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MORPHOGENESIS OF THE DOWN FEATHER
IN THE PRESENCE OF PYRIMIDINES, A
RIBOSIDE, AND RELATED COMPOUNDS.

Iowa State University of Science and Technology
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MORPHOGENESIS OF THE DOWN FEATHER IN THE PRESENCE
OF PYRIMIDINES, A RIBOSIDE, AND RELATED COMPOUNDS

by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	4
MATERIALS AND METHODS	14
RESULTS	18
DISCUSSION	33
THE NUCLEOLUS, GROWTH, AND DIFFERENTIATION	44
SUMMARY	49
LITERATURE CITED	52
ACKNOWLEDGEMENTS	63
PLATES	64

INTRODUCTION

Antimetabolites and structural analogues of normal cell components were used as tools in the study of development by Brachet (1945, 1946, 1947) who studied the effects of acriflavine, barbituric acid, and benzimidazole on the processes of gastrulation and neurulation in the frog embryo. Hisaoka and Hopper (1957) explored the effects of barbituric acid and one of its derivatives on the development of the zebra fish, Brachyanio rerio. Derivatives of nucleic acids have also been employed in the study of the embryology of higher animals. For example, 8-azaguanine produced abnormalities of the skeletal system in developing mice (Kelton and Woodside, 1955; Nishimura and Nimura, 1958). Frair and Woodside (1956) and Waddington and Perry (1958) used 8-azaguanine in their studies on the growth and development of early chick embryos.

The present study is an outgrowth of an earlier one (Gibley and Hamilton, 1959) in which the developing down feather was grown in the presence of certain compounds structurally related to the purine and pyrimidine bases of nucleic acids. These experiments tested the effects of the growth-promoting substance, orotic acid, and the purine and pyrimidine analogues, isoguanine sulfate, 8-azaguanine, thiouracil, and 2-amino-4-hydroxy-6-methylpyrimidine on organogenesis and differentiation. Briefly, orotic acid and the guanine derivatives inhibited the production of feathers at concentrations

of 333 and 166 $\mu\text{g/ml}$. They also disturbed the pattern of alkaline phosphatase and the reaction for ribonucleic acid. The enzyme was either not present, or, if so, diffused throughout the tissue with no localization in feather loci. Thiouracil increased the reaction for alkaline phosphatase, particularly in the outlying fibroblasts.

A striking effect of the analogues was noted in the nucleoli. These were enlarged or vacuolated, irregular in size and shape, and even occurred in chains. The principal effect seemed to be upon nucleolar RNA, although there was good correlation between the activity of phosphatase and growth of feathers.

The aim of the present investigation was to employ additional compounds related to components of nucleic acids to see how they would affect development of the feather. These included such relatives of uracil as 5-bromouracil, 5-nitro-uracil, and barbituric acid; the pyrimidine analogues, dithiopyrimidine, isoorotic acid, and 2,4,6-triaminopyrimidine; inhibitors of the synthesis of ribonucleic acid, diethylbarbituric acid, and 4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole; and, an inhibitor of protein synthesis, puromycin. Specifically, we noted the effects of these compounds on alkaline phosphatase and ribonucleic acid, since these two substances always seem to be active during the development of the down feather (Koning and Hamilton,

1954; Hamilton and Koning, 1956; Fabiny, 1959). Where applicable, tests were also made for protein. In addition, we measured the total growth of treated cultures as compared with controls. Because of our previous interest in the nucleolus (Gibley and Hamilton, 1959) this structure was examined closely in an effort to determine, what role, if any, it plays in the morphogenesis and differentiation of the down feather.

REVIEW OF LITERATURE

Barbituric acid (2,4,6-trihydroxypyrimidine) has been applied to amphibian development by Brachet (1945). Frog embryos in 0.3% barbituric acid showed a marked retardation of development, particularly when late blastula and early gastrula stages were so treated. Specific abnormalities included the inhibition of the nervous system and notochord, and anomalies of the pronephros and circulatory system. Hisaoka and Hopper (1957) found that all stages of development of the zebra fish were completely inhibited in 0.2% barbituric acid. Death occurred within six hours.

Barbituric acid also has some antibacterial activity (Woods, 1941). Weak solutions of this acid retarded the growth of root seedlings of Zea mays (Seiler, 1954). In a study of the effects of various biochemical compounds on the synthesis of glutamine by brain cells in vitro, Messer (1958) discovered that barbituric acid reduced the synthesis of glutamine and also inhibited respiration.

Diethylbarbituric acid (barbital) was also used in the study of the embryology of the zebra fish by Hisaoka and Hopper (1957). It produced abnormalities in the embryo, including edema, cessation of circulation, structural irregularities of the brain, spinal cord, and sense organs, and retarded development of the gut, air bladder, pancreas, and liver. Histochemically, it inhibited the synthesis of

ribonucleic acid in the central nervous system and sense organs, and to some extent in the liver.

Barbital has been found to stimulate the organism, Streptomycin, at low concentrations (Ferguson, Huang and Davisson, 1957) by promoting the development of the enzyme which catalyzes the biosynthesis of streptomycin. Kirihara (1931) found that diethylbarbituric acid depressed growth of fibroblast cultures. Related barbiturates inhibited the outgrowth of nerve fibers in hanging drop cultures of spinal cord from chick embryos (Pomerat, Drager and Painter, 1946).

The effects of barbital on some enzyme systems have been investigated. Subcutaneous injection of barbital into mice had no effect on the parenchymal alkaline phosphatase reaction of liver cells (Williams and Aronsohn, 1951). Diethylbarbituric acid activated the acid phosphatase reaction in the dog liver (Bertran and Sanchez, 1951).

5-bromouracil inhibited the growth of Lactobacillus casei in a basal medium supplemented with thymine (Hitchings, Falco, and Sherwood, 1945). In the absence of thymine or folic acid, bromouracil supported limited growth of the microorganism. Weygand, Wacker, and Grisebach (1951) found that bromouracil inhibited the growth of Streptococcus faecalis in a basal medium supplemented with thymine. Later work by Puleston, Poe, and Witt (1955) showed that bromouracil had neither a stimulatory nor an inhibitory effect on the growth of Streptococcus faecalis R. Bromouracil also suppressed growth in

Escherichia coli 15T⁻ (Dunn and Smith, 1957) and in Lactobacillus leichmannii (Bardos, Levin, Herr, and Gordon, 1955).

Bacteria have not been the only organisms affected by bromouracil. Thompson et al., (1949) found that bromouracil prohibited the multiplication of Vaccinia virus. Tomisek, Reid, Short, and Skipper (1957) observed that 5-bromouracil inhibited the growth of Chlorella pyrenoidosa up to 95-99%.

5-nitrouracil also suppressed growth of various microorganisms: Lactobacillus casei (Ågren, 1954; Shive, 1950; Hitchings, Elion, and Vanderwerff, 1948; Hitchings, Elion, and Falco, 1950; Hitchings et al., 1950); Streptococcus faecalis R, (Puleston, Poe, and Witt, 1955); Escherichia coli, (Zamenhof and Griboff, 1954). Teramoto, Hashida, and Mukai (1958) showed that nitrouracil inhibited the growth of hiochi bacteria completely.

Thompson et al., (1949) found nitrouracil to be effective against the multiplication of virus. In a study of the chemical inhibition of regeneration in Stentor coeruleus, Weisz (1955) showed that various analogues of nucleic acids, including nitrouracil, produced good inhibition fairly consistently.

4,5,6(5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole (TRB). Benzimidazole and its derivatives have been used on a variety of organisms. Brachet (1947) used benzimidazole on the frog embryo in a study of gastrulation and

neurulation. Certain benzimidazoles have been found to have a lethal effect on the early chick embryo (Blackwood and Shorb, 1958). Alkyl derivatives of benzimidazole inhibited the multiplication of influenza virus (Tamm et al., 1953).

TRB and its relative, the dichloro form, greatly suppressed the multiplication of influenza virus (Tamm, 1954; Tamm, Folkers, Shunk, and Horsfall, 1954). Recently, Tamm (1958) has compiled a review of the effects of these compounds on viral multiplication. DRB inhibited the uptake of alanine- 1-C^{14} in isolated cell nuclei (Allfrey, Mirsky, and Osawa, 1957). DRB and other halogenated β -D-ribofuranosyl benzimidazoles are thought to interfere with the metabolism of ribonucleic acid (Tamm, Folkers, and Shunk, 1956a, 1956b). The dichloro form also caused a 50% inhibition of the incorporation of adenine 8-C^{14} into RNA of the chorioallantoic membrane from 10-to 11-day chick embryos (Tamm, 1957).

Puromycin, an antibiotic isolated from Streptomyces alboniger, had a wide range of activity against various types of bacteria, both gram-positive and gram-negative (Porter et al., 1952). It also had definite amoebicidal effects on Endamoeba histolytica (Alba, Artigas, and Otto, 1955; Nakamura and Jonsson, 1957). Bortle and Oleson (1955) showed that puromycin inhibited the growth of Tetrahymena. In addition, it was effective against some tumors (Troy et al., 1953; Sugiura, Stock, Reilly, and Schmid, 1958).

Puromycin has proven useful in the experimental chemotherapy of trypanosomiasis. It was very effective against mice and rabbits infected with Trypanosoma equiperdum and T. cruzi (Hewitt et al., 1953). In vivo development of T. equinum, T. evansi, T. rhodesiense, and T. gambiense was prevented or suppressed by puromycin depending on whether the compound was administered four hours after inoculation or at the height of the infection (Tobie, 1954). Trinc o et al. (1955, 1956) have found the antibiotic effective in human sleeping sickness. Chemical studies have shown that puromycin inhibits in vitro carbohydrate metabolism of T. equiperdum (Agosin and von Brand, 1954).

Puromycin is not limited in effectiveness to single-celled parasites; Gumble, Hewitt, Taylor, and Wallace (1956) have demonstrated its efficacy against multicellular organisms such as oxyurids and tapeworms. For a review of the biological activity of puromycin and the effect of chemical alteration of the molecule on such activity, see Hutchings (1957). He also discusses some experiments relating to its possible mechanism of action.

No pertinent literature was found on isoorotic acid, dithiopyrimidine, or 2,4,6-triaminopyrimidine.

Alkaline Phosphatase

A comprehensive review of the literature concerning the localization of alkaline phosphatase in developing tissues and

organs and summarizing the theories on its possible function (s) has been presented in an earlier paper (Gibley, 1959). Only the recent reports will be summarized here.

Fleisch and Neuman (1960) have shown that alkaline phosphatase may play a very important role in biological calcification. The enzyme removes substances present in serum which are inhibitory to the seeding of calcium phosphate crystals by collagen. Malone (1960) has correlated alkaline phosphatase with the synthetic mechanisms of luteal growth in rats. It has also been implicated in the absorption and in the synthesis of the jelly substance in the eggs of Bufo gargarizans (Chu and Chin, 1958). Mellander, Bennich, and Fölsch (1959) found that intestinal alkaline phosphatase will readily hydrolyze O-phosphorylates derivatives of serine. Ikeda (1959) correlated RNA and alkaline phosphatase with the site of rapid synthesis of protein. The relationship of alkaline phosphatase, morphological differentiation, and the synthesis of ribonucleic acid and proteins has been discussed recently by Rogers et al. (1960) in their investigations of enzymes in the differentiation of the chick brain. For a more complete discussion of this relationship, see Gibley (1959).

Ribonucleic Acid

A previous report (Gibley, 1959) reviewed the evidence for relating RNA to the synthesis of protein. Since then, considerably more literature on the subject has appeared. As

Brachet (1960) has so aptly stated "we can thus from now on consider the correlation between ribonucleic acid and protein synthesis as a well-established fact, and we can draw the conclusion that ribonucleic acid somehow takes a direct role in protein synthesis". Just what role the nucleic acid plays in this process has been discussed in the excellent reviews of Simkin (1959); Hoagland, Zamecnik, and Stephenson (1959). These and many other contributions provide evidence that ribonucleic acid participates in at least two ways in the synthesis of protein: (1) soluble RNA is involved at some stage in the transport of activated amino acid residues to the site of protein synthesis; (2) RNA bound to protein is involved at the site of synthesis (Simkin, 1959).

The Nucleolus

The literature on the structure, chemical composition, and function of the nucleolus has increased precipitously in recent years. The following discussion will be limited to some biochemical and functional studies of the nucleolus which have a bearing on the present investigation. Excellent reviews on the nucleolus have been presented by Lin (1955), Vincent (1955), and Swift (1959).

Chemical composition

Ever since the pioneer work of Montgomery (1898) the nucleolus has been the subject of chemical investigation. As crude as Montgomery's dye mixtures were at the time, they

still indicated that the nucleolus contained protein. With the advent of refinements in technique came more positive identification of the chemical components. Ribonucleic acid was identified in the nucleolus by Caspersson and Schultz (1940) and Brachet (1940). At the present time there is little doubt that the nucleolus contains some RNA, but the amount varies with the material used. Vincent (1955) found that nucleoli from Drosophila salivary gland, cat axon, and starfish oocytes contained from 2 to 5% ribonucleic acid. Litt, Monty, and Dounce (1952) analyzed preparations of rat liver nucleoli and found only a few percent RNA. Monty, Litt, Kay, and Dounce (1956) found that liver cell nucleoli contained from 1.0 to 1.5% RNA on a dry weight basis. According to Vincent (1955), "the RNA content of the nucleolus is rarely greater than 5% and probably considerably less than that in most cells."

There is some evidence that the nucleolus contains two types of RNA, one which is fairly acid-soluble, and one which is bound to the structural framework of the nucleolus (Pollister and Leuchtenberger, 1949). In addition, the recent work of Love and Bharadwaj (1959) indicates two types of ribonucleoprotein in the nucleolus of mammalian cells.

Actual values referable to protein in the nucleolus range from 40-90% and greater (Vincent, 1955). Vincent also reports the presence of the following substances in the nucleolus:

lipoidal material, minerals (sulfur, calcium and potassium), adenosinetriphosphatase, phosphorylase, aldolase, arginase, and catalase. Bradfield (1951) and Danielli (1953) have established the presence of alkaline phosphatase in the nucleolus. Tandler (1960) also found orthophosphate in the nucleolus.

Function

Monty et al. (1956) speculated that the nucleolus contains the special DNA- or RNA-protein template necessary for the synthesis of the special enzymes and proteins involved solely in the mitotic apparatus. Stich (1956) presented evidence that the nucleolus is a place of protein synthesis. Autoradiography of nucleolar contents has provided further evidence for the involvement of the nucleolus in the metabolism of nucleic acids and proteins (Sirlin, 1958). Changes in nucleolar form and composition, associated with altered synthetic states of the cell, have led Swift (1959) to the conclusion that the nucleolus is a site of protein synthesis. Wainwright (1959) also associated the nucleolus with the formation of proteins. The initiation of the synthesis of hemoglobin has been attributed to the nucleolar region (O'Brian, 1960).

Irradiation of the nucleolus reduced the amount of nucleic acid formed in the nucleus 3-7 hours after irradiation (Seed, 1960). It was concluded that the nucleolus is important

in synthetic activities. Among the numerous reports supporting the theory that the nucleolus is a site of synthesis of RNA are those of Allfrey and Mirsky (1959); Perry, Hell, and Errera (1960); McMaster-Kaye (1960); Brachet (1960) and Errera and Brachet (1960). Polymerization of ribonucleic acid has been found in the nuclei of Zea mays (Pollister and Leuchtenberger, 1949). Vincent and Baltus (1960) had the following comments concerning the function of the nucleolus:

The data reported here, and many other observations in the literature, are consistent with the following hypothesis: a major function of the nucleolus is the provision of "soluble" or "activation" RNA for use in the synthesis of cellular proteins. The "activation" RNA provided by the nucleolus is bound to a carrier protein, likewise nucleolar in origin; this protein is considered to be essential for the integrity of the RNA.

MATERIALS AND METHODS

Pieces of skin were removed under sterile conditions from the backs of White Leghorn chick embryos between the stages of 29⁺ and 33⁻ (6-8 days; Hamburger and Hamilton, 1951). Pairs of nearly identical pieces of tissue, about 1 mm. square, were isolated in chick Ringer's solution in the following manner: a strip of dorsal skin about 2 mm. wide was isolated by cutting along either side of the mid-dorsal line from the region of the shoulder to the base of the tail, undermining the skin with a needle, and severing the strip at its two ends. This piece of tissue was then cut several times at right angles to its length and each rectangular piece was divided into bilateral halves along the mid-dorsal line. Each embryo yielded from four to eight such bilateral pairs. One member of each pair was placed in a tissue culture of the hanging-drop type consisting of one drop of embryonic extract, one drop of heparinized chicken plasma and one drop of Pannett-Compton saline solution. The corresponding half was placed in a similar mixture containing one drop of the test solution in place of the saline solution. The number of drops of Pannett-Compton solution and test solution was varied to obtain a variety of final concentrations. Cultures were incubated at 37°C for two to four days, examined microscopically, and treated according to the histochemical techniques described below.

The embryonic extract was prepared by passing 9- or 10-day-old chick embryos through a 5-ml syringe and diluting them with twice their volume of Pannett-Compton solution; the diluted brei was then centrifuged to remove tissue debris, and the supernatant fluid was used in making the cultures.

The test solution was prepared by weighing the required amount of the chemical in a clean, dry, test tube and diluting to the desired concentration with Pannett-Compton solution. The resulting solution was sterilized by pressing it through a Seitz filter into a previously sterilized test tube. All solutions used in culturing were prepared with Pyrex distilled water which was also run through an ion exchange column.

Histochemical Techniques

Cultures to be stained for alkaline phosphatase by the Gomori method were fixed in cold acetone for at least 24 hours. After washing with water, the control and treated cultures were placed in a substrate mixture containing 25 ml of distilled water and the following amounts of 2% solutions: 10 ml sodium barbital, 10 ml sodium glycerophosphate, 4 ml calcium chloride, and 1 ml magnesium sulfate. The cultures were incubated at 37°C for 45 minutes. They were then washed with distilled water, treated with 2% cobalt chloride, washed again, and treated with a dilute solution of ammonium hydro-sulfide to develop the precipitate of cobalt sulfide at the sites of enzymatic activity. The stained cultures were

dehydrated in a series of alcohols, cleared in xylol, and mounted in Hartman-Leddon's Synthetic Resin (H.S.R.).

Cultures to be sectioned were fixed in cold absolute alcohol containing 5% glacial acetic acid (Wolman and Behar, 1952). They were embedded in a mixture of 10 parts paraffin (M.P. 56-58°C) and one part bayberry wax. Sections were cut at 7 μ and mounted on albumenized slides. The slides were passed through xylol and a graded series of alcohols to water and then stained for 15 minutes in a 0.05% solution of toluidine blue in 5% ethyl alcohol. They were destained in tertiary butyl alcohol overnight.

Preparatory to staining, some sections were incubated in a 0.1% solution of ribonuclease in distilled water for three hours at 59°C, while others were incubated for the same length of time in distilled water alone. The basophilic material removed by such enzymatic activity was presumed to be ribonucleic acid. Sections were then cleared in xylol and mounted in H.S.R.

Cultures to be stained for protein were fixed and sectioned in the same manner as for toluidine blue. The slides were passed through xylol, absolute alcohol, and 95% alcohol. They were then stained in a solution of mercuric bromphenol blue (Mazia, Brewer, and Alfert, 1953) for 15 minutes, washed in 0.5% acetic acid, differentiated in 95% alcohol, dehydrated in absolute alcohol, cleared in xylol, and mounted in H.S.R.

Some sections were pre-treated with ribonuclease to remove nucleic acids that might bind basic groups and interfere with the interpretation of the protein stain. Some staining was performed without mercury in the solution of bromphenol blue, in an attempt to determine what kind of protein was being stained. (Mazia et al., 1953).

RESULTS

The concentrations of the chemicals in the cultures were varied between 1666 and 1/6 $\mu\text{g/ml}$ to determine the lowest concentration which would affect the development of the feathers. The cultures were incubated for two to four days and then examined microscopically. The results obtained from the living cultures are summarized in Table 1. 2,4,6-triaminopyrimidine produced no significant effect except at high concentrations (1666 and 833 $\mu\text{g/ml}$). At such levels it was probably toxic. 5-bromouracil, 5-nitrouracil, diethylbarbituric acid, and isoorotic acid had no substantial effect at any of the concentrations tested. Barbituric acid, dithiopyrimidine, puromycin, and 4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole all produced various degrees of feather inhibition depending on the concentrations used. The optimal level for inhibition by barbituric acid and dithiopyrimidine was high (833 $\mu\text{g/ml}$ and 333 $\mu\text{g/ml}$, respectively) when compared with that for TRB and puromycin (20 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$, respectively).

Barbituric acid (BA). This close relative of uracil was inhibitory only at high concentrations (833 and 333 $\mu\text{g/ml}$) and then not consistently (see Table 1). The most striking effects were produced at 833 $\mu\text{g/ml}$. Feathers were prevented from forming in about 35% of the explants. In another 27% of the cases the feathers in the treated cultures were less

Table 1. Effect of analogues on the growth and development of feathers

Chemical	Conc. in $\mu\text{g/ml}$	Total no.		Average diameter		Average no. feathers		ND	S	C	Stim.	Percent inhibition of feathers
		em-bryos	cul-tures	control	treated	control	treated					
BA	833	18	104	3.16	2.98	4.0	2.3	19	29	37	19	63.5
BA	333	5	24	4.47	3.96	4.9	3.5	2	14	2	6	66.6
BA	166	7	35	3.82	3.46	3.94	2.83	6	9	10	10	54.2
BA	33	12	74	3.90	3.81	4.28	4.04	21	22	3	28	33.7
TRB	83.3	2	9	3.01	1.00	3.77	0.00	0	0	9	0	100
TRB	41.6	2	9	2.85	1.00	4.00	0.00	0	0	9	0	100
TRB	20.8	16	74	3.41	2.16	4.41	0.12	0	4	70	0	100
TRB	10.4	5	31	3.08	2.69	5.25	2.38	4	13	11	3	77.3
TRB	5.2	2	15	2.83	2.70	5.26	5.06	0	9	0	6	60
DTP	333	9	57	4.42	3.77	4.71	0.43	1	7	48	1	96.5
DTP	166	8	55	4.53	4.49	4.56	2.60	9	25	14	7	71.0
DTP	83.3	7	51	4.46	4.44	5.27	4.25	15	26	2	8	54.9
DTP	41.6	2	15	3.86	3.84	4.26	3.80	3	8	0	4	53.3
DTP	20.8	2	14	3.64	4.30	5.85	5.85	6	5	0	3	35.7
T	1666	2	12	5.45	1.00	3.91	0.00	0	0	12	0	100
T	833	3	15	4.52	2.60	3.33	0.00	0	0	15	0	100
T	333	8	50	4.46	3.72	4.54	1.94	6	25	17	2	84
T	166	6	31	4.63	4.26	4.63	2.61	5	16	7	3	74.2
T	83.3	5	34	4.47	4.53	4.71	4.36	12	12	3	7	44.2
T	33.3	11	66	4.42	4.65	4.51	3.42	19	26	5	16	47
T	16.6	7	46	4.94	5.29	5.15	4.59	7	21	2	16	50

Table 1. (Continued)

Chem- ical	Conc. in µg/ml	Total no.		Average diameter		Average no. feathers		ND	S	C	Stim.	Percent inhibition of feathers
		em- bryos	cul- tures	control	treated	control	treated					
B	500	2	15	2.77	2.59	4.53	3.60	4	8	0	3	53.3
B	333	2	9	3.06	3.04	5.77	5.66	1	5	0	3	55
B	166	3	16	3.42	3.56	6.06	5.55	1	6	2	7	50
B	83.3	2	12	2.80	3.17	5.41	5.75	3	4	0	5	33
B	41.6	2	16	2.62	2.70	6.06	6.31	6	4	0	6	25
NU	500	2	10	3.30	3.21	3.60	2.00	2	2	4	2	60
NU	333	5	38	3.04	2.90	3.65	2.07	7	16	10	5	68.5
NU	166	4	22	3.40	3.27	3.13	1.86	3	8	6	5	63.5
NU	125	1	6	3.00	3.03	4.00	4.00	3	0	1	2	16.6
NU	83.3	2	12	3.36	3.43	3.25	2.33	5	3	2	2	41.7
IO	500	3	22	3.02	2.83	3.50	2.09	4	10	4	4	63.5
IO	333	3	21	2.99	2.90	3.14	3.28	5	7	1	8	38.1
IO	166	3	20	2.85	2.55	3.95	2.20	7	10	3	0	65
IO	83.3	1	8	3.25	3.10	2.50	2.12	4	2	1	1	37.5
P	333	1	7	3.42	1.0	3.58	0.00	0	0	7	0	100
P	166	1	7	3.27	1.0	2.14	0.00	0	0	7	0	100
P	83.3	1	7	2.78	1.0	3.86	0.00	0	0	7	0	100
P	41.6	1	10	3.49	1.0	3.60	0.00	0	0	10	0	100
P	20.8	2	13	3.15	1.0	3.15	0.00	0	0	13	0	100
P	10.41	1	7	2.92	1.0	3.00	0.00	0	0	7	0	100
P	5.20	2	11	3.23	1.0	3.64	0.00	0	0	11	0	100
P	1.7	3	13	2.83	1.0	3.22	0.00	0	0	13	0	100
P	1.06	13	69	2.80	1.01	3.67	0.00	0	0	69	0	100
P	.53	2	15	3.16	2.66	5.80	3.67	0	11	1	3	80
P	1/3	2	9	2.58	1.86	2.67	1.33	1	2	4	2	66
P	1/6	1	6	2.20	2.40	2.50	1.87	1	2	1	2	50

Table 1. (Continued)

Chemical	Conc. in ug/ml	Total no.		Average diameter		Average no. feathers		ND	S	C	Stim.	Percent inhibition of feathers
		em-bryos	cul-tures	control	treated	control	treated					
DB	1666	3	19	2.78	2.82	4.32	4.26	5	8	0	6	42.1
DB	833	3	15	3.26	3.12	4.73	4.60	7	4	1	3	33
DB	500	3	16	2.53	2.56	2.74	2.56	8	4	1	3	31.2
DB	333	3	14	2.75	2.74	2.28	2.64	5	3	0	6	21.4
DB	166	2	9	2.78	2.75	3.22	2.78	3	2	1	3	33
DB	83.3	2	11	2.96	2.93	3.64	2.92	1	5	1	4	54

ND = no difference

S = reduction in size and number

C = complete absence of feathers

Stim. = better growth of feathers

BA = barbituric acid

TRB = 4,5,6-(5,6,7)-trichloro-1-(3-D-ribofuranosyl)-benzimidazole

DTP = dithiopyrimidine

T = 2,4,6-triaminopyrimidine

B = bromouracil

NU = nitrouracil

IO = isoorotic acid

P = puromycin

DB = diethylbarbituric acid

numerous and smaller than the controls. Alkaline phosphatase was active, but splotchy, in the centers of affected loci. Histologically, phosphatase-positive granules were found between cells. The nuclei were also phosphatase-positive. Dot-like deposits of the enzyme appeared where there should have been a build-up of alkaline phosphatase into a feather locus. Peripherally, each feather locus seemed to be broken into smaller centers of phosphatase activity, as though the morphogenetic field had lost control over the outlying zones (Figs. 5-7).

Serial sections of cultures treated with barbituric acid showed just the beginnings of feathers. There was some outgrowth, but there seemed to be a lack of organization into definitive feather germs. The distribution of ribonucleic acid was comparable to the control. The most striking effect was found in the nucleoli. Normally, there are two per nucleus, small and spherical or ovoid in a control feather (Fig. 1). In treated cultures the nucleoli of the epidermis were very prominent, typically single and enlarged (Fig. 2). The same situation was seen in the nucleoli of the pulp, but not as often. Multiple nucleoli were also observed (Fig. 3). They were smaller and not as prominent as the single type.

Dithiopyrimidine (DTP) arrested growth of feathers at a concentration of 333 µg/ml. Lower levels had no significant effect. Treated cultures showed a variety of conditions:

(1) some positive reaction for alkaline phosphatase in the fibroblasts, (2) localization of the phosphatase within a few feather loci, (3) the explant completely negative for alkaline phosphatase. The most prevalent condition was an inhibition of feathers with a corresponding diminution in the amount of alkaline phosphatase (Figs. 9 and 10).

In contrast to barbituric acid, sectioned material of cultures treated with DTP showed no effect on the nucleoli. The amount of ribonucleic acid was also comparable to the control. There was considerable necrotic material localized in the pulp. The organization of the prospective feather germ was very poor. Cells were haphazardly arranged, not organized into structural units. Typically, the epidermis had begun to protrude to form a feather, but there was little organization within it. The destructive effect seemed to be concentrated in the pulp, with a concomitant lack of organization in the epidermis (Fig. 4).

4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole (TRB). Concentrations of 83.3 and 41.6 $\mu\text{g/ml}$ completely stopped growth and differentiation of feathers. Alkaline phosphatase was present but not localized in feather loci (Figs. 8 and 11). Lower levels (20 $\mu\text{g/ml}$) permitted growth but totally suppressed the development of feathers. Phosphatase was diffuse throughout the original explant. A few feather areas were present, but they were splotchy in

appearance. The feather locus appeared broken up into little centers within a larger one (Figs. 12-14), as though there had been some weakening and subdivision of the original field. Concentrations of 10 $\mu\text{g/ml}$ still produced some inhibition, but not as much as the higher levels. At this level feather growth and the appearance of phosphatase approached the conditions in the control. Some feathers were nearly normal in morphology and histology. Others had definite centers surrounded by an encampment of little centers much the same as occurred at 20 $\mu\text{g/ml}$. Levels below 10 $\mu\text{g/ml}$ produced no significant effect; growth, morphology, and histochemistry of feathers appeared normal.

Sectioned material of cultures treated with TRB (20 $\mu\text{g/ml}$) showed a much-thickened epidermis above the pulp. The only sign of an epidermal response to the underlying pulp was a nodular aggregation of cells above the dermis (Figs. 15 and 16). Sections stained with toluidine blue contained basophilic material in both the epidermis and the pulp (compare with the control, Fig. 15). The pulp was intact, not necrotic.

The nuclei were elongated and boat-shaped, not characteristically oval in shape. The nucleoli were single and multiple (Figs. 30-32). The epidermal nuclei contained single large and many smaller nucleoli. The majority of the nuclei of the epidermis and pulp contained 4-6 nucleoli; they were

small and sometimes lined up in chains. The epidermal nucleoli were not as large and prominent as those seen in the cultures treated with barbituric acid.

A striking situation resulted when sections were treated with ribonuclease prior to staining with toluidine blue. In a control section stained with toluidine blue alone (Fig. 17) the epidermis was heavily basophilic, mainly in the cytoplasm. There was quite an accumulation of basophilia in the cytoplasm of those epidermal cells adjacent to the pulp. The cytoplasm of the cells in the pulp was lightly stained. Nuclear (and nucleolar) basophilia was about as intense in the epidermis as in the pulp. In a control feather treated with ribonuclease and then toluidine blue (Fig. 18), all of the cytoplasmic basophilia next to the basement membrane had disappeared, indicating that this material was ribonucleic acid. This is in accord with the findings of Koning and Hamilton (1954). The remaining portion of the epidermal cytoplasm was devoid of basophilia, as was that of the pulp. In the latter case there was strong metachromasia in the cytoplasm in the form of very fine strands. There was a certain amount of residual basophilia left after treatment with the enzyme; this resided in the nuclei of both the epidermis and the pulp, but was stronger in the epidermis. The nucleolus was positive too, although this was sometimes obscured by the strong basophilic reaction throughout the nucleus itself. In a culture treated with TRB

and stained with toluidine blue, the basophilia was localized in the nucleus, and little was present in the cytoplasm (Fig. 19). The nuclei were positive, including the nucleoli. The small amount of basophilia found in the cytoplasm was less than that of the control. There was no build-up of basophilic material next to the basement membrane comparable to the control (compare Figs. 17 and 19). Nuclear basophilia was also less than in the control. Treatment of TRB sections with ribonuclease removed almost all of the basophilia. The cytoplasm was completely negative (Fig. 20). Nuclear basophilia was absent except for that found in the nucleoli, and even there was not as prominent as before treatment with ribonuclease.

Proteins. A control feather stained with mercuric bromophenol blue showed a general distribution of protein. It was present in approximately equal quantities in the cytoplasm and the nuclei. Because of this it was difficult to see the nucleus. The distribution of protein seemed heavier in the epidermis, with slightly less in the pulp. A treated culture contained about the same amount of protein. The thickened epidermis was slightly darker than the underlying dermis. In general, the amount, distribution, and localization of protein in a TRB-treated culture were comparable to a control. Treatment of sections with ribonuclease prior to application of the protein stain did not result in a significant difference

between these and the normally stained sections. If anything, the sections treated with ribonuclease were a little darker. Sections stained with bromphenol blue (without mercury) were equal in color to those stained with mercuric bromphenol blue.

Puromycin stopped growth at concentrations of 333, 166, 83.3, 41.6, 20.8, 10.4, 5.2, and 1.7 $\mu\text{g/ml}$ (see Table 1). Such levels were probably toxic. At 1 $\mu\text{g/ml}$ there was some "growth" (outward migration of cells), but no differentiation. Lower levels (1/2; 1/3 and 1/6 $\mu\text{g/ml}$) were less effective. Growth was comparable to that of the control and the feathers were normal in their morphology and distribution.

The levels of puromycin that were toxic still exhibited a positive reaction for alkaline phosphatase. Histologically, the explants were nothing more than cellular debris. Some whole cells were present, but most of the tissue was degenerate. At 1.7 $\mu\text{g/ml}$ there was some indication of cellular survival. Tissue debris remained, but not nearly the amount found at the higher concentrations. The cells were elongated instead of rounded and necrotic, thus showing that they were still living. The alkaline phosphatase was that of living cells, not necrotic ones. This was the first level where there was a good reaction for the enzyme in feather centers. Cultures treated with 1 $\mu\text{g/ml}$ contained no feathers, but did show a little growth. The cells were intact and organized. Some phosphatase was evident in definitive feather loci

(Figs. 25 and 26). Levels below 1 $\mu\text{g}/\text{ml}$ permitted normal growth and differentiation.

Sections treated with puromycin exhibited various abnormalities including: (1) necrosis in the pulp, indicative of preferential destruction of this layer, (2) strong basophilia in the epidermis, mostly of nuclear origin with little in the cytoplasm, (3) an intact, but definitely thickened epidermis (Figs. 21 and 22) which showed some outgrowth (at least an indication of the epidermis pushing out from the skin). In such cases there was no accumulation of basophilia (RNA) in the cytoplasm of the epidermis (Figs. 23 and 24). In the only case which had a feather in the treated culture, the basophilia of the epidermis was definitely nuclear, with almost none in the cytoplasm. Many of the epidermal nucleoli were single and enlarged (Fig. 28). Numerous nuclei of the pulp contained multiple nucleoli (Fig. 29). These conditions were in contrast to the usual appearance of control nucleoli (Fig. 27).

Ribonuclease-treated sections of control feathers exhibited the same picture previously described for control feathers under the TRB section. The main points of this description include: (1) strong build-up of RNA in the cytoplasm of the epidermal cells adjacent to the pulp, (2) residual basophilia (pale blue in color) localized in the nuclei of the pulp and the epidermis. Ribonuclease removed almost all of

the basophilia from cultures treated with puromycin. Some residual basophilia was left in the nuclei. The intensity and distribution of this basophilia were comparable to the control.

As previously mentioned, protein was of general distribution in a control feather, but a little darker in the epidermis. Cultures treated with puromycin presented the same histological picture whether stained with toluidine blue or mercuric bromphenol blue. The thickened epidermis was heavily colored by the protein stain, indicating the presence of a considerable amount of substance. The intensity and localization of this stain were comparable to the control.

2,4,6-triaminopyrimidine completely suppressed the growth of feathers at high concentrations (1666 and 833 $\mu\text{g/ml}$). At such levels the chemical was probably toxic. Evidence for this is seen in Table 1. The overall growth of the treated culture was much below that of the control. Total inhibition of feathers was accompanied by a negative reaction for alkaline phosphatase. Lower concentrations produced no outstanding differences between the control and treated cultures. There was some inhibition of feathers at the lower levels, but this was slight. Such cases of inhibition showed a corresponding diminution of alkaline phosphatase.

Bromouracil, nitrouracil, isoorotic acid and diethylbarbituric acid were inhibitory in only about fifty percent of

the cases regardless of the level tested. Even high concentrations of the chemicals had no marked effect (Table 1). Histologically, the control and treated feathers were similar in their number, distribution and morphology. Histochemically, there was no difference in the two types of cultures.

Counteracting the Effects of TRB and Puromycin

Attempts were made to counteract the effects of TRB and puromycin with the naturally occurring nucleic acid components, adenosine and adenylic acid. In these experiments four pieces of tissue constituted one set, viz: a control, a piece treated with TRB or puromycin, a piece treated with adenosine or adenylic acid, and finally, a culture treated with TRB plus adenosine or puromycin plus adenylic acid.

Eleven sets of cultures containing TRB and adenosine were made with equivalent concentrations of each chemical (25 $\mu\text{g}/\text{ml}$). In these instances there was neither growth nor differentiation in the culture containing TRB and adenosine (see Table 2). Sixteen additional sets of cultures were made containing TRB and adenosine, with the concentrations of TRB and adenosine equalling 25 and 50 $\mu\text{g}/\text{ml}$, respectively. Raising the concentration of adenosine to this amount permitted some growth, but no development of feathers, in the presence of both chemicals. There was no growth in the cultures containing only TRB.

Thirteen sets of cultures contained final concentrations

Table 2. Counteracting the effects of TRB and puromycin

Number of em- cases bryos		Average number of feathers				Average diameter of culture				Conc. of chem. in $\mu\text{g/ml}$		Better growth diff.	
		con- trol	TRB	A	T+A	con- trol	TRB	A	T+A	TRB	A		
11	3	3.36	0	3.36	0	2.85	1.0	2.95	1.0	25	25	none	none
16	4	2.17	0	1.81	0.12	2.66	1.14	2.73	1.67	25	50	13/16	1/16
em- cases bryos		con- trol	P	AA	P+A	con- trol	P	AA	P+A	P	A		
13	4	3.70	0.31	3.10	0.77	2.67	1.03	2.60	1.47	1	1	11/13	5/13

TRB = 4,5,6 (5,6,7)-trichloro-1(β -D-ribofuranosyl)-benzimidazole

A = adenosine

T+A = TRB and adenosine in the same culture

P = puromycin

AA = adenylic acid

P+A = puromycin and adenylic acid in the same culture

or $-1 \mu\text{g/ml}$ each of puromycin and adenylic acid. There was better growth of the explant in the majority of cases (see Table 2), but the differentiation of feathers was improved in only five out of thirteen cases.

DISCUSSION

Barbituric acid (BA). As was noted previously, barbituric acid has been found to retard the development of frog embryos, particularly the central nervous system (Brachet, 1945, 1947). Histochemical tests showed less ribonucleic acid than normal in the tissues of the treated embryos. Hisaoka and Hopper (1957) were unable to repeat these results using barbituric acid on the zebra fish. The slight response was attributed to poor penetration of the chemical through the chorion.

Other barbiturates have been used in a study of the alkaline phosphatase activity of various tissues of the rat (Manzini and Zoboli, 1953). The compounds increased activity of the enzyme in the brain and liver, but decreased it in the kidney.

Curtis (1947) employed several derivatives of barbituric acid in an investigation of the development of orchid embryos in a culture medium. Many of the treated embryos produced large, undifferentiated, cell masses instead of normally-organized seedlings. The nuclei of the treated cells were much larger and stained less intensely than in the normal embryos, and the total cell mass was considerably larger.

Feathers grown in tissue cultures containing barbituric acid produced results which agreed with some of the above literature and others that were entirely unexpected. Although

the total amount of enzyme activity was diminished, the principal effect of barbituric acid was on the distribution rather than the amount of alkaline phosphatase. The enzyme was localized in feather loci. However, within individual feather fields there were smaller centers of phosphatase activity (Figs. 5-7), giving the appearance that the original morphogenetic field had broken up and lost control over the outlying zones. None of the smaller centers appeared stronger than its neighbors. Contrary to the undifferentiated condition found in the orchid embryos, there was differentiation of feathers in our explants. There was, however, a lack of organization into definitive feather germs in some of the feather loci.

Sections of treated cultures stained for ribonucleic acid showed a considerable change in the nucleoli: in contrast to Brachet's work, there was no decrease or inhibition of ribonucleic acid, but the nucleoli were either single and enlarged or multiple (Figs. 2 and 3). These results might indicate a disturbance in the metabolism of RNA (see later discussion). Structurally, barbituric acid is a close relative of the naturally-occurring pyrimidine base, uracil. It is possible that such an analogue might have substituted for uracil and formed a special type of RNA, which could not be used by the feather for normal growth and differentiation.

Dithiopyrimidine (DTP) is also an analogue of uracil. It did not increase the reaction for alkaline phosphatase in

either the original explant or the peripheral fibroblasts, but did arrest the growth of feathers at a concentration of 333 $\mu\text{g/ml}$ (Figs. 9 and 10). There was a slight decrease in enzyme activity, but this was probably due to the decrease in the number of feathers. In contrast, the monosulfur derivative, thiouracil, greatly increased the reaction for alkaline phosphatase in the outlying fibroblasts, but had no effect on the development of feathers (Gibley and Hamilton, 1959).

As with thiouracil, DTP had no effect on the nucleoli or the content of ribonucleic acid in sectioned material. It did, however, cause destruction of part of the pulp, accompanied by degenerative basophilia. Since the mesoderm controls events in the epidermis, it is not surprising that the organization of feathers in the presence of DTP was poor. The ectoderm had begun to protrude to form a feather, but it lacked the capacity to continue (Fig. 4, note the necrosis in the pulp). Exactly how dithiopyrimidine affected the pulp cannot be stated at this time. As in the case of thiouracil, it might have interfered with the metabolism of nucleic acids by forming an aberrant RNA. Such a molecule, because of the substitution of the analogue in its nucleotidic sequence, might be unable to direct synthesis of proteins of the right type for continued differentiation.

4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole (TRB), and the dichloro form, DRB, have been used to

inhibit the multiplication of influenza virus (Tamm, 1954). On a molar basis, TRB had eight times greater activity than DRB. It has been suggested that DRB affected the metabolism of nucleic acid (Tamm, Folkers, Shunk and Horsfall, 1954). In 1957 Tamm discovered that DRB interfered with the metabolism of RNA by reducing the uptake of 8-C¹⁴ adenosine into RNA. The effect on the synthesis of protein was slight. Allfrey, Mirsky, and Osawa (1957) also reported that DRB interfered with the formation of ribonucleic acid. They suggested that DRB inhibited the synthesis of protein by interfering with a preliminary synthesis of RNA. DRB has been reported to inhibit the reproduction of influenza virus by disturbing the metabolism of RNA in infected tissues (Tamm, Folkers, and Shunk, 1956a).

In our experiments, high concentrations of TRB (83.3 and 41.6 µg/ml) stopped growth and differentiation of feathers. Such levels were probably toxic. The chemical did not appear to inhibit the production of alkaline phosphatase. However, at lower concentrations (20 and 10 µg/ml) it did interfere with the distribution of the enzyme in a manner similar to barbituric acid. The feather locus contained little centers of phosphatase activity as though there had been a weakening and subdivision of the original field (Figs. 12-14).

Sectioned material showed that there had been some reaction of the epidermis to the underlying pulp. The epidermis

was thickened and there was a small nodular mass of cells wholly above the basement membrane in the center of the feather locus (Figs. 15 and 16). The structure of the nucleus was changed as shown by the appearance of single and multiple nucleoli in the treated cultures (Figs. 30-32). Despite the observation that halogenated derivatives of benzimidazole interfere with the metabolism of ribonucleic acid (Tamm, Folkers and Shunk, 1956b) a complete inhibition of RNA was not noted in our experiments. True, RNA did not accumulate next to the basement membrane as in the control (compare Figs. 17 and 19). However, some RNA was present because a considerable amount of basophilic material was removed from TRB cultures treated with ribonuclease (compare Figs. 19 and 20). Most of the RNA appeared localized within the nuclei. In summary, cultures treated with TRB contained some nuclear RNA, but practically no cytoplasmic ribonucleic acid. Evidently, there had been some synthesis of RNA in the nucleus and either no movement of it to the cytoplasm or no independent synthesis of RNA in the cytoplasm. (For a discussion of nuclear-cytoplasmic relationships see a later part of this discussion).

Another peculiarity of cultures treated with TRB was the absence of residual basophilia after treatment with ribonuclease (except for the nucleoli) (Fig. 20). A control feather has a certain amount of basophilic material left in the nuclei of the epidermis and pulp (Fig. 18). Just what

constitutes this basophilia has not been ascertained at the present time, although it might be some substance of an acidic nature such as acid protein.

The amount and distribution of proteins were comparable in controls and cultures treated with TRB. At first glance it would seem that TRB had no effect on the synthesis of protein. However, it is possible that the protein stained in the treated cultures was already present before the addition of the chemical. Addition of TRB could have prevented the formation of new proteins, thereby stopping further morphogenesis. No difference was noted between staining with mercuric bromphenol blue and bromphenol blue alone. Without mercury, a typical picture of acid staining is observed (Mazia et al., 1953), and only basic protein is stained. Since there appeared to be no difference with either method, it seems that only basic protein was present. This contradicts the above-mentioned possibility that the residual basophilia might be due to acid proteins. However, we should not overlook the possibility that the amount of acid protein present might be insufficient to give a difference with either stain.

The suggestion has been made that TRB interfered with the appearance of RNA in the cytoplasm either by preventing the release of RNA from the nucleus or by stopping the formation of RNA directly in the cytoplasm itself. It was also suggested that the synthesis of new protein was inhibited. How

might these possibilities be explained?

DRB and TRB are structurally related to adenosine, the normal nucleoside derivative of RNA (Tamm, 1957, 1958). Adenosine has been found to block the action of DRB (Tamm, 1957), indicating that DRB and adenosine are antagonists. In our experiments there was some indication that adenosine could prevent the effects of TRB, because additions of adenosine permitted growth in the TRB-treated cultures. It would seem, then, that TRB acted as an antimetabolic agent. Being an antagonist of the naturally occurring nucleoside, adenosine, TRB could have been incorporated into RNA, forming an aberrant molecule incapable of performing the usual metabolic functions of RNA. Such an abnormal RNA could have stopped the transfer of materials from the nucleus to the cytoplasm, and prevented the accumulation of ribonucleic acid normally seen along the basement membrane in a control feather. This could have restricted the synthesis of new protein. If, on the other hand, there was an independent synthesis of RNA in the cytoplasm, and no transfer from the nucleus, how did TRB affect cytoplasmic synthesis? Previous research (see beginning of this discussion) has shown that DRB (and possibly TRB) directly interfere with the incorporation of various nucleic acid components into the RNA molecule. If this were true in the cytoplasm of our cultures, then TRB might have stopped the production of RNA within the cytoplasm itself and prevented the

accumulation near the basement membrane. Whatever the mechanism, the diminution of RNA in the cytoplasm could easily limit the production of new proteins and stop morphogenesis.

Puromycin. As mentioned earlier, puromycin inhibited the growth and multiplication of Endamoeba histolytica (Nakamura and Jonsson, 1957). The inhibition was completely reversed with adenylic acid, partially with adenine, not at all with guanine. It was suggested that puromycin blocked the synthesis of nucleic acids in the amoeba. The inhibition of the growth of Tetrahymena by puromycin (Bortle and Oleson, 1955) was reversed by guanylic acid. It would thus appear that the metabolic block is a competitive action and is present in the direct pathway of the synthesis of nucleic acid by this protozoan. Additional investigations (Agosin and von Brand, 1954; Hewitt et al., 1954; Hutchings, 1957) have provided further evidence for the hypothesis that puromycin interferes with purine metabolism.

In this investigation puromycin stopped growth at concentrations of 333 to 1.7 $\mu\text{g/ml}$. Undoubtedly, such levels were toxic, because histological examination showed nothing except cellular debris. At 1 $\mu\text{g/ml}$, growth had been initiated, but there was little differentiation. This concentration seemed to be optimal for inhibiting feathers; at lower levels, growth and differentiation were normal. None of the concentrations stopped the activity of alkaline phosphatase, although there

was a definite decrease in the amount and only a slight localization of the enzyme within feather loci.

Sectioned material showed a decrease in RNA. This was most evident in the epidermis. In most cases the treated cultures had a thickened epidermis (Fig. 22); others showed some outgrowth of feathers. Even in those instances where there had been outgrowth, there was a lack of basophilia (RNA) in the epidermis, when compared with a control of the same stage of development (Figs. 23-24). Coincident with this decrease in the amount of RNA were abnormalities of the nucleoli (Figs. 27-29). Once again there seems to be a parallel relationship between an upset in the RNA content and changes in nucleolar structure.

In Pseudomonas fluorescens, strain A₃₋₁₂, puromycin did not inhibit the synthesis of ribonucleic acid, whereas it completely abolished the formation of protein (Takeda, Hayashi, Nakagawa, and Suzuki, 1960). The incorporation of P³²-orthophosphate into RNA was not affected significantly. The RNA formed in the presence of puromycin was stable in growing and resting cells. Contrary to expectation, there did not appear to be less protein in our treated cultures than in the controls. Apparently initial induction had taken place, for the epidermis was thickened and showed some feather outgrowth. Consequently, there had to be some formation of protein (probably from RNA that was present before the chemical was added

to the system). The addition of puromycin might have stopped the synthesis of protein after the initial stock of RNA was exhausted. This would, in turn, affect further growth and morphogenesis.

Attempts to counteract the effects of puromycin with adenylic acid were only partially successful. There was a definite indication of better growth, and some signs of better differentiation, when the adenylic acid was added to the same culture containing puromycin. Thus, it appeared that the antibiotic was antagonistic to adenylic acid. Such antagonism might have affected the incorporation of nucleic acid components into RNA. This could have halted the formation of new ribonucleic acid and secondarily limited the synthesis of new proteins.

5-nitrouracil has been found to be antagonistic to either uracil or thymine in Streptococcus (Puleston, Poe, and Witt, 1955). Nitrouracil has also been found to affect folic acid, while only slightly affecting thymine (Hitchings, Elion, and Falco, 1950). The inhibition of the growth of L. casei by nitrouracil has been attributed to folic acid antagonism (Hitchings et al., 1950). Shive (1950) counteracted the effect of nitrouracil in L. casei with uracil, rather than folic acid.

In our experiments nitrouracil had no significant effect on the growth and differentiation of feathers at any of the

concentrations tested. Although nitrouracil has been found to be antagonistic to uracil, it would appear that this was not the case in this investigation. If, on the other hand, nitrouracil were antagonistic to folic acid, it would not be expected to affect uracil or RNA directly. As a result, normal growth and differentiation would continue in the presence of nitrouracil.

5-bromouracil. The inhibition of E. coli 15T⁻ due to bromouracil was counteracted by thymine (Dunn and Smith, 1957). These authors also found that bromouracil had been incorporated into the DNA of the inhibited bacteria. Similar results have been obtained in other strains of E. coli by Zamenhof and Griboff (1954); Zamenhof, DeGiovanni and Rich (1956). In addition, bromouracil has been found to be antagonistic to thymine in other bacteria; Lactobacillus leichmannii (Bardos, Levin, Herr, and Gordon, 1955); L. casei (Hitchings et al., 1950). Weygand, Wacker, and Dellweg (1952) also observed that bromouracil was incorporated into bacterial DNA.

From the literature it appears that bromouracil is an antagonist of thymine and can be incorporated into DNA, but not RNA. The latter seems to play the important role in the formation of feathers. This might explain why the chemical had no effect on the development of feathers at any of the concentrations tested, in the short time that the cultures were under observation. It probably affected the DNA, not the RNA.

THE NUCLEOLUS, GROWTH, AND DIFFERENTIATION

Probably the most striking effect of the analogues used in this and a previous investigation (Gibley and Hamilton, 1959) was the change in the structure of the nucleolus. Most of the compounds tested (orotic acid, 8-azaguanine, isoguanine sulfate, barbituric acid, TRB, and puromycin) changed the shape, size, or number of the nucleoli in the cells of the treated cultures. These chemicals also had an effect on the metabolism of ribonucleic acid. Some of them decreased the amount of RNA in the cell; others may have produced an unnatural RNA molecule. Similar results have been obtained by other investigators. β -2-thienylalanine (an analogue of phenylalanine) produced large, swollen nucleoli in the epidermal cells of inhibited feathers (Fabiny, 1959). Kischer (1960) observed large nucleoli in feather cultures treated with sodium cyanide.

In viral studies, the nucleoli of infected cells treated with 5-fluorouracil were enlarged (Pollard, Starr, Tanami, and Elliott, 1960). It was suggested that fluorouracil caused accumulations of abnormal RNA-staining material which inhibited multiplication of the virus. The incorporation of fluorouracil into RNA to form an unnatural nucleotide has also been proposed by Dagg (1960). Actinomycin decreased the RNA in non-malignant and malignant cells (Rounds, Nakanishi, and Pomerat, 1960). There was a corresponding reduction in the

size of the nucleoli. An enlarged nucleolus has been considered a major criterion of a rapidly growing tumor (MacCarty, 1936). Inhibition of cellular growth by ribonuclease also produced modifications of the nucleolus while repressing or completely preventing nucleolar and cytoplasmic basophilia (Chévremont, Chévremont-Comhaire, and Firket, 1958). Variations in metabolism have produced changes in the size and number of nucleoli (Swift, 1959).

These results seem to indicate that modifications of the nucleolus accompany changes in the normal growth and differentiation of the cell. Usually, there is also a corresponding effect on ribonucleic acid. Apparently, the nucleolus and RNA are involved in the growth and differentiation of the cell. The close relationship between RNA and the nucleolus in these processes is not too surprising since there is increasing evidence that some synthesis of RNA takes place in the nucleolus.

How could modifications in the metabolism of RNA manifest themselves in structural abnormalities of the nucleolus? Antimetabolites and analogues of naturally-occurring metabolites could cause an accumulation of aberrant RNA in the nucleolus which might lead to its enlargement. Similar suggestions have been made by Fabiny (1959) and Pollard et al., (1960). In addition to the change in size, other abnormalities of the nucleolus found in our experiments included a change in shape and vacuolization. If an abnormal RNA were

formed by these analogues, this could change the structure of the nucleolus. Of the two types of RNA found in the nucleolus, one was bound to the structural framework (Pollister and Leuchtenberger, 1949). The other type was soluble in acid solutions. An abnormal RNA could form in either type. This could produce structural changes in the nucleolus. If the abnormal, soluble type passed on to the cytoplasm, it could very well interfere with normal growth and differentiation. It is apparent from the literature that the nucleolus has a high synthetic activity and a rapid turnover rate. For example, the nucleolus incorporated glycine about a hundred times more rapidly than the cytoplasm in starfish oocytes (Ficq, 1953). With such a constant turnover it would not be difficult to visualize the incorporation of analogues into both types of nucleolar RNA.

The next question to be answered is how nuclear RNA could affect growth and differentiation via the cytoplasm? There is increasing evidence in the recent literature for the movement of materials (including RNA) from the nucleus to the cytoplasm; and also, that nuclear RNA is the precursor of cytoplasmic RNA.

Cytological studies have shown that nucleolar material is transferred to the cytoplasm either in the form of small or middle-sized nucleoli or by the emptying, through the nuclear membrane, of the complex nucleoli (Dalcq, 1955). There are

numerous reports of the synthesis of RNA in the nucleus and its subsequent movement to the cytoplasm (Paul and Hagiwara, 1960; Perry, 1960; Perry, Hell, and Errera, 1960; Plaut, 1960). Radioautographic studies lend support to the hypothesis that the RNA of the nucleolus is the precursor of cytoplasmic RNA (Goldstein and Micou, 1958, 1959; Leblond and Amano, 1960; Woods and Taylor, 1959).

In the present study it was suggested that an abnormal RNA formed in the nucleolus could alter the control that the nucleus normally has over the cytoplasm. Such a "different" RNA might misdirect the synthesis of cytoplasmic constituents, especially the proper type of protein, or stop the formation of protein altogether, thus prohibiting further growth and differentiation. Brachet (1957) has suggested that cancer cells contain a special type of RNA, the presence of which would lead to alterations in the mechanisms of protein synthesis. The possibility that the nucleus exerts such control over development has also been suggested by Sirlin (1959). In a study of the differentiation of nuclei in the amphibian blastopore he discovered that these nuclei had an enhanced metabolism of RNA. He suggested that the nuclei of the blastopore might initiate the differentiation attributed from classical embryological experiments to the region as a whole.

In conclusion, there appears to be a correlation involving the nucleolus and RNA in the growth and differentiation of

the cell. The probability is good that the nucleolus (acting through RNA) plays a role in the normal embryological processes of growth and differentiation of the down feather.

SUMMARY

1. The growth of feathers was inhibited by the addition of barbituric acid, dithiopyrimidine, puromycin, and 4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole to tissue cultures in concentrations varying from 833 μ g/ml to 1 μ g/ml. Isoorotic acid, 2,4,6-triaminopyrimidine, 5-bromouracil, 5-nitrouracil, and diethylbarbituric acid had no significant effect.
2. Barbituric acid was inhibitory only at high concentrations (833 μ g/ml), and then not consistently. Alkaline phosphatase was active, but splotchy, in the centers of affected feather loci. Peripherally, each feather area was broken into smaller centers of phosphatase activity, as though the morphogenetic field had lost control over outlying zones. The distribution of ribonucleic acid was comparable to the control. The most striking effect was on the nucleoli. These were either single and enlarged or multiple. One possible explanation for these effects is that barbituric acid substituted for uracil and formed a special type of RNA which could not be used by the feather for normal growth and differentiation.
3. Dithiopyrimidine arrested the growth of feathers with a corresponding diminution in alkaline phosphatase. It had no effect on the nucleoli or the content of ribonucleic acid. The destructive effect was concentrated in the

pulp, with concomitant lack of organization in the epidermis. Dithiopyrimidine might have interfered with the metabolism of nucleic acids, forming an aberrant RNA. Such a molecule, because of the substitution of the analogue in its nucleotidic sequence, might be unable to direct the synthesis of proteins of the right type for continued differentiation.

4. 4,5,6 (5,6,7)-trichloro-1-(β -D-ribofuranosyl)-benzimidazole stopped growth at higher concentrations (83.3 and 41.6 $\mu\text{g/ml}$). Phosphatase was diffuse throughout the original explant. Lower concentrations (20 $\mu\text{g/ml}$) permitted growth, but not differentiation. Many feather loci were subdivided into smaller centers, indicating weakening of the original field. The nucleoli were either single or multiple. TRB decreased the amount of ribonucleic acid in the nucleus and cytoplasm of treated cultures. It was suggested that the analogue interfered directly with the synthesis of RNA or formed an aberrant molecule incapable of performing the usual metabolic functions of RNA. In either case the diminution of RNA could easily limit the production of new proteins and stop morphogenesis.
5. Puromycin stopped growth at concentrations of 333 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$. Undoubtedly, such levels were toxic. At 1 $\mu\text{g/ml}$ growth had been initiated, but there was little differentiation. Alkaline phosphatase was active, but there was

a decrease in the amount and only a slight localization of the enzyme within feather loci. Treated cultures showed a decrease in RNA, particularly in the epidermis. Nucleoli were single and enlarged or multiple. Puromycin stopped the formation of feathers after initial induction. The addition of the puromycin might have halted the formation of new RNA and secondarily limited the synthesis of new proteins.

6. The effects of TRB and puromycin were only partially counteracted by the addition of adenosine and adenylic acid, respectively.
7. The results of this and a previous investigation indicate that modifications of the nucleolus accompany changes in the normal growth and differentiation of the cell. Usually, there is a corresponding effect on ribonucleic acid. It may well be that the nucleolus (acting through RNA) plays a role in the normal embryological processes of growth and differentiation in the down feather.

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PLATES

Plate 1

Explanation of Figures

- Fig. 1. Part of a cross section of a feather from a control culture of skin from an embryo of stage 31⁺. Sectioned at 7 μ and stained with toluidine blue. The nucleoli, which usually number two per nucleus, are small and ovoid. X1890.
- Fig. 2. Part of a cross section of a feather from the corresponding bilateral half of the piece of skin shown in Figure 1, grown in the presence of barbituric acid (833 $\mu\text{g/ml}$). Note the single nucleolus in each nucleus. X1890.
- Fig. 3. Part of a cross section of the same piece of tissue shown in Figure 2. Note the numerous nucleoli within the nucleus. X1890.
- Fig. 4. Sagittal section of a piece of tissue from an embryo of stage 32⁻, grown in the presence of dithiopyrimidine (333 $\mu\text{g/ml}$). The pulp is necrotic. X270.
- Fig. 5. Control explant of skin from the back of an embryo of stage 31. Note the strong reaction for alkaline phosphatase in the pulp of each feather, immediately below the growing epidermal tip. X70.
- Fig. 6. The corresponding bilateral half of the piece of skin shown in Figure 5, grown in the presence of barbituric acid (833 $\mu\text{g/ml}$). Peripherally, each feather locus appears broken into smaller centers of phosphatase activity. X70.
- Fig. 7. A higher magnification of the large feather locus shown in the center of Figure 6. This shows the subdivision of the area more clearly. X270.
- Fig. 8. Control explant of skin from the back of an embryo of stage 30⁻, showing normal feathers and a strong phosphatase reaction within the feather germs. X70.
- Fig. 9. The corresponding bilateral half of the piece of tissue shown in Figure 10, grown in the presence of dithiopyrimidine (333 $\mu\text{g/ml}$). There is an inhibition of feathers and a diminution in the activity of alkaline phosphatase. X70.

Plate 1 (Continued)

Fig. 10. Control explant of skin from an embryo of stage 31⁻, showing normal feathers and phosphatase reaction.
X70.



Plate 2

Explanation of Figures

- Fig. 11. The corresponding bilateral half of the piece of skin shown in Figure 8, grown in the presence of TRB (41.6 $\mu\text{g/ml}$). Alkaline phosphatase is present, but not localized within feather loci. X70.
- Fig. 12. Control explant from the back of an embryo of stage 32⁻, showing the strong reaction for phosphatase within the feathers. X70.
- Fig. 13. The corresponding bilateral half of the piece of tissue shown in Figure 12, grown in the presence of TRB (20 $\mu\text{g/ml}$). The phosphatase is localized within feather loci. X70.
- Fig. 14. A higher magnification of one of the feather loci shown in Figure 13. Note the subdivision of the area into smaller centers of phosphatase activity. X270.
- Fig. 15. Sagittal section of a control feather from skin explanted from an embryo of stage 31. Sectioned at 7 μ and stained with toluidine blