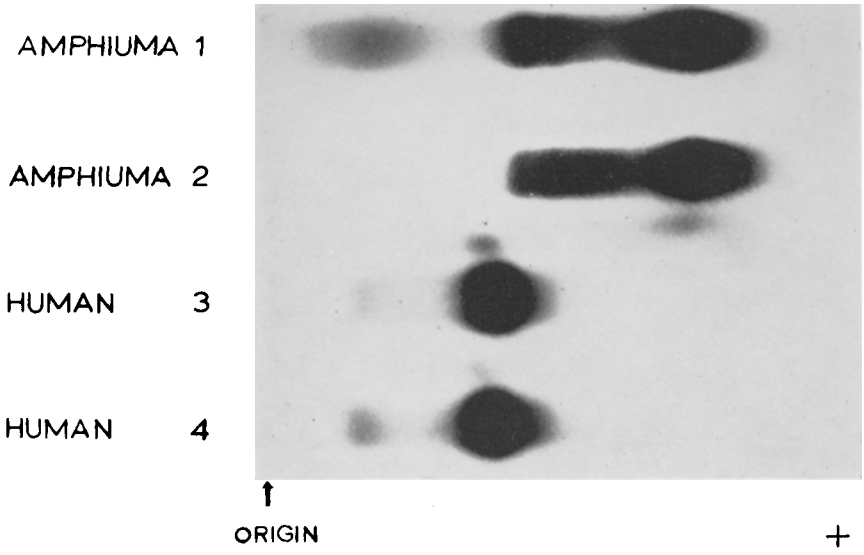

Fig. 6. Electrophoresis of Amphiuma and human hemoglobin (0.9 M tris, 0.02 M EDTA, 0.5 M boric acid, *p*H 8.6). Electrophoresis at 120 v for 15 hr. The gels were stained with benzidine. The hemolysates of Amphiuma 1 and human 3 were treated with iodoacetate according to the technique of Riggs (1965).

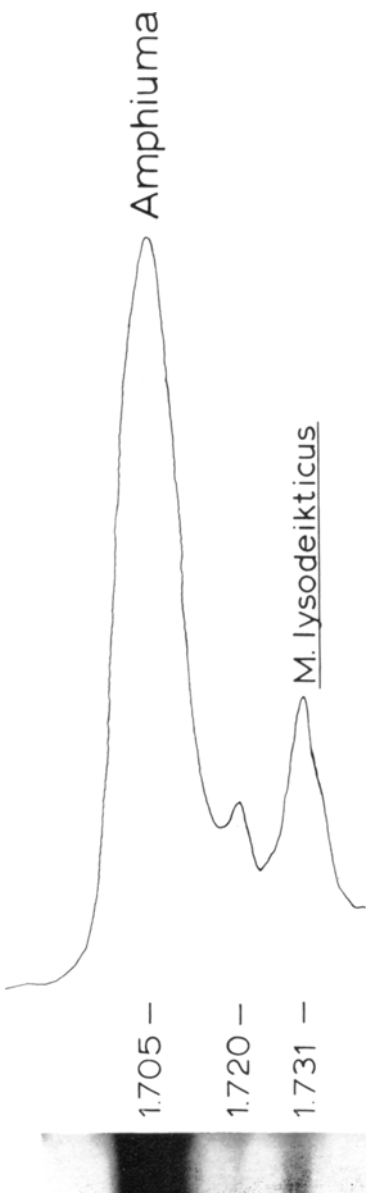

Fig. 7. Microdensitometry tracing of a UV photograph of an analytical ultracentrifugation of 8 μg/ml of Amphiuma red blood cell DNA and 1.0 μg/ml of *Micrococcus lysodeikticus*.

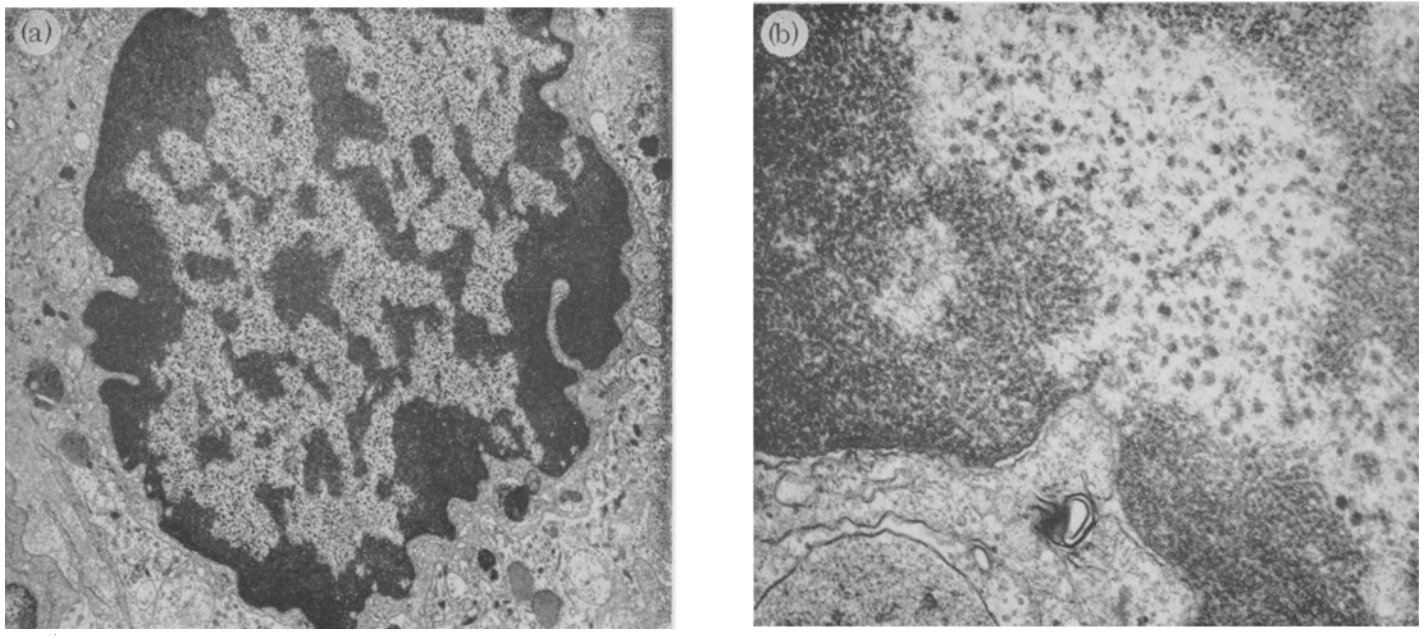

Fig. 8. Electron microscopy of liver nuclei of Amphiuma. (a) 5250 ×. A considerable amount of condensed chromatin is evident. (b) 37,000 ×. Large intranuclear granules are present in the euchromatin. Linear structures within the condensed chromatin parallel to the nuclear membrane as described by Davies (1967) are evident.

