Biological Specificity and Growth

EDITED BY ELMER G. BUTLER

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FOREWORD

THE Society for the Study of Development and Growth annually conducts a symposium which brings together for several days a group of active investigators to discuss important problems in the field of growth. Participants represent many scientific disciplines, including not only biology but also the related fields of chemistry, physics, and the medical sciences. Guest speakers present prepared papers in which they report recent research in their laboratories, review progress in the general area with which they are concerned, and often point the way to fresh interpretations and new paths of investigation. Problems of both abnormal and normal growth receive attention. So far as possible, the symposia are conducted in an informal manner; vigorous discussion in the lecture hall is often continued at the dinner table and later in informal groups on the grass under the trees. The prepared papers, frequently revised in the light of discussions at the time of the symposium, become the chapters of the symposium volume.

The topic chosen for the Twelfth Growth Symposium was *Biological Specificity and Growth*. In developmental biology the phenomenon of specificity occupies a prominent position. When it can be determined that interactions among components of a biological system are such that specific activities occur, or when a particular stimulus, internal or external, evokes a specific response, it becomes of considerable importance to discover the basis for the selectivity and specificity concerned. It is with an examination and evaluation of the role of specificity in growth and differentiation that this volume deals. The participants in the symposium have approached the problem at different levels of organization and by the use of a variety of biological materials and techniques. Taken together the chapters of this volume represent an assessment of our present knowledge of the subject.

This symposium was planned jointly by the Society for the Study of Development and Growth and the Committee on Developmental Biology of the National Research Council. It was held at the University of New Hampshire, Durham, June 14-22, 1953. Grateful acknowledgment is made to the members of the biological faculty and to the administration of the university for their hospitality and for the excellent facilities they so generously provided. Financial support which made the symposium possible was received from the American Cancer Society, acting through the Committee on Growth of the National Research Council; the National Cancer Institute of the National Institutes of Health; the National

Science Foundation; and the Committee on Developmental Biology of the National Research Council.

The appearance of growth symposia in book form began with the Eleventh Symposium on the *Dynamics of Growth Processes,* published by the Princeton University Press in 1954. Earlier symposia appeared as supplements to the journal *Growth.* Limited quantities of these supplements are still available from the editors of that periodical.

All of the initial editorial work on this volume was carried out by Dr. David R. Goddard, University of Pennsylvania. Unfortunately, because of the press of other duties he was prevented from continuing with the editorship. Dr. John T. Bonner, Princeton University, has rendered considerable assistance at various stages in the preparation of this book. Thanks are also due to Miss Suzanne H. Eldredge, Princeton University, for continued assistance with manuscript and proof. All those concerned with the preparation of this volume acknowledge with gratitude the cooperation of Mr. Herbert S. Bailey, Jr., of the Princeton University Press.

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Princeton University
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BIOLOGICAL SPECIFICITY AND GROWTH

I. ONTOGENY OF THE BLOOD AND RELATED ANTIGENS AND THEIR SIGNIFICANCE FOR THE THEORY OF DIFFERENTIATION

BY A. M. SCHECHTMAN[1]

I. THE NATURE OF IMMUNO-EMBRYOLOGICAL INVESTIGATION

ALL MODERN HYPOTHESES concerning differentiation mechanisms are concerned in the last analysis with molecular specificity. It is interesting to note that most of our recent hypotheses concerning the creation of new molecular specificities stem from work done in other fields of biology which just a few years ago had no apparent relationship to embryonic development. These hypotheses suggest possible means by which molecular specificities may be altered, abolished, or created. Some, like the concept of antibody formation applied to development, have already stimulated experimentation. Others, like the concept of adaptive enzymes, have hardly advanced beyond the "talking stage" insofar as embryonic differentiation is concerned. The speculations of Weiss (1947), Tyler (1947), Brachet (1949), Sonneborn (1947), Schultz (1952), and others have helped greatly to clarify the essence of the problems of differentiation as well as to suggest processes which possibly go on within the embryo. The purpose and design of experimental work for many years to come will doubtlessly spring from these speculations.

The patterns of molecular specificities, established by means as yet highly hypothetical, constitute the groundwork of differentiation and are expressed according to the means of analysis at our disposal: as morphological differentiation, biochemical change, specific physiological functions, embryonic competences, etc. With such assumptions as to the fundamental nature of differentiation, it is little wonder that immunological phenomena have become of greater interest to the embryologist as well as to others concerned with the creation of new features by living materials. A process which permits the production of apparently endless numbers of specific molecules in the form of antibodies, and these specificities resident in protein molecules which may be chemically identical, would seem to have possibilities leading toward the understanding of causal factors in the production of new features in the developing organism.

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However, it must be said that during the past fifty years or so in which it has been applied to developmental problems, immunology has been used largely as a tool for the investigation of chemical epigenesis rather than the causes of epigenesis. In this respect it has been used like the microscope, which revealed the details of morphological epigenesis. Immuno-embryology has shown little of the approach characteristic of *Entwicklungsmechanik,* which deliberately seeks causal factors in epigenesis. The time of appearance or disappearance of antigens and the occurrence of certain antigens throughout the life history of the organism are descriptive chemical embryology and are equivalent to studies on the appearance or disappearance of morphological structures such as the notochord or pronephros. Of themselves they suggest, but they do little to elucidate the mechanisms by which specificity is established. Such studies certainly give us important correlations: an antigenic change precedes or is concomitant with a morphological change. I have no intention of depreciating the significance of these studies. The application of immunology to chemical epigenesis is a vital part of embryological investigation because (1) it demonstrates an order of chemical specificities which thus far are not detectable by any other methods and (2) it brings us closer to differentiation on the molecular level. A start has been made in the application of immunology to causal aspects of development in such experiments as those of Weiss (reviewed, 1950) and Ebert (1950). However, it is important to realize clearly the nature of the results thus far obtained by this method as it has been applied in experimental work. Only then can we apply immunological concepts and methods toward causal analysis of development.

II. THE SIGNIFICANCE OF MACROMOLECULAR TRANSFER

The main objectives of the present discussion are to show (1) that molecular epigenesis occurs during development and (2) that the new macromolecules which appear are not necessarily the products of the embryo's own synthesis. The specific data cited are largely derived from studies of the embryonic blood and related antigens. These provide the clearest though not the only evidence that new molecules like new morphological structures make their appearance during the course of development. It is equally clear that the *de novo* appearance of antigens or other molecules tends to create a false impression of the synthesizing capacities of the embryonic organism. The embryo is definitely deficient in some types of molecular synthesis and is dependent upon presynthesized molecules supplied normally by the maternal body. Of course it is obvious

that in one way or another the organic materials required by the early embryo are of maternal origin. But it has been assumed generally that the materials transmitted are of a rather simple nature, the kind that readily pass through the plasma membrane of most adult cells. It has been assumed further that such small molecules are nothing more than simple structural units or sources of energy which provide some of the "conditions of life" that permit the embryo to elaborate its own specific macromolecules. On this basis the mechanisms of differentiation reside wholly in the embryo. The situation assumes a totally different aspect when we realize that *the embryo admits complex macromolecules and that such admission occurs prior to and during the time when the basic differentiations of the vertebrate body are established.* We have, therefore, the essential relationships observed in certain microorganisms in which a macromolecular constituent, apparently desoxyribose nucleic acid, induces a permanent alteration in the synthesizing capacities of the organisms (McCarty and Avery, 1946, on *Pneumococcus;* Boivin et al., 1945, on *E. coli*). However, the great distinction between the status of bacterial transformation and embryonic differentiation lies in the fact that there is good evidence for a causal relationship between the macromolecule and transformation, whereas the uptake of macromolecules and embryonic differentiation have not yet been related causally.

In the following we shall refer to macromolecular substances elaborated by the embryo as autosynthetic and to those produced by the adult organism and transferred to the embryo as heterosynthetic.

III. EPIGENESIS OF RED BLOOD CELL ANTIGENS

It is a curious fact that embryologists are not inclined to attribute causal differentiative roles to the antigens of the red blood corpuscles. On the other hand it has been assumed that antigens appearing, for example, during the development of nervous, spleen, or cardiac tissue may have some causal role in the differentiation of these tissues. The reasons for this discrimination seem to be that blood cells differentiate whether or not they contain certain specific antigens; in addition much of the available evidence has indicated that the antigens are present from the earliest stages studied and therefore must be independent of morphological or physiological differentiation. Thus the A and B antigens were reported in the 37-day human fetus (Kemp, 1930), the Rh antigens in the fetus weighing only 8 grams (see Levene, 1948). Bornstein and Israel (1942) found agglutinogens A,B,M,N, and Rh positive in 7-21 cm. human fetuses, and Keeler and Castle (1933, 1934) found two anti-

gens (H_1 and H_2) in the nucleated red cells of the 4 mm. rabbit embryo.

However, there are indications that certain of the human antigens are not static but undergo change in development. Thus the A and B antigens seem to be "incompletely developed" in the red cells at birth (Wiener, 1946). If we are looking for correlations we have one in this instance, for the maturation of the A and B antigens occurs concomitantly with the gradual disappearance of fetal hemoglobin and the rise of adult hemoglobin. The Lewis factor (Le^a) also apparently differs in the infant and adult. Antisera react with the adult factor only if it is in homozygous cells whereas the factor is detectable in both homozygous and heterozygous infants (Andresen, 1947).

Epigenicity is more clearly discernible in certain blood cell antigens of birds. Certain chicken red cell antigens are present in very early development whereas others do not appear until after hatching (Briles, McGibbon, and Irwin, 1948). Six alleles of the B series of antigens occur in the 3-day embryo. In the D series one factor is found in the 3-day embryo whereas three others are detectable only after hatching and may then undergo change until they reach the degree of agglutinability found in the adult. In *Columba guinea,* Miller (1953) found the antigens A,B,C,E, and F in 29-somite embryos at 68-72 hours of incubation. However, embryonic cells seem to be slower to agglutinate. The A and F antigens appear to develop gradually after hatching, for the cells of squabs containing A and F tend to show weaker agglutination and titer than the adult cells. Burke et al. (1944) prepared antisera against adult chicken red blood cells and carried out complement fixation tests with blood cells from successive stages of embryonic development. Complement was first fixed by cells from the 96-hour embryo. Unfortunately, interpretation of the latter results is doubtful since the data and experimental conditions are presented in very brief form and it seems that individual blood groups were not taken into consideration. Indeed the comparison of adult and embryonic cellular antigens is beset with technical difficulties because of the high degree of antigenic individuality of each bird and the fact that samples of blood are not taken from the same individual embryos. It has been possible to distinguish each chick in three different families of birds by means of the absorptive qualities of their red cells. Various aspects of blood cell serology in genetics and embryology are available in reviews by Irwin (1949, 1951).

Since the present evidence shows that at least some of the red cell antigens are epigenetic, we have no good reason for considering them any more or less significant as possible factors in differentiation than the

antigens arising in other tissues or at certain stages in the development of the sea urchin, frog, and chick. Moreover the blood cell antigens show the same general features as other tissue antigens. Some (like Rh) are specific to the red cells, whereas others (like A and B of man and K_5 of rabbits) are held in common with other tissues. Of course on the mere grounds of epigenicity and distribution we have no reason for attributing to them any causal role in the differentiation of the cells, but this applies with equal force to other antigens shown to arise during differentiation. In the case of the red cells, however, we are aware of a causal factor in the differentiation of the antigens. *The relationships of the red cell antigens to genic factors suggest that they are end products of differentiation rather than parts of the mechanism of differentiation.* We have at present no reason to assume that any other antigen of development is any different.

IV. EPIGENESIS OF HEMOGLOBINS

Chemical epigenesis has no better illustration than the hemoglobins. The first hemoglobin to appear is certainly a product of the embryo's own synthesis (autosynthetic), since unincubated eggs contain no iron heme (Hill, 1931). Ramsay (1951) states that nonheme iron accounts for 99 to 100.5 per cent of total iron in unincubated eggs. After the formation of this first hemoglobin there is a gap in our knowledge, for most of the work on fetal hemoglobins of vertebrates begins at the midfetal period. Within this advanced period in man, sheep, goat, cattle, and rabbit there is a large body of evidence for the existence of a fetal type of hemoglobin which is lost sometime after birth, and an adult type which gradually replaces it. The fetal type, at least in man, can be distinguished serologically from the adult type (Darrow, Novakovsky, and Austin, 1940; Chernoff, 1952). In addition the two hemoglobins can be distinguished on the basis of their solubility-temperature curves, their different crystal systems, electrophoretic mobility, amino acid composition, oxygen dissociation, resistance to alkaline denaturation, and other properties (Roughton and Kendrew, 1949, and Kendrew, 1949). The existence of an embryonic hemoglobin has not been shown in the chicken, but the studies of Hall (1934-1935) and Boyer (1950) on O_2 affinity and O_2 consumption of embryonic red cells are suggestive. Both sets of data are consistent with the assumption that an embryonic type of hemoglobin is gradually replaced by an adult type, although the evidence is insufficient to establish the fact for the chick embryo.

In the instance of the hemoglobins we have a tissue-specific and embryo-

specific antigen (fetal hemoglobin) and the appearance of new antigens (fetal and adult-type hemoglobins) during the course of development. Their epigenetic and autosynthetic character is clear. It happens that we know something of the nature and functions of these antigens and their control by genic factors. Again the antigens are end products of differentiation.

V. EPIGENESIS OF THE BLOOD PLASMA PROTEINS

The plasma proteins of the developing organism show the epigenetic features apparent in blood cell antigens, hemoglobins, tissue antigens, and in histological and morphological differentiation generally. The evidence at the present time is sufficient to show that new proteins appear while others disappear, that some proteins are specific to the embryonic stages whereas others are continuously present throughout the life history. The relative proportions of the various components of the plasma are as characteristic as the changing proportions of the morphological units of the body.

The remarkable developmental changes in the plasma have not been apparent because so much of the work has been limited to the advanced fetus and postnatal life. In this period the general tendency is for the total protein to increase in concentration while the proportion of albumin decreases. Summaries of these investigations are given in Windle (1940), Barcroft (1947), and Smith (1951). In some instances the serum differences are very striking as in the newborn horse, which has little or no detectable γ-globulin before ingestion of colostrum, and the newborn rabbit, with only 0.2 per cent globulin (Haurowitz, 1950, p. 155).

However, these changes, important as they are, are largely of a quantitative nature without apparent *de novo* accessions or losses. The same may be said of most studies of the blood-clotting mechanism. It has long been known that the mammalian fetus has relatively long blood-clotting times and that the human newborn frequently requires 10-12 minutes. These differences have been correlated with quantitative changes in fibrinogen, calcium, prothrombin, or other elements of the clotting mechanism. (See Needham, 1931, and Smith, 1951, for reviews.) However, Pickering and Gladstone (1925) showed that the absence of clotting in chick embryo blood prior to the twelfth day of incubation is associated with the absence of both fibrinogen and prothrombin. At later stages the blood clots slowly because of quantitative differences in the clotting components. It seems clear that the proteins of the clotting mechanism show epigenetic development, but we must restrain any assumptions that the early clotting proteins are necessarily autosynthetic products.

Sherman (1919) could demonstrate complement in the chick's blood at 17 days of incubation but not in the unincubated egg. This is certainly an epigenetic component but it is not at all clear that it is synthesized by the embryo. In some of the earliest work Sachs (1903) found that several fetal pigs had only about one-twentieth of the complement present in adult pigs. On the other hand the newborn guinea pig has only slightly less complement than the adult. The events in man are in line with those in the guinea pig, as might be expected from the reduced type of placenta found in both. The human 14-week fetus shows low complement titers but the 28-week fetus shows titers close to those of the newborn and adult (Sölling, 1937). These data suggest that in the mammals complement, like antibody, is transmitted from the mother to the offspring according to placental type. The situation in the chick is not clear. From our knowledge of antibody transmission in the chick it would seem probable that complement also is a product of maternal synthesis. Sherman's failure to demonstrate complement in yolk requires that the subject be reinvestigated with emphasis on anticomplementary substances and "masked" complement of yolk and embryonic tissue.

The "natural" lysin for rabbit erythrocytes which occurs in chicken blood was found by Sherman (1919) in the 21-day chick (at hatching) but not in the 19-day chick. Rywosch (1907) also found that these lysins appear late in development, but shortly after hatching, according to her results. Again we have a clearly epigenetic event but the conclusion that the lysin is "natural" in the sense of human isoagglutinins is not warranted. As will be indicated below, immune factors transferred from the hen may be conserved in yolk and tissue of the embryo and released to the blood only in advanced stages of development.

Duran-Reynals (1940) showed that fowl serum contains substances, probably globulins, which flocculate saline tissue extracts of many species. These substances first appear 3 to 4 weeks after hatching when bacterial agglutinins are also appearing. They are of low specificity since they not only react with tissue components of many species but also may be increased by injecting a variety of antigens. The importance of this instance is that it illustrates the uncertainty involved in classifying certain serum constituents as "natural antibodies," presumably produced without the stimulus of homologous antigen. The factors described by Duran-Reynals seem to be antibodies of low specificity produced by active immunization of the chick.

In the case of the human isoagglutinins we are apparently on firmer ground when we think of them as natural components of the serum not requiring the stimulus of an exogenous antigen. Only about 50 per cent

of newborn infants show such agglutinins although the human blood cell isoagglutinogens are present very early in development (Wiener, 1943). Isoagglutinin titers of the newborn are probably of maternal origin and, like many antibodies, enter the fetal circulation via the placenta; this is suggested by the fact that the congenital isoagglutinins disappear from the blood during the first two weeks after birth and their place is taken by the later isoagglutinins synthesized by the infant's body (see Thomsen and Kettel, 1929; Wiener, 1943).

It is evident from consideration of the clotting proteins, complement, "natural" lysins, and isoagglutinins that the mechanisms for their synthesis come into active production at relatively advanced stages of life. These macromolecules are certainly of epigenetic character but it is still doubtful that the first molecules which appear in the blood are products of the embryo's own synthesis despite their late appearance. The organism's own synthesizing mechanism in some instances come into play still later, and the heterosynthetic molecules are then replaced by those of autosynthetic origin.

The serum proteins of the chick embryo have for obvious reasons been studied over a wider range of life history than in any other vertebrate. Serologically one can detect constituents with the antigenicity of adult serum fractions at successive stages of development. Thus the specific antigenicity of albumin, α-β-globulin, and γ-globulin are detectable in about 4 to 5, 6, and 9 to 12 days respectively (Nace, 1953). The earliest blood samples obtained in sufficient quantity for serological analysis (3 to 4 days incubation) react with the antisera versus adult serum. If the antiadult serum is absorbed with yolk its reactivity for these early blood samples disappears completely. Nevertheless the antisera still react with blood extracts from the fifth day of development and with all older embryos. This has been interpreted to mean that the very early blood is composed largely of one or more of the soluble proteins present in that fraction of the yolk known as livitin (Nace and Schechtman, 1948). Serum proteins having the antigenicity of adult proteins but not of yolk proteins (nonvitelloid constituents) are regularly detectable on the fifth to sixth days of incubation. It is of course possible that they occur in the earlier blood but in concentrations too low to react with the antisera. Nevertheless it is remarkable that the blood shows this antigenic change at a time when the primitive blood cells are disappearing and large numbers of disintegrating cells are seen in the circulating blood. At this same stage substances having the specific antigenicity of the α-β-globulins of adult blood are also detectable for the first time (Schechtman and

Hoffman, 1952). Components with the electrophoretic mobility of α-β-globulins are prominent in extracts of various adult tissues; their rise in the embryonic blood coincident with extensive cytolysis supports the view that disintegrating blood cells make an appreciable contribution to the plasma proteins. This had been suggested earlier from observations on plasma formation in the blood islands of the chick (Sabin, 1920) and a good deal of evidence indicates that similar processes occur in some adult mammals (Sabin, 1939; White and Dougherty, 1946). It should be noted that none of these considerations establishes any presumption in favor of the autosynthetic character of the substances released.

Proteins antigenically similar to or identical with certain yolk proteins occur in chicken blood throughout the entire life history. Antisera prepared against the water-soluble (livetin) fraction of yolk show clear reactions with the serum of 80-hour embryos (the earliest stages from which blood uncontaminated by other tissues could be obtained) and with all other developmental and post-hatching stages tested, including adult males and females (Schechtman, Nace, and Nishihara, 1953). The same conclusions are indicated by the use of antisera against adult serum fractions (Nace, 1953). Marshall and Deutsch (1951), using quantitative precipitin tests, have presented strong evidence that ovalbumin of egg white is very similar to yolk albumin and to adult serum albumin. Also conalbumin of egg white is very similar to and perhaps identical with components found in both yolk and adult serum. Finally proteins similar to or identical with conalbumin and ovalbumin are present in the serum of the 13-day chick embryo on the basis of both electrophoretic and serological studies. Ovomucoid and lysozyme of egg white could not be detected in the yolk extract, as prepared by Marshall and Deutsch, nor in embryonic serum or adult serum.

The most abundant type of protein in the yolk is a phosphoprotein (or complex of several phosphoproteins) designated as vitelline, ovovitelline, or lipovitelline. This is probably completely hydrolyzed by the embryo, since little if any *protein* phosphorus can be found in the embryonic serum (Marshall and Deutsch, 1950). It is either absent or present in very low concentrations in the immature hen and the cock but is easily identified in the serum of the laying hen (Roepke and Bushnell, 1936). The vitelline content of adult blood can be increased in immature birds by injections of androgens or estrogens (Common, Rutledge, and Bolton, 1947). We have recently repeated experiments like those of Roepke and Bushnell and found that it is possible to identify the serum of the laying hen by its serological reaction with antivitelline

(Schechtman, Nace, and Nishihara, 1953). In addition antivitelline sera were found to react with embryonic serum from the earliest samples tested (80-hour chick) throughout embryonic development and post-hatching life. However, since it was impossible to obtain an antivitelline which would not react with livetin, even after extensive absorption, it must still be considered most probable that phosphoprotein is probably not present in the serum in appreciable concentrations until sometime before egg laying commences.

Among the electrophoretic components of embryonic chick serum there are two or three which migrate ahead of albumin and which vanish by the third day after hatching (Marshall and Deutsch, 1950). These fast proteins have a high phospholipid content. Their loss shortly after hatching would indicate they are embryonic proteins in the same sense that certain structures like the notochord of higher vertebrates are embryonic. For a while their embryonic character was doubtful since Brandt, Clegg, and Andrews (1950) found a faster-than-albumin component in the serum of the laying hen by the use of borate buffer rather than the phosphate and veronal buffers more commonly used. They could not find the fast component in sera of the cockerell and nonlaying hen. Heim (1953) has used the borate buffer and has verified the findings of Brandt, Clegg, and Andrews on adult birds. The same buffer was used in electrophoretic studies of the serum proteins from the eighth day of incubation to the adult. It was found that two or three faster-than-albumin components are present throughout the embryonic period. This does not of course establish the identity of the fast embryonic and adult components. Heim has pointed out that the components can hardly be identical since veronal buffer brings them out in the embryonic but usually not the adult serum, whereas borate buffer is effective for both. Moreover the faster-than-albumin components have thus far not been discernible in the electrophoretic patterns of yolk extracts and egg white.

The evidence of embryo-specific proteins is not limited to electrophoretic studies. Serological tests indicate the same conclusion. Rabbit antisera against the serum of 10-day embryos were absorbed to completion with adult laying-hen serum. Such antisera retained activity for the 10-day embryo serum (Schjeide, 1953). Specific anti-10-day sera reacted with all embryonic sera from the tenth incubation day to several days after hatching. The correspondence of these serological results with the electrophoretic data of Marshall and Deutsch, and Heim, is most striking.