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11. Hydration dates are figured at 260 years to the micron (to the nearest 1/10 micron). For consistency with the radiocarbon dates, the obsidian ages are subtracted from 1950 and are rounded off to the nearest 10 years.
12. The San Sebastian obsidian readings yield "short" dates, presumably because the specimens had been placed in a deep (5 m), sealed tomb shortly after their manufacture. Deep burial of this type preserves the obsidian in a more constant, somewhat cooler environment than that characteristic of surface sites and, as a result, the hydration rate of tomb obsidian is apparently considerably slower.

13. We are indebted to the radiocarbon laboratory at U.C.L.A. for the extensive set of radiocarbon determinations which made the present study possible, and to Rainer Berger and Ervin Taylor of that laboratory for their advice and assistance. Helpful comments on the manuscript were received from Clifford Evans, I. Friedman, and Joseph Michels. These studies were supported by NSF grant GS-911.

Assay Systems for the Study of Gene Function

Two assay systems, the syntheses of RNA and of protein, are described; their virtues and drawbacks are discussed.

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One of the major goals of developmental biology has been to find out how a cell of a higher organism acquires its structural and functional characteristics as it develops, and how the different cell types of an organism become different from one another during embryonic development. In recent years, the results obtained by molecular geneticists have had a strong impact on the thinking of developmental biologists. "The point of faith is: make the polypeptide sequences at the right time and in the right amounts, and the organization will take care of itself" (*1*).

All cell types do not contain the same array of proteins. Hemoglobin, to choose a well-known example, is present in red blood cells, but not in the cells that produce the black pigment of hair and skin. These pigment-producing cells in turn contain tyrosinase, an enzyme necessary for pigment formation; this enzyme is not found in red blood cells.

The simplest hypothesis to account for cellular specialization at this level of organization is that all genetic information does not effectively reach all cells. This concept of "differential gene function" dates back to Theodor Boveri (*2*). It could be due to at least three mechanisms. First, the different cell types could differ in their genetic constitution. If such differences were qualitative, this would mean that some cells contained some genes that were lacking in others. This is almost certainly not the case, for there are a number of instances where a population of already specialized cells can give rise, in the course of regeneration, to a variety of cell types (*3*). Also, when an individual cell nucleus is removed from a differentiated cell and placed into an egg, this artificial embryo can divide, grow, and develop into a mature organism containing all the cell types typical for a normal sib (*4*). But maybe genetic differences between cell types are quantitative only. In fact, it has been found that amphibian oocytes contain far more copies of the genes coding for ribosomal RNA than differentiated cells of the same organism do (*5*). It remains to be shown whether such quantitative differences also exist among nonribosomal RNA genes. Second, the genetic constitution of all cell types could be identical, but only those genes whose products are needed in a particular cell type would be transcribed. Thus, the various cell types would differ in the quality and quantity of RNA copies of the common DNA templates. Cellular differentiation in this case would result from a mechanism regulating genetic transcription. Third, the RNA copies produced during transcription could be utilized differentially; in this scheme, a given informational RNA copy would be translated in one cell, but not in another. Regulation of translation would therefore account for differentiation.

Probably more different routes of attack have been chosen than there are laboratories trying to unravel these fundamental regulatory processes. In this article we restrict ourselves to describing the experimental systems

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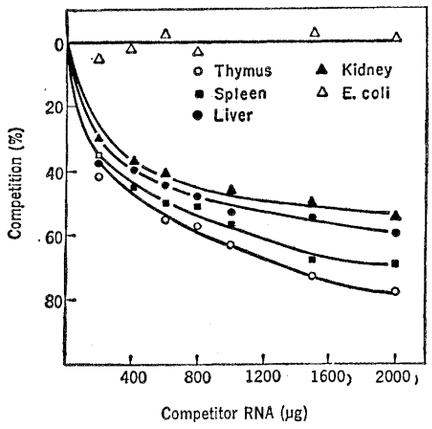


Fig. 1. Competition of unlabeled RNA's from various tissues with labeled RNA synthesized by isolated thymus nuclei. Reaction was between 40 μg of labeled RNA and 100 μg of DNA, for 18 hours, at 65°C, in 4 × SSC buffer (*22*). Percent competition refers to the amount of labeled RNA bound in the presence of unlabeled RNA, relative to that bound when no competitor RNA was added. [Adapted from (*6*)]

that have been used in our laboratory recently. We report some results that have already been obtained and then estimate how much additional information we may be able to get out of these assay systems in the future.

RNA Synthesis by Isolated Nuclei

In the first set of experiments we asked specifically whether an isolated cell nucleus expresses genetic activity qualitatively similar to that in vivo (*6*). Nuclei were isolated from steer thymus and liver, in a sucrose medium. These preparations consisted primarily of nuclei but were contaminated by small thymocytes; on the average, this contamination amounts to 3 to 4 percent. These nuclear preparations, in the presence of the RNA precursor molecule uridine (which was labeled with tritium) were incubated for RNA synthesis in a medium containing sucrose, divalent cations, and buffer. The antibiotics penicillin and streptomycin were also added, at concentrations high enough to minimize bacterial contamination. Incubation was continued for 30 minutes at 37°C, and the amount of RNA synthesized under these conditions was then determined from the radioactivity of material precipitable with trichloroacetic acid. The measured radioactivity was indeed contained in RNA. For when RNA from the incubation mixture was hydrolyzed in alkali, and the hydrolyzate was acidified with perchloric acid, neutralized, and chromatographed on a Dowex-formate column, close to 90 percent of the radioactive label was recovered as uridine monophosphate (UMP). Another way of demonstrating the RNA nature of the product was by elution from a hydroxyapatite column with different concentrations of phosphate buffer. The labeled material was eluted when the buffer concentration was 0.2*M*, which is characteristic for RNA; the material in this peak also showed a positive colorimetric reaction in the orcinol test, which specifically demonstrates the presence of RNA.

That the RNA made under these conditions originates in the isolated nuclei was demonstrated by autoradiography. When a photographic emulsion was spread over a microscopic preparation of nuclei from the incubation mixture and exposed in complete darkness, subsequent development of the emulsion showed silver grains over the nuclei (*7*). As expected, the contaminating intact thymocytes (3 to 4 percent) were radioactive also, but not more so than isolated nuclei. Consequently 96 to 97 percent of the RNA made was synthesized by the isolated nuclei.

The important aspect of the work now was to determine the kinds of RNA synthesized by isolated nuclei. Do these thymus nuclei in the test tube make RNA that they also synthesize in the intact tissue? Or, alternatively, are we simply observing general, nonspecific RNA synthesis? If not, can we tell the difference between RNA made by isolated thymus nuclei and that found in the liver nucleus? Techniques for DNA-RNA hybridization were used to find answers to these questions. These techniques measure the degree of complementarity of nucleotide sequences. Bovine DNA, made single-stranded by heating and rapid cooling, was baked onto membrane filters (*8*). Radioactively labeled RNA that had been synthesized by isolated thymus nuclei was extracted with hot phenol and detergent (*9*) and purified with deoxyribonuclease, pronase, and then by passage through a hydroxyapatite column. Such RNA was added to a solution that contained the membrane

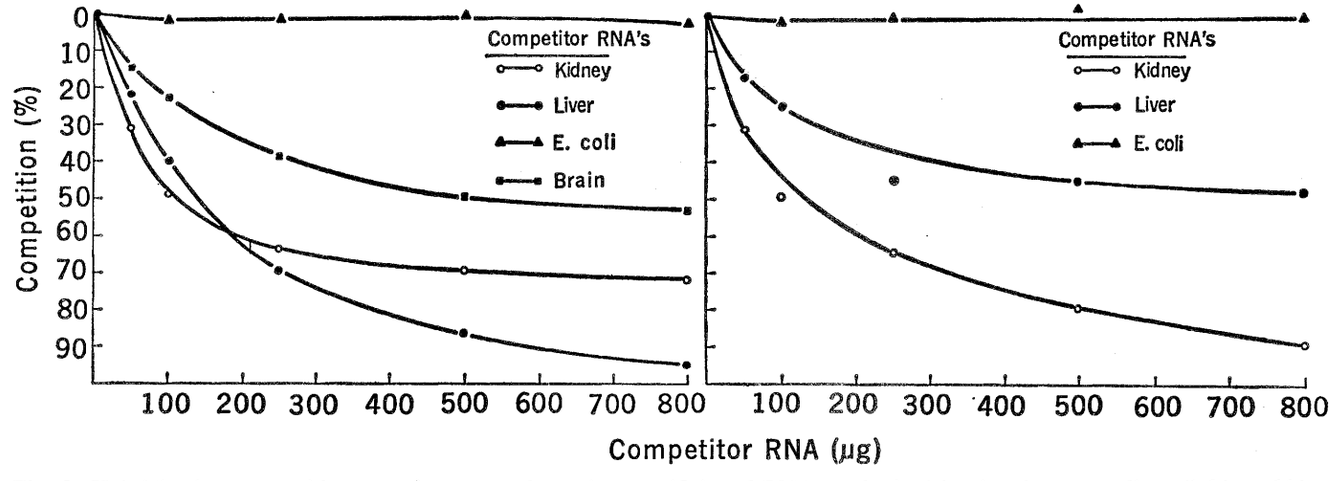


Fig. 2. Hybridization competition experiments to determine specificity of RNA synthesized in vitro by mouse liver (left) or kidney (right) chromatin. The four nucleoside triphosphates (H^3-cytidine triphosphate) were incorporated into high-molecular-weight RNA for 10 minutes in the presence of mouse RNA polymerase. RNA isolated from various mouse tissues or *Escherichia coli* was used as competitor in the hybridization of in vitro synthesized RNA to DNA. For hybridization, 12 μg of DNA, 20 μg of labeled RNA, and increasing amounts of unlabeled competitor in 0.2 ml of 2 × SSC at 67°C for 18 hours were used.

filters, and the hybridization reaction was permitted to go on for 18 hours, at 65°C. During this interval, radioactive nucleotide sequences in the RNA find their complementary sequences on the DNA and become trapped on the membrane filter. The amount of bound RNA can thus be measured by determining the radioactivity of the membrane filter. A modification of the same experiment can give information on the similarity of two preparations of RNA; the modified experiment is called a competition-annealing experiment. In this procedure, the radioactive RNA synthesized by the isolated nuclei was incubated with the DNA filters in the presence of unlabeled "competitor" RNA. If this competitor RNA has nucleotide sequences very similar to those of the labeled RNA, then a decreasing amount of radioactivity is found on the filters as one increases the concentration of unlabeled RNA, simply because unlabeled sequences also become bound to the DNA on the filter. On the other hand, if the nucleotide sequences of the competitor RNA are quite different from those of the labeled RNA, then the presence of the unlabeled RNA does not affect the binding of the labeled RNA to the DNA filters.

In our experiments, we prepared unlabeled RNA from thymus cells, for example. This RNA is a very effective competitor for the binding of RNA made by isolated thymus nuclei (Fig. 1), an indication that the RNA synthesized in vitro has similar nucleotide sequences to that found in yivo. RNA extracted from spleen, liver, kidney, and *Escherichia coli* are less effective competitors, in this order. Of the RNA made in vivo by thymus, that prepared from cell nuclei is a better competitor than that obtained from cytoplasm.

These findings make isolated nuclei an interesting assay system for the study of gene function. For they mean that cell nuclei retain some of their developmental bias even when separated from their cytoplasmic environment. Many examples of cytoplasmic influences upon activity of nuclei have been described. If we assume, for the moment, that differential transcription is a way in which genes are differentially expressed, and that it is regulated by cytoplasmic components, then the isolated nucleus can serve as an assay system for the interaction of nucleus and cytoplasm. We then could determine whether the source of cytoplasm—thymus or liver, for example—is decisive in determining the nature of RNA synthesized by isolated nuclei. We could test, under well-controlled conditions, how stable the developmental bias of a nucleus is, and whether it can be altered by foreign cytoplasm.

Although investigations of this nature are now technically feasible, there are a number of difficulties in the interpretation of results. How reliably may we conclude from competition experiments that RNA made by isolated thymus nuclei resembles RNA made by thymus in vivo? Recent research indicates that in the mammalian genome some annealing sequences are present in thousands, perhaps millions of copies (*10*). Other sequences occur in fewer, maybe only single copies. The conditions in our RNA-DNA hybridization experiments permit detection only of the redundant, or repetitive, portion of the genome, but not of that portion of the genome which occurs in only a few copies. Thus competition experiments may at present give only an incomplete picture of the similarity or dissimilarity of RNA from various sources. Furthermore the relation between "genes" and "annealing sequences" is not known in our system. Thus by demonstrating similarities in annealing properties, we do not really measure similarities in transcriptional activity. In other words, we have no way of identifying the biological function of the RNA that is singled out in the hybridization experiment. Thus, although gene action is measured at its first step, these experiments do not permit an identification of products of known genetic loci.

This reservation also applies to the next assay system to be discussed. In this, we asked ourselves whether chromatin, not only nuclei, retained its developmental bias after isolation (*11*).

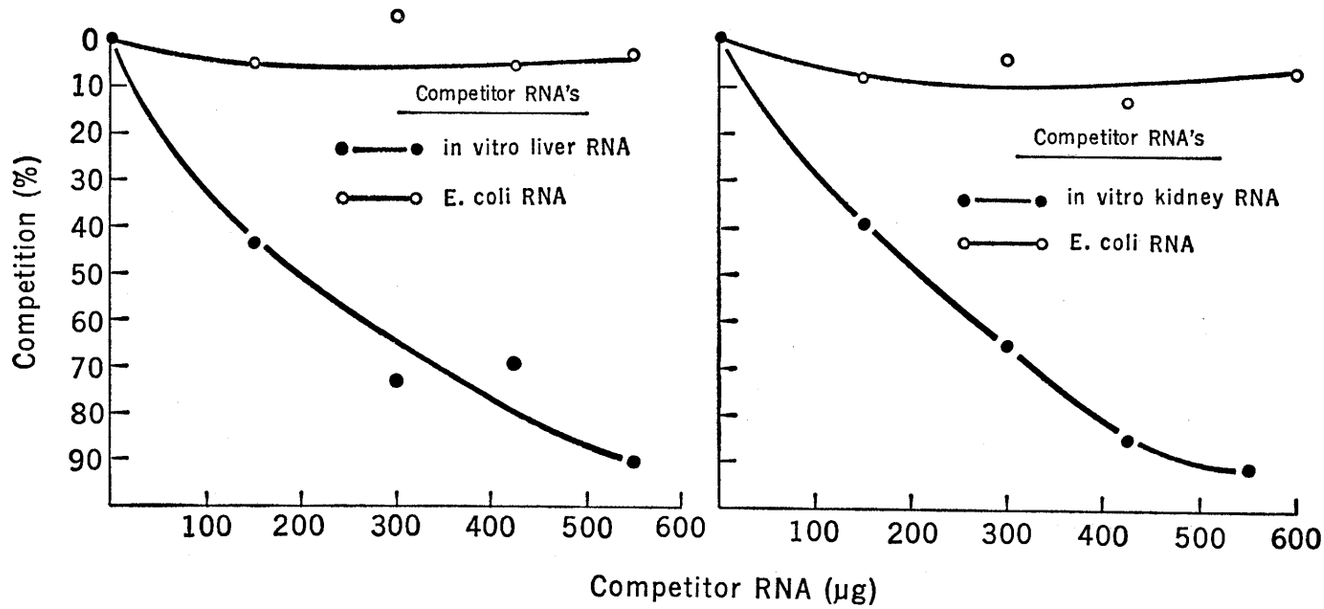


Fig. 3. RNA, labeled in vivo for 1 hour with phosphate, was isolated from mouse liver (left) and a kidney (right). RNA synthesized in vitro with liver (left) or kidney (right) chromatin was used as competitor for the hybridization of the in vivo labeled RNA to DNA. Hybridization was with 20 μg of DNA, 10 μg of labeled RNA and increasing amounts of unlabeled competitor RNA in 0.2 ml of 4 × SSC at 65°C for 18 hours.

RNA Synthesis by Isolated Chromatin

These experiments were done in collaboration with B. J. McCarthy and R. B. Church of the University of Washington. Labeled RNA was synthesized in vitro, with chromatin from liver, kidney, or spleen as a template in the presence of exogenous RNA polymerase. Unlabeled RNA, isolated from either intact liver, kidney, spleen, lung, brain, or *Escherichia coli* was used as competitor. In each case the RNA from the tissue homologous to the chromatin was the most effective competitor. RNA from the heterologous tissues gave only partial competition (Fig. 2). These finding agree with those obtained with RNA labeled in vivo (*12*). Since the competition by the RNA in vivo is essentially complete, the synthesizing system in vitro has not generated any RNA molecules which are not normally present in the intact tissue. That a complete array of RNA molecules is synthesized in vitro is shown in Fig. 3. Unlabeled RNA synthesized in vitro from either a kidney or a liver chromatin template was used in competition for either kidney or liver RNA labeled in vivo. Again, the homologous RNA gives the most effective competition as expected. Since competition in this case is also essentially complete, there is no RNA present in the intact tissue which is not also synthesized in the synthesizing system in vitro. Similar findings have been reported by others (*13*).

Thus, within the limits of our hybridization assay, the template specificity associated with chromosomes in vivo is retained by isolated chromatin. From these results, it seems clear that the structural organization of the chromosome accounts for the specificity of transcription associated with specific cell types, and that this system will provide an excellent assay for investigating the mechanism involved in establishing and maintaining this specificity.

The major difficulty in all the foregoing experiments is the evaluation of the biological function of the molecules whose synthesis is being measured. In this sense the study of genetically controlled enzymes is more meaningful.

Gene Function Measured at the Protein Level

When Beadle (*14*) formulated the "one-gene one-enzyme" theory, it immediately became clear that the study of proteins in differentiating organisms would be a way for ascertaining the functioning of genes in development. However most of the organisms used in embryological research did not lend themselves readily to genetic analysis. Yet a genetic analysis of proteins during development seems promising, especially in organisms with giant chromosomes in which genes are cytologically manifested. In such cases it is possible to study the relationship between the appearance of a protein in the cell and the transcriptional activity of its structural locus on the chromosome (*15*).

With this in mind we have screened the fruitfly *Drosophila* for enzymes that are suitable for genetic and developmental study. A prerequisite for cytogenetic analysis is the availability of structural enzyme mutants. If these are found, the locus responsible for the production of the enzyme can be determined on the genetic map by appropriate crosses, with the use of the techniques of Mendelian genetics. This has recently been carried out for several enzymes. Figure 4 shows two electrophoretic forms of the enzyme aldehyde oxidase, discovered in two different *Drosophila* species. The offspring of a cross between these two species contains three forms of the enzyme, the two parental forms and a third, hybrid enzyme. The method of detection of the enzyme phenotype is simple and sensitive enough to permit analysis of a single fly. A fly is homogenized in a few microliters of buffer, applied to the origin of an agar gel supported on a glass plate, and subjected to electrophoresis for some 20 minutes. The gel is then immersed in a histochemical staining mixture containing all the components necessary for the enzymatic reaction of aldehyde oxidase to occur. One of the products of this reaction is used to reduce and precipitate the dye in the reaction mixture. Thus formazan, the purple product, is deposited only at those regions of the gel that contain aldehyde oxidase, when aldehydes are used as substrates. If, on the other hand, an alcohol is used as the substrate, alcohol dehydrogenase (ADH) can be visualized. As seen in Fig. 5, mutants were also found for ADH, and again hybrid flies were readily detected upon electrophoretic analysis. In this case, the structural locus was determined to be at 50.1 on the second chromosome, in the region 34 E3/35 D1 of the cytogenetic map (*16*) (Fig. 6). A different enzyme, octanol dehydrogenase, was mapped at 49.2 on the third chromosome, the same chromosome that also carries the locus for aldehyde oxidase, although in a different position (*17*).

Stage-Specificity of Proteins

With respect to the ontogeny of enzymes, our attention has been focused on ADH and aldehyde oxidase. Both of these enzymes undergo fluctuations during development. For this study it was necessary to grow *Drosophila* in synchrony, and then to harvest larvae,

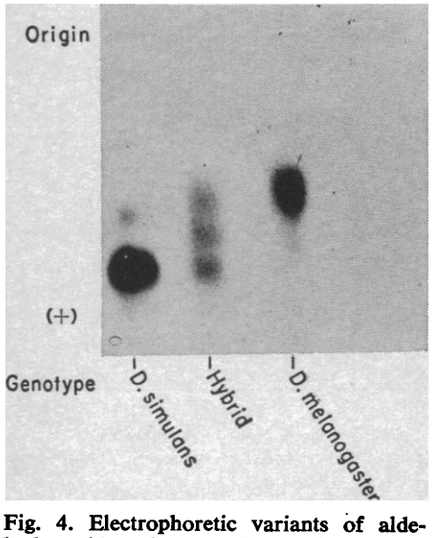


Fig. 4. Electrophoretic variants of aldehyde oxidase in two *Drosophila* species. [Adapted from (*18*)]

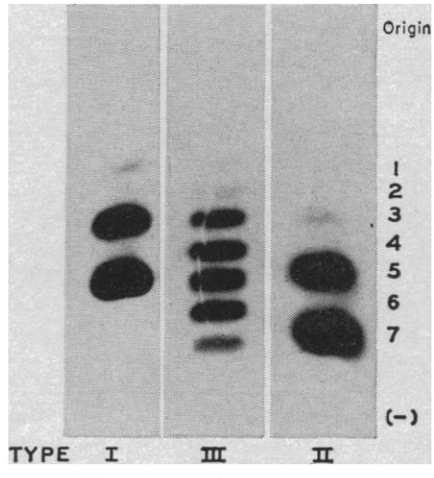


Fig. 5. Electrophoretic variants of alcohol dehydrogenase in *Drosophila melanogaster*. Type III is a hybrid offspring from a cross, type I × type II. [From Fig. 1, Ursprung and Leone (*16*)]

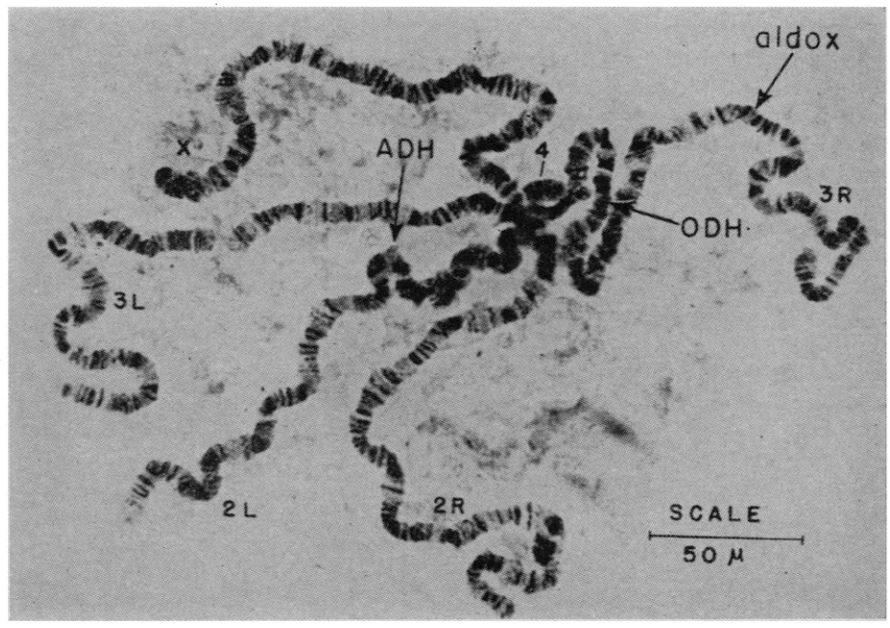
Fig. 6. The salivary gland chromosomes of *Drosophila melanogaster*, with an approximate indication of the structural loci for alcohol dehydrogenase (ADH), aldehyde oxidase (aldox) and octanol dehydrogenase (ODH). Photograph of chromosomes, a modified version of Fig. 9 in *Drosophila Guide*. [M. Demerec and B. P. Kaufmann (Carnegie Institution of Washington, D.C., ed. 8, 1967)].

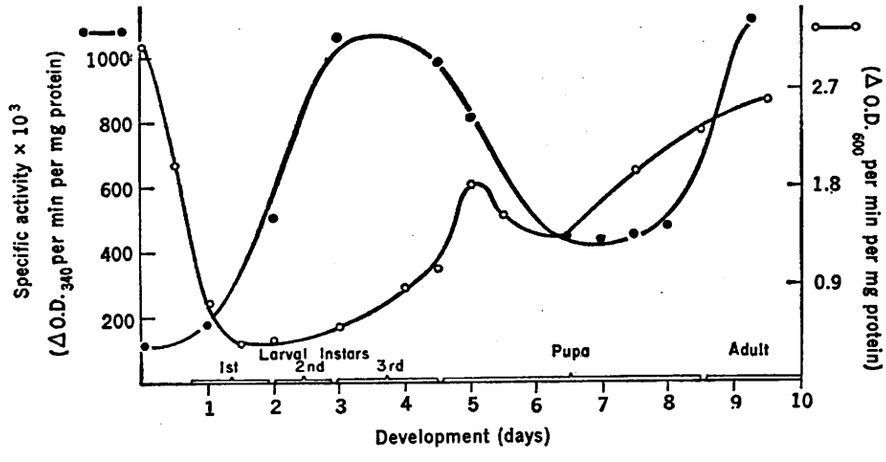
Fig 7. Development of alcohol dehydrogenase (solid circles) and aldehyde oxidase (open circles) in *Drosophila melanogaster*. The data for aldehyde oxidase were made available by W. J. Dickinson (unpublished).

pupae, or adults at different stages of their development. The organisms were homogenized, and both enzyme activity and protein concentration were determined. Figure 7 shows the fluctuation of the specific activities of ADH and aldehyde oxidase during normal development. Both enzymes undergo fluctuations in specific activity, but the developmental patterns of the two enzymes are very different. Throughout larval life ADH increases steadily, decreases sharply after the formation of the pupal case, and rises again late during metamorphosis and after hatching of the adult fly.

This time course suggests that ADH is synthesized throughout the larval stages, but that it is then degraded rapidly during early pupal life and again synthesized toward the end of metamorphosis. We do not yet know whether this is indeed the case. But we have tools available to test this hypothesis. Large quantities of flies were grown in culture boxes, collected, and homogenized; and ADH was purified to the stage where it migrates as a single band in ultracentrifugal analysis and in acrylamide-gel electrophoresis. This preparation was used for producing antibodies directed specifically at ADH. If we now raise *Drosophila* at given developmental stages on food containing radioactive amino acids, the newly synthesized protein in those insects will become labeled. We can single out ADH from among the radioactive proteins contained in a crude fly homogenate by antibody precipitation. In this manner, it should be possible to obtain an answer concerning the rates of synthesis of ADH during development.

It will be interesting to carry out the exact same set of experiments on aldehyde oxidase, which has a different





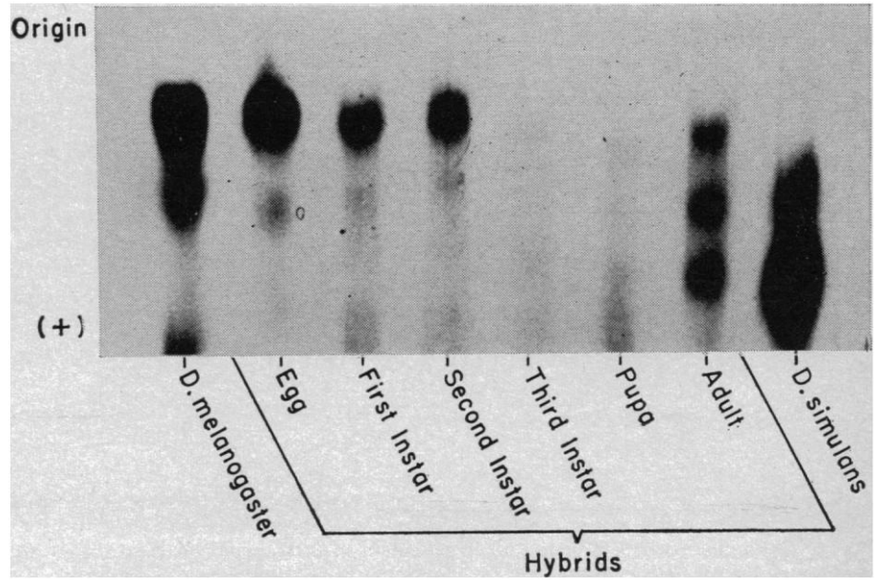


Fig. 8. Maternal transmission of aldehyde oxidase in an interspecies cross between *Drosophila melanogaster* (♀) and *Drosophila simulans* (♂). The enzyme present in the egg is of the maternal type. [Adapted from (*18*)]

time course of specific activities (Fig. 7). Its specific activity is very high in the egg, drops sharply, and only begins to rise toward the end of larval life. We already have a partial explanation for the sudden drop early in development. When the progeny of a cross of the two structural aldehyde oxidase mutants was analyzed by gel electrophoresis (*18*), it became immediately apparent that the egg enzyme was entirely of the maternal type (Fig. 8). Only much later was the paternal type expressed. Most likely, synthesis *de novo* of aldehyde oxidase starts much later than ADH synthesis. But again the more critical antibody technique of labeled protein precipitation will have to be used before we can draw this conclusion more firmly.

Tissue-Specificity of Proteins

These results will not be very illuminating with respect to the problem of cellular differentiation, since homogenates of entire organisms are used. But we already know the tissue localization of several enzymes. For example, ADH occurs at high concentration in the fat body of *Drosophila*; some is also found in the Malpighian tubules, but none in the salivary glands, the brain, or the imaginal disks. The latter fact is particularly interesting. Imaginal disks are the larval primordia of most adult structures of flies. The genital imaginal disk is essentially a simple epithelium, from which in normal development a variety of organs are formed: a sperm pump, two paragonia, a ductus ejaculatorius, a piece of the intestine, and the external genitalia. These organs are characterized by cells of different structure and function. The adult genital apparatus does contain ADH. At what developmental stage the enzyme is first made in the various cell lines leading from the primordium to the finished organ is not yet known, but newly developed techniques of electron-microscope histochemistry will give us this information (*19*). We already have good reason to believe that an increase of synthetic activities in the imaginal disk cells occurs shortly after pupation. Electron microscopy of sections through various stages shows that the incidence of rough endoplasmic reticulum increases as development proceeds (*20*) (Fig. 9). Study of the appearance of enzymes in various cell lines derived from larval primordia will enable us to establish catalogs of biochemical events associated with cellular differentiation. Those tissues containing giant chromosomes should permit us to ascertain whether and when RNA synthesis occurs at the cognate genetic loci.

With such facts at hand, we have a handle for studying the next important question, that of regulation. Why does a fat-body cell contain or synthesize ADH, but not a salivary gland cell? Would a nucleus of a salivary-gland cell produce the RNA necessary for specifying the synthesis of ADH, if it were guided by fat-body cytoplasm? In other words, is the primary gene function controlled by interaction of nucleus and cytoplasm? In elegant experiments on hybridization of somatic cells in vertebrates (*21*), there is evidence that the cytoplasm does indeed exert a regulatory effect on the expression of genetic information. Here again, for studying genetic activity at known structural loci, the choice of *Drosophila* as an experimental system has unique advantage provided that *Drosophila* cells lend themselves to somatic hybridization.

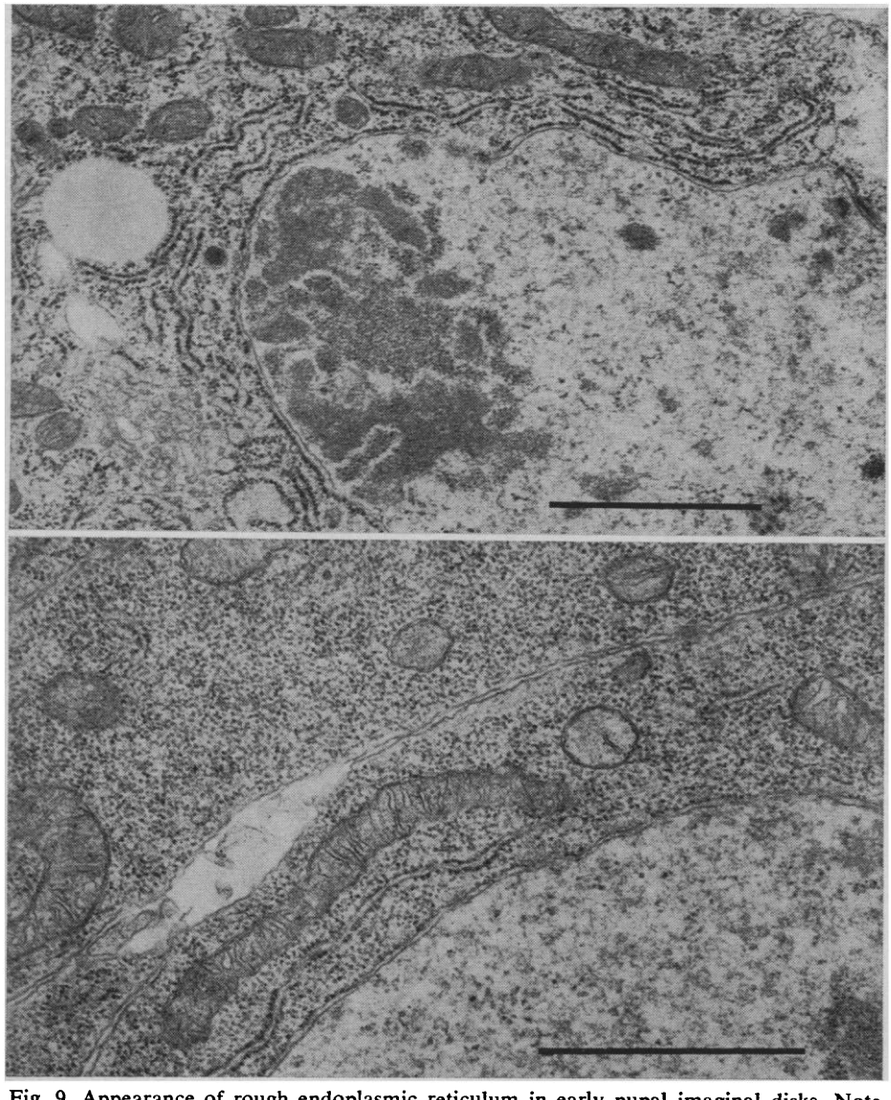


Fig. 9. Appearance of rough endoplasmic reticulum in early pupal imaginal disks. Note the predominantly free ribosomes in the younger disk (bottom, fixed 75 minutes after puparium formation) and the abundant rough endoplasmic reticulum in somewhat older disks (top, fixed 6½ hours after puparium formation). The bars represent 1 μ. [From (*20*)]

Summary

The study of genetic regulatory mechanisms operating in plants and animals is of paramount importance in contemporary biology. A precise understanding of the mechanisms that underlie normal cellular differentiation is a prerequisite for understanding neoplastic transformation and genetic disease. At present, we are not aware of a single assay system that can give

answers to all questions we are already able to pose. Studies of RNA synthesis are valuable because they provide a direct measurement of transcriptional activity. But these studies remain incomplete until we succeed in unraveling the metabolic roles of the molecules whose synthesis we study. In this respect, the study of enzyme synthesis represents a better defined assay system, although the interpretation of observed fluctuations in synthetic rates is made difficult by the many steps that intervene between the genes and their finished protein products. We propose that a combination of protein biosynthetic and cytogenetic analysis is a promising assay system for further investigation.

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23. Supported by NSF grants GB-4451 (H.U.), GB-5629 (K.D.S.), PHS grants 1-F2-GM-24, 115 (W.H.S.) and 1 TI HO-139 (D.T.S.).

Dental Research: The Past Two Decades

National Institute of Dental Research interdisciplinary programs have broadened the base of dental science.

Alvin L. Morris and Richard C. Greulich

Twenty years ago an act of Congress created the National Institute of Dental Research (NIDR) in recognition of the fact that dental disease is a threat to the nation's health, well-being, and productivity. In the intervening period there has evolved a generation of dental practitioners and researchers whose concepts of dental disease, dental practice, and related research are so modified as to add an entirely new dimension to their functional role as health specialists. In earlier efforts of the profession to establish a separate identity in the field of health, the mouth was considered, mistakenly, a biologic entity. Largely through the influence of NIDR, dental science has moved from a mechanical to a biological orientation, and the oral cavity is again looked upon as an integral component of the body. In the process, by shedding a narrow identification and aligning itself with a broad front of basic sciences, dental science has made exciting progress toward an understanding of oral disorders. At the same time, it has increasingly provided information of fundamental importance to other fields of scientific endeavor. The occasion of the 20th anniversary of the creation of NIDR, the major source of support for oral health research and research training in the United States, affords an opportunity to assess where the dental sciences stand today in a broad spectrum of research whose full import is yet to be realized.

The Scope of Dental Sciences

It is not possible to accurately define the boundaries of dental research. While there are those who, in the past, have related it only to investigations whose titles include the words *tooth* or *mouth*, such a restricted viewpoint is totally inappropriate today. The biochemist studying the cross-linkage of collagen, the microbiologist studying the polysaccharide coating of streptococci, the metallurgist investigating the phenomenon of corrosion, the crystallographer examining the structure of the apatite crystal are all engaged in work of vital interest to dentistry. This statement can be expanded to include the research of scientists from 30 discrete disciplines.

That research involving the oral cavity must inevitably implicate a wide range of science disciplines is obvious when the total oral environment is considered. Anatomically the mouth includes all basic tissue elements. The penetration of the teeth through the oral mucosa is the only example of the loss of continuity of the protective layer of skin or mucosa which lines all body surfaces or cavities. The nature of the union between the soft tissue of the oral cavity and the tooth structure in an erupted tooth is anatomically unique, as is the suspensory ligament by which the tooth is anchored in bone. The salivary glands, functioning under neural and endocrine control, excrete each day approximately 1500 milliliters of fluid containing minerals, glycoprotein, and enzymes. There is evidence in animals which suggests an endocrine function for these glands. The oral cavity has a rich microflora which includes bacteria with a full range of oxygen dependency. Commonly present in the oral cavity are various dental-restorative

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