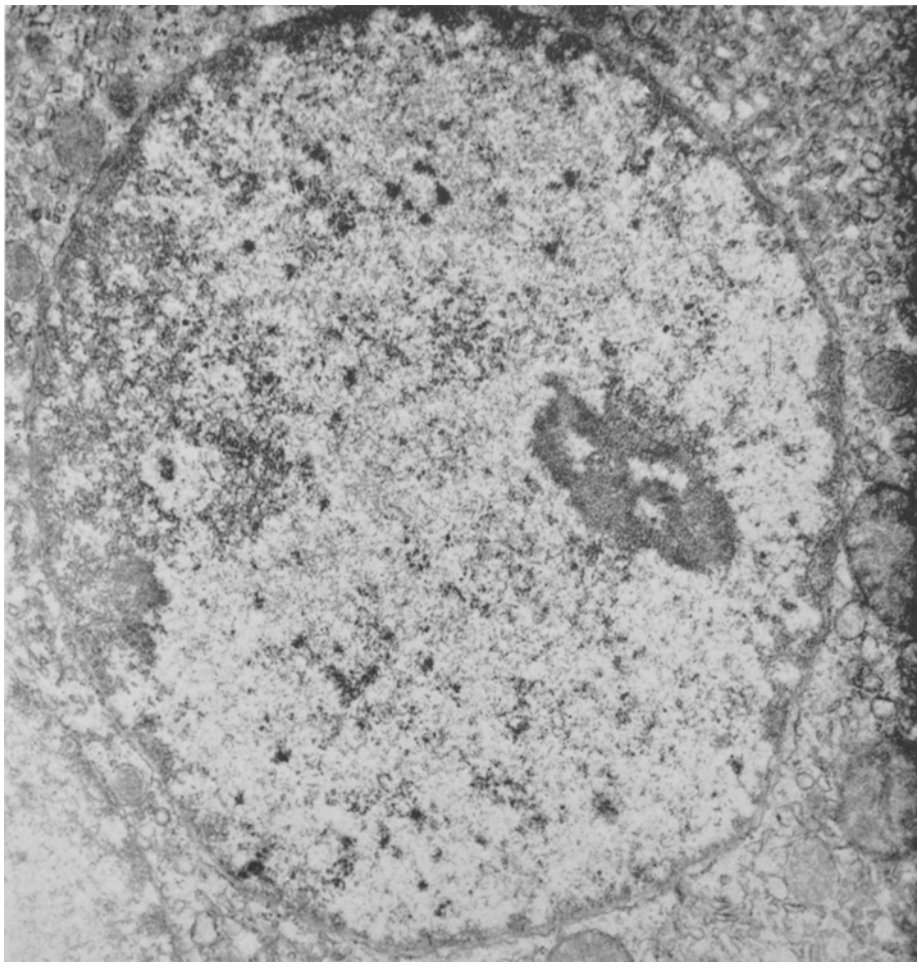

Fig. 9. Electron microscopy of a liver nucleus of the field vole *Microtus agresti*. Even though this organism has a large amount of X-chromosomal heterochromatin, the total amount of condensed nuclear chromatin is much less than in Amphiuma. 15,000 × ; reduced 35% for reproduction.

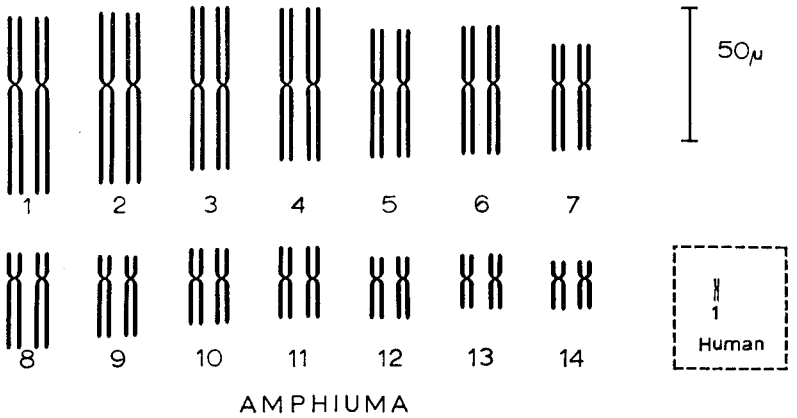

Fig. 10. Comparative size of the Amphiuma and human chromosomes. Redrawn from Donnelly and Sparrow (1963).

chromosome (#1) is shown for comparison. Amphiuma chromosomes have the appearance that might be expected if each chromosome contained an unusually long, single folded (DuPraw, 1965) DNA double helix. The total haploid length of an anaphase set of chromatids of Amphiuma is approximately 5.8 times that of the human, and the chromatid width is approximately 3.6 times that of humans. Thus, the increased length alone of the Amphiuma chromosomes can account for 5.8-fold of its 22-fold excess of DNA. Some increase in width of the chromatid would be expected on the basis of increased folding of a longer DNA helix. Although chromosomal proteins complicate these considerations, this comparative chromosomal geometry suggests there is room at most for a twofold increase in polyteny of Amphiuma over human chromosomes, and it is possible that there is no difference in the degree of strandedness of the basic chromosomal unit of these two species. If polyploidy was the only mechanism, then every gene would have been duplicated approximately six times, but the retention of the relatively low chromosome number would have required extensive concomitant chromosome fusion. Although localized gene duplication would be consistent with the relatively small number of very long chromosomes, both polyploidy and localized gene duplication were probably involved in the evolution of Amphiuma.

The results of this study can be most simply stated as follows: Of the systems that were tested in Amphiuma there was no evidence for unusual multiplicity of functional gene products. Although there are a number of possible reasons why this was so, two major ones are most apparent.

1. Although duplications of these genes occurred they were inactivated either by sustaining new mutations or by control mechanisms within the cell. The electron microscopy studies of the liver nuclei showed an amount of condensed chromatin which seemed proportionately greater than that seen in mammalian liver nuclei. This may represent some of the "excess" DNA in a genetically inactive state.

2. Only a portion of the genome underwent extensive gene duplication and that portion represented genes other than those studied. This would be consistent with the studies of Britten and Kohne (1966), which demonstrated that a large fraction of the DNA complement of all the higher organisms they studied (including Amphiuma) reassociated very rapidly, indicating a large population of repetitious sequences. These represented from 10,000 to 1,000,000 copies of sequences similar enough to reassociate with each other. Thus, a few sequences were copied many times (saltatory replication) but a majority of sequences were copied little, if at all. Britten and Kohne reported greater sequence similarity between related species in the portion of DNA containing repeated sequences than among the nonrepeated sequences. If the hemoglobin and enzyme proteins were coded by genes in the nonrepeated portion of DNA, multiple gene products would not be expected. The absence of multiple or abnormally large satellite bands indicates that if only a portion of the DNA underwent repetitious duplication, that portion did not possess a unique base composition.

The proportionate increase in the amount of gene product (Table I) implies that either a single gene is producing more gene product, or that the gene product is catabolized more slowly, or that a series of duplicated genes are producing an identical gene product. The latter possibility would be consistent with, but certainly does not prove, the possible existence of a master gene with multiple duplicated slave genes (Callen, 1967; Whitehouse, 1967).

REFERENCES

Atkin, N. B., and Ohno, S. (1967). DNA values of four primitive chordates. *Chromosoma* **23**: 1.

Atkin, N. B., Mattinson, G., Becak, W., and Ohno, S. (1965). The comparative DNA content of 19 species of placental mammals, reptiles, and birds. *Chromosoma Berl.* **17**: 1.

Birnstiel, M. L., Wallace, H., Sirlin, J. L., and Fischberg, M. (1965). Localization of the ribosomal DNA complements in the nucleolar organizer region of *Xenopus laevis*. *Natl. Cancer Inst. Monograph* **23**: 431.

Britten, R. J., and Kohne, D. E. (1965–66). Nucleotide sequence repetition in DNA. *Carnegie Inst. Wash. Year Book*, pp. 78–106.

Callen, H. (1967). The organization of genetic units in chromosomes. *J. Cell Sci.* **2**: 1.

Davies, H. G. (1967). Fine structure of heterochromatin in certain cell nuclei. *Nature* **214**: 208.

Davies, H. G., and Tooze, J. (1966). Electron- and light-microscope observations on the spleen of the newt *Triturus cristatus*: The surface topography of the mitotic chromosomes. *J. Cell Sci.* **1**: 331.

Donnelly, G. M., and Sparrow, A. H. (1963). Karyotype and revised chromosome number of *Amphiuma*. *Nature* **199**: 1207.

Donnelly, G. M., and Sparrow, A. H. (1965). Mitotic and meiotic chromosomes of Amphiuma. *J. Heredity* **56**: 91.

DuPraw, E. J. (1965). Macromolecular organization of nuclei and chromosomes: A folded fiber model based on whole-mount electron microscopy. *Nature* **206**: 338.

Glock, G. E., and McLean, P. (1953). Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. *Biochem. J.* **55**: 400.

Hopkins, D. A., Spencer, N., and Harris, H. (1964). Genetical studies on human red cell acid phosphatase. *Am. J. Human Genet.* **16**: 141.

Markert, C. L., and Faulhaber, I. (1965). Lactate dehydrogenase isozyme patterns of fish. *J. Exptl. Zool.* **159**: 319.

Mathai, C. K., Ohno, S., and Beutler, E. (1966). Sex-linkage of the glucose-6-phosphate dehydrogenase gene in Equidae. *Nature* **210**: 115.

Mirsky, A. E., and Ris, H. (1951). The deoxyribonucleic acid content of animal cells and its evolutionary significance. *J. Gen. Physiol.* **34**: 451.

Ohno, S., Klein, J., Poole, J., Harris, C., Destree, A., and Morrison, M. (1967). Genetic control of lactate dehydrogenase formation in the hagfish *Eptatretus stoutii*. *Science* **156**: 96.

Ohno, S., Wolf, U., and Atkin, N. B. (1968). Evolution from fish to mammals by gene duplication. *Hereditas* **59**: 169.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**: 208.

Riggs, A. (1965). Hemoglobin polymerization in mice. *Science* **147**: 621.

Schildkraut, C. L., Marmur, J., and Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4**: 430.

Smithies, O. (1955). Zone electrophoresis in starch gel, group variations in the serum proteins of normal human adults. *Biochem. J.* **61**: 629.

Vendrely, R. (1955). The deoxyribonucleic acid content of the nucleus. *In The Nucleic Acids*, Vol. 2, edited by E. Chargaff and J. N. Davidson, Academic Press, New York, pp. 155–180.

Wallace, H., and Birnstiel, M. L. (1966). Ribosomal cistron and the nucleolar organizer. *Biochim. Biophys. Acta* **114**: 296.

Whitehouse, H. L. K. (1967). A cycloid model for the chromosome. *J. Cell Sci.* **2**: 9.