

The Development and Role of p-Aminobenzoic Acid in the Early Chick Embryo¹

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Morphogenesis, cellular differentiation and growth are the result of an ordered sequence of physical and chemical transformations. Contrary to older opinion, the complement of proteins and conjugated proteins of the early embryo (e.g., the chick embryo at the definitive primitive streak stage) is comprehensive and highly complex, as revealed by immunochemical and enzymatic analyses (10, 16). In the course of further development, the protein complement of the embryo undergoes a progressive change resulting from the operation of at least three distinct processes, viz., (1) structural transformations of proteins; (2) purely quantitative changes in the number of protein molecules; and (3) qualitative changes in the kinds of proteins. At the outset of the discussion, care must be taken to emphasize that mechanisms two and three may be subtractive as well as additive. For example, many enzymes are more active in early than in later stages of development. To cite a case in point, Levy and Palmer (14) have convincingly shown that during the first few days of development embryonic tissue becomes poorer, not richer, in aminopeptidase. Moreover, although certain tissue-specific antigens are added during the course of development, other, transitory, antigens disappear (9, 10). The present investigation is directed toward an understanding of one link in the chain of metabolic processes underlying protein synthesis in the early chick embryo. The conjugated protein, hemoglobin, has been selected for analysis, for the following reasons: (1) relatively early appearance during development (blood islands are present in the posterior half of the blastoderm at the four somite stage), and (2) striking visual criteria of identification (appearance of characteristic red color and specific staining with patent blue [7]). Moreover, considerable information is available on the nature of hemoglobin in vertebrate embryos, young animals, and adults. Embryonic hemoglobin has been shown to be different from that of the adult, for they behave as different antigens (6), have different solubilities (29), sedimentation constants and electrophoretic mobilities (1) and osmotic pressures (15). While investigators generally agree that it is the globin moiety which differs in the embryo from the adult, Riggs (18) has recently proposed that the difference may be due to a change in the mode of linkage between the heme and globin components. In addition there is considerable information (largely mutually corroborative) in regard to the time course of accumulation of hemoglobin in the embryo (17). There is, however, a dearth of information concerning the factors controlling its synthesis.

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The approach to the problem adopted in the present investigation was suggested by Needham (17) as early as 1942. Following a lucid discussion of the history of liver therapy in macrocytic anemias, he reviewed the evidence presented by Wintrobe and Shumacker (25) which suggested that the hematopoietic tissues of the embryo are influenced by the anti-anemia factor of liver extracts. Knowledge of the chemistry of the liver anti-anemia principle was meagre at that time. Yet from a review of the available information, particularly that concerning the occurrence of pterines (complex purines) in the liver extracts, and from a consideration of the known functions of the purines, Needham suggested that the probable role of the pterines might be in the formation of the active anti-anemia principle. He concluded (p. 650), "in the light of all these facts, it is clear that the pterine metabolism of developing embryos in full hematopoiesis urgently invites study."

Despite the early statement of the problem, suitable methods for its attack have been developed only recently, through advances in two fields, namely: (1) The demonstration by Spratt (20-22) that one may gain insight into the control mechanisms which characterize the processes of differentiation and morphogenesis by an examination of the nutritional requirements of blastoderms in progressive stages of development, *in vitro*; and (2) the application of antimetabolites has provided a keen tool for the study of biochemical processes, particularly in studies of biosynthesis. A combined approach to the problem of the role of nutritional factors in the synthesis of hemoglobin is, therefore, available. The specific factors to be considered here are folic acid (folacin, pteroylglutamic acid, PGA) and its integral structural moiety, p-aminobenzoic acid (PABA).

The Folic Acid—p-Aminobenzoic Acid Relationship

That folic acid (or its conjugates or derivatives) is intimately involved in erythropoiesis has been demonstrated convincingly in both clinical and experimental studies. Remission of symptoms involving hematopoietic failure in certain members of the "family" of macrocytic anemias is regularly obtained by administration of folic acid (neurologic lesions are alleviated only by administration of vitamin B-12; the exact relationship between folic acid and vitamin B-12 has not been elucidated). Shive (19) has summarized the evidence that folic acid (probably as folinic acid or a coenzyme form of it) functions in the synthesis of purines and the pyrimidine, thymine. At the time that the structure and synthesis of folic acid were announced, the antimetabolite concept was well established. The synthesis of folic acid was followed by the synthesis of a large number of related compounds for study as antimetabolites. Structural analogues of PABA also are available; in fact, the classical analysis of the PABA-sulfonamide relationship by Woods and Fildes (26) first led to the realization that compounds structurally similar to metabolites have broad application in chemotherapy and related fields and prompted a widespread search for inhibitory analogues of metabolites. Further emphasis of the importance of PABA came with the proof of its presence in the folic acid molecule. PABA is undoubtedly a precursor of folic acid. Woolley (27) has, in fact, proposed that sulfonamide effectiveness is due primarily to interference with folic acid synthesis. On the other hand, the

excellent series of inhibition analyses by Shive and his collaborators clearly shows that PABA is involved not only in the synthesis of folic acid, purines and thymine, but of methionine and serine as well. The interrelationships of PGA and PABA cannot be fully interpreted on the basis of present knowledge. Shive has advanced two possible explanations of their relationship: (1) a single coenzyme may be formed from PABA and PGA, and the organisms which utilize PABA for the formation of the coenzyme may differ in their ability to convert PGA to the coenzyme. Folic acid may then be utilized only slowly by an organism which utilizes PABA rapidly. (2) The alternative view would be that more than one coenzyme is formed. If such is the case, organisms utilizing folic acid must have the ability to convert folic acid to these coenzymes by processes not affected by sulfonamides.

The Effects of Sulfonamides on the Development, in vitro, of the Early Chick Blastoderm

Copenhaver and Detwiler (4), have reported the results of an investigation of the effects of sulfonamides on the development of *Amblystoma punctatum*. Embryos were reared in solutions of sulfadiazine (0.12 to 2.0 per cent) and sulfanilamide (0.25 to 1.0 per cent). Embryos were placed in the solutions at the following stages (Harrison): 8, 12, 25 and 28. Embryos at the earlier stages rarely survived to the stage of yolk resorption when reared in the 2 per cent sulfadiazine solution. When embryos were reared in either inhibitor in concentrations of 1 per cent, or less, there were few toxic effects in the early stages. In embryos reared until late stages (40 to 46) there were pathological changes resembling those reported from the application of sulfonamides in clinical usage (23), among the most frequent being anemia. Bass, Yntema, and Hammond (2) have described briefly the effects of sulfonamides on the development of the mouse and chick embryos. No details are given, but in the latter case it was stated that "the drug appeared to interfere with the development of the vascular system." These results suggest that the sulfonamides, generally believed to distinguish bacterial metabolism from that of vertebrate tissues, are, in fact, effective antimetabolites in embryonic metabolism. It follows, therefore, that PABA is an essential metabolite during embryonic development. The findings of the present analysis support this view.

The dependence of the developmental processes upon an appropriate supply of exogenous nutrients provides the opportunity to isolate and cultivate the embryo, in vitro, in a suitable nutrient environment containing antimetabolites, both in the presence and absence of the specific metabolites under consideration. At the outset of the experiments described below, early chick blastoderms were cultivated on either (1) a medium composed of saline-agar + albumen extract, or (2) a chemically defined complete (synthetic) medium. Directions for preparation of the media and complete operative procedures have been described in earlier reports (8, 20). Both of the media tested in the preliminary experiments were adequate for the initiation and continuation of morphogenesis and differentiation of the early blastoderm. Only the albumen extract medium

supported hemoglobin formation consistently, however; thus it was the medium employed in all the experiments to be described.

In all tests employing antimetabolites, a measured amount of a sterilized stock solution of the inhibitor was added to the medium after it had cooled to 40-45° C. The pH of the medium was measured with the Beckman pHmeter at the beginning and end of each experiment, and was found to remain relatively constant (8.3-8.5). In the experiments in which the extent of protection against the analogue by added vitamin was determined, a measured amount of a sterilized stock solution of the vitamin was added together with the inhibitor. A total of 324 chick blastoderms (stages 4 to 8 of the Hamburger-Hamilton series [11]) was explanted to the control medium and to media containing sulfanilamide, either alone or in combination with one of the growth factors; an additional 42 embryos were cultured in media to which one of the growth factors alone was added. The results are summarized in table 1. (Another group of 30 blastoderms was cultured in media containing sodium sulfadiazine; these data are not included in the tables.)

TABLE I

Development on media containing sulfanilamide
with or without added vitamins

details in text					
Medium Saline-Agar + Albumen Extract	No. of Blastoderms Explanted	Stage of Blastoderms Explanted	Survival	Growth	Hemoglobin formation after 20 40 60 hours in vitro
alone	30	4-5	29	+	— + +
alone	33	6-8	31	+	+ + +
+ sulfanilamide					
10-50 micrograms/ml.	47	4-8	0		
+ sulfanilamide					
10-50 micrograms/ml.	51	6-8	46	retarded	— — —
+ sulfanilamide					
over 50 micrograms/ml.	39	6-8	25	greatly retarded	— — —
+ PABA/sulfanilamide					
1/100-1/2500	34	4-5	0		
+ PABA/sulfanilamide					
1/100-1/2500	37	6-8	35	retarded	+ + +
PABA alone					
10-100 micrograms/ml.	19	4-8	19	+	+ + +
+ PGA/sulfanilamide					
1/10-1/200	31	4-5	0		
+ PGA/sulfanilamide					
1/10-1/200	22	6-8	22	retarded	+ + +
+ PGA alone					
10-250 micrograms/ml.	23	4-8	21	+	+ + +

The effects produced by the antimetabolite are related directly to the stage of development at the time of explantation. Let us consider first the effects of sulfanilamide on embryos explanted at stages 4 and 5 (definitive primitive streak and head process blastoderms). Even the lowest concentration of sulfanilamide indicated in table 1, 10 micrograms per milliliter of medium, completely blocks the development of the embryo. Within an hour after explantation, the embryos were opaque; the condition persisted for as long as 10 hours before degenerative changes were observed. In addition to the inhibition of morphogenetic and differentiative mechanisms, growth was completely inhibited. Seventeen additional embryos explanted to media containing sulfanilamide in a final concentration of 5 micrograms per milliliter underwent essentially normal morphogenesis and differentiation, but exhibited the generalized retardation of growth. The complete inhibition of growth and development produced by sulfanilamide resembles the marked effect obtained when media are purposely made toxic in various ways (too high an osmotic pressure, etc.) (20). That the sulfanilamide inhibition at these stages is, indeed, of a general nature, probably not due to a specific antimetabolic effect of the inhibitor, is further indicated by the fact that the inhibition is not prevented by either PABA or PGA, in final vitamin: inhibitor ratios as high as 1:100 and 1:10 respectively, ratios far greater than those required to prevent inhibition in all other organisms studied.

The findings at stages 6 to 8 differ sharply from the foregoing. Concentrations of sulfanilamide between 10 and 50 gammas produce a relatively specific effect on the embryo, viz., an inhibition of hemoglobin formation. The morphogenesis and differentiation of the embryo are relatively normal; the nervous system is well organized, a pulsating heart develops. Yet hemoglobin is not formed; embryos were observed routinely at 20, 40 and 60 hours after explantation, and in some instances at 100 hours after explantation. In addition to the specific effect of the inhibitor, growth was retarded. Concentrations of sulfanilamide above 50 gammas (120 gammas per milliliter being the highest concentration tested), while affecting hemoglobin synthesis, also inhibit morphogenesis and differentiation. Of the 25 embryos which survived the higher concentrations of the inhibitor, 21 exhibited gross structural abnormalities, including the failure of the mesoderm to differentiate into clearly demarcated somites. Growth was completely retarded, dwarf embryos being produced. The findings further show that the effect on hemoglobin synthesis is a direct result of the antimetabolic function of sulfanilamide. The inhibition of hemoglobin production at stages 6 to 8 is readily prevented by the addition of either PABA or PGA to the medium, at vitamin/sulfanilamide ratios as low as 1/2500 and 1/200 respectively. Only the effect on hemoglobin formation is prevented by the vitamins, however; the generalized retardation of growth is not prevented.

From the foregoing discussion, the following tentative conclusions may be derived: (1) the complete inhibition of all developmental activity obtained with sulfanilamide at stages 4 and 5, and the generalized growth retardation observed at stages 6 to 8 are apparently not related to the usual antimetabolic role of sulfanilamide, since the inhibition cannot be prevented with either PABA or PGA. The results of other, preliminary,

TABLE II
p-Aminobenzoic acid in the early chick embryo.

Sample: Blastoderms at	No. of Embryos	Weight of Sample in milligrams	PABA in Sample in micrograms	PABA/mg. of dry weight in micrograms
Stage 4	65	2.3	.0031	.0013
4	64	2.7	.0039	.0014
6	59	3.1	.0049	.0016
6	59	3.3	.0047	.0014
8	51	3.8	.0060	.0016
8	50	4.3	.0056	.0013
YOLK	—	6.1	.0067	.0011
YOLK	—	6.2	.0063	.0010
ALBUMEN	—	6.0	.0030	.0005
ALBUMEN	—	6.3	.0027	.0004

experiments further indicate that these effects are not prevented by added methionine. Serine has not been tested. (2) The relatively specific inhibition of hemoglobin production at stages 6 to 8 is due to the specific anti-metabolic role of sulfanilamide. Sulfanilamide is, in effect, blocking folic acid synthesis, which consequently results in the failure of the embryo to produce hemoglobin. The inhibition is prevented by either PABA or PGA, as expected. The results lead to a further question. Since sulfanilamide becomes effective as an antimetabolite only after stage 5, it would appear that either (1) PABA is not present in the embryo prior to stage 6 or (2) PABA may be present but has no role, i.e. that it becomes functional only at stage 6 and thereafter. It is possible to answer the question in favor of the second alternative as a result of the experiments to be discussed below.

p-Aminobenzoic Acid Concentration in the Early Embryo

The concentration of PABA in the early chick embryo at progressive stages of development was determined by a standard microbiological assay technique. The microbiological assay method of Landy and Dicken (13), as modified by Cheldelin and Bennett (3) proved to be sufficiently sensitive and hence was adopted. The test organism was *Acetobacter suboxydans* (ATTC 621). The technique was used exactly as described by its authors; hence details of procedure need not be stated here. Following determination of the standard curve the following samples were assayed: embryos at stages 4, 6, 8, fresh yolk, fresh albumen. Embryos were rapidly removed from the yolk in the usual manner, carefully trimmed of all adhering yolk, washed in three changes of sterile chick Ringer's solution, drained, and dried over calcium chloride, *in vacuo*, for 36 hours at 4° C. The samples were weighed rapidly on an analytical balance, ground in a Ten Brock glass tissue grinder in 2 ml. of glass distilled water, and autoclaved for 30 minutes at 120° C., 15 pounds pressure. The extract

was centrifuged at 3600 r.p.m. for 30 minutes; the centrifugate was discarded. The supernatant was diluted to 5 ml. with glass distilled water and added to the standard assay medium. Following inoculation from a washed 24-hour culture, the flasks were incubated at 30° C. for 48 hours. After that time the flasks were shaken and the optical density read directly in the Coleman spectrophotometer at 540 millimicrons. The quantity of the vitamin in the sample was determined by comparison with the standard curve. The results of the determinations, in which a total of 348 embryos was assayed, are given in table 2.

Clearly, PABA is present in the blastoderm as early as stage 4, and remains relatively constant in amount per milligram of dry weight through stage 8. It follows, therefore, that PABA has no essential role in folic acid synthesis until the beginning of stage 6. The nature of the generalized growth retardation which cannot be prevented by PABA administration remains unanswered. Zwilling and DeBell (30) have reported that injections of sulfanilamide into the yolk sac of developing embryos (as early as 30 hours) produce micromelia, parrot-beak and growth retardation. PABA did not prevent the effects of sulfanilamide. In fact it exaggerated the condition. On the latter point, the present findings are not in agreement. It is probable that the discrepancy may be explained by the difference in concentration of PABA employed. The smallest amount administered by Zwilling and DeBell was reported as 5 mg., 50 times greater than the highest concentration reported here.

Other lines of evidence indicate that folic acid is essential for embryonic development. The studies of Karnofsky, Patterson, and Ridgway (12) and Cravens (5), for example, have shown that high concentrations of the folic acid analogue, aminopterin (4 amino folic acid), injected into the egg cause mortality in the developing chick, resulting from a profound inhibition of growth and of hemoglobin formation. Wagley and Morgan (24) have reported that aminopterin similarly injected, causes marked changes in the yolk sac blood islets, as revealed by cytological studies. In each case, the inhibition is not prevented by folic acid, but is prevented by folinic acid, indicating that aminopterin functions by interfering with the conversion of folic acid to folinic acid. The results of nutritional experiments with breeding hens also support the view that folic acid is essential for development. Feeding the hen a diet low in PGA results in a marked reduction in hatchability, due to 2 peaks of mortality, one during the first week of incubation, the other just prior to hatching. The injection of PGA into deficient eggs prior to the fourth day of incubation permits normal development (5).

The evidence obtained from analyses of the effects of folic acid analogues lends support to the idea presented here, i.e. that sulfanilamide inhibition of hemoglobin formation is achieved through interference with folic acid synthesis. On the other hand the growth retardation obtained with sulfanilamide apparently does not involve the same mechanism as does the growth retardation achieved with aminopterin. The latter effect can be prevented by folinic acid or by purines and the pyrimidine, thymine. Since the purines and pyrimidines are precursors of nucleic acids, it has been concluded that aminopterin inhibition of growth is due to a failure of nucleic acid synthesis (28). The sulfanilamide inhibition of growth

cannot be prevented, however, with the corresponding metabolites. Although it would be unwise to state that the mechanism of sulfanilamide inhibition of growth clearly does not involve an inhibition of nucleic acid synthesis, the evidence does indicate that another pathway is more likely involved.

Summary

Evidence is presented that p-aminobenzoic acid (PABA) is present in the chick embryo as early as the definitive primitive streak stage, and remains relatively constant in amount per milligram of dry weight through the early somite stages. The results further indicate, however, that PABA may have no essential role in the metabolism of hemoglobin formation until the head fold stage, for it is only at that time that it is first possible to reversibly inhibit hemoglobin synthesis with sulfanilamide (SAN). Inhibition during this period is prevented by both PABA and folic acid; complete reversal is obtained with PABA at a PABA/SAN ratio of 1/2500; reversal with folic acid is achieved only with large quantities of the vitamin (index 1/200). Inhibition of development prior to the head fold stage, and growth retardation at all stages cannot be reversed with either PABA or folic acid.

Literature Cited

1. ANDERSCH, M. A., D. A. WILSON, and M. L. MENTEN. 1944. Sedimentation constants and electrophoretic mobilities of adult and fetal carbonylhemoglobin. *J. Biol. Chem.*, **153**:301.
2. BASS, A. D., C. L. YNTEMA and W. S. HAMMOND. 1949. Effect of sulfadiazine on survival of the mammalian embryo. *Science*, **110**:527.
3. CHELDELIN, V., and M. BENNETT. 1945. Modifications in the *Acetobacter suboxydans* assay for p-aminobenzoic acid. *J. Biol. Chem.*, **161**:751.
4. COPENHAVER, W. M., and S. R. DETWILER. 1948. The effects of sulfonamides on *Amblystoma* embryos, with particular reference to blood development. *J. Exp. Zool.*, **109**:239.
5. CRAVENS, W. W. 1952. Vitamin deficiencies and antagonists. *Annals N. Y. Acad. Sci.*, **55**:188.
6. DARROW, R. R., S. NOWAKOVSKY and M. AUSTIN. 1940. Specificity of fetal and adult hemoglobin precipitins. *Arch. Path.*, **30**:873.
7. DUNN, R. C. 1946. A hemoglobin stain for histologic use based on the cyanol-hemoglobin reaction. *Arch. Path.*, **41**:676.
8. EBERT, J. D. 1950. Analysis of the effects of anti-organ sera on the development, in vitro, of the early chick blastoderm. *J. Exp. Zool.*, **115**:351.
9. EBERT, J. D. 1951. Ontogenetic change in the antigenic specificity of the chick spleen. *Physiol. Zool.*, **24**:20.
10. EBERT, J. D. 1952. Appearance of tissue-specific proteins during development. *Annals N. Y. Acad. Sci.*, **55**:67.
11. HAMBURGER, V., and H. L. HAMILTON. 1951. A series of normal stages in the development of the chick embryo. *J. Morph.*, **88**:49.
12. KARNOFSKY, D. A., P. PATTERSON and L. RIDGWAY. 1949. Effect of folic acid, "4-amino" folic acids and related substances on growth of chick embryo. *Proc. Soc. Exp. Biol. Med.*, **71**:447.
13. LANDY, M., and D. DICKEN. 1942. A microbiological method for the determination of p-aminobenzoic acid. *J. Biol. Chem.*, **146**:109.

14. LEVY, M., and A. H. PALMER. 1943. Chemistry of the chick embryo. IV. Amino-peptidase. *J. Biol. Chem.*, **150**:271.
15. MCCARTHY, E., and G. POPJAK. 1947. Osmotic pressure of fetal sheep hemoglobin. *Nature*, **159**:198.
16. MOOG, F. 1952. The differentiation of enzymes in relation to the functional activities of the developing embryo. *Annals N. Y. Acad. Sci.*, **55**:57.
17. NEEDHAM, J. 1942. *Biochemistry and Morphogenesis*. University Press. Cambridge.
18. RIGGS, A. 1951. The metamorphosis of hemoglobin in the bullfrog. *J. Gen. Physiol.*, **35**:23.
19. SHIVE, W. 1951. The functions of B-vitamins in the biosynthesis of purines and pyrimidines. *Vitamins and Hormones*, **9**:76.
20. SPRATT, N. T. 1947. Development in vitro of the early chick blastoderm explanted on yolk and albumen extract saline-agar substrata. *J. Exp. Zool.*, **106**:345.
21. SPRATT, N. T. 1948. Development of the early chick blastoderm on synthetic media. *J. Exp. Zool.*, **107**:39.
22. SPRATT, N. T. 1952. Metabolism of the early embryo. *Annals N. Y. Acad. Sci.*, **55**:40.
23. VAN DYKE, H. B. 1943. The toxic effects of sulfonamides. *Annals N. Y. Acad. Sci.*, **44**:477.
24. WAGLEY, P. F., and H. MORGAN. 1948. Observations on the effects of folic acid antagonists, folic acid, liver extract, and vitamin B-12 on embryonated eggs. *Bull. Johns Hopkins Hosp.*, **83**:275.
25. WINTROBE, M. M., and H. SHUMACKER. 1936. Erythrocyte studies in the mammalian fetus and newborn. *Am. J. Anat.*, **58**:313.
26. WOODS, D. D. 1947. Bacterial metabolism. *Ann. Rev. Biochem.*, **16**:605.
27. WOOLLEY, D. W. 1947. Water-soluble vitamins. *Ann. Rev. Biochem.*, **16**:359.
28. WRIGHT, L. D. 1951. Antimetabolites of nucleic acid metabolism. *Vitamins and Hormones*, **9**:131.
29. WYMAN, J., J. RAFFERTY and E. INGALLS. 1944. Solubility of adult and fetal carbonylhemoglobin of the cow. *J. Biol. Chem.*, **153**:275.
30. ZWILLING, E., and J. DEBELL. 1950. Micromelia and growth retardation as independent effects of sulfanilamide in chick embryos. *J. Exp. Zool.*, **115**:59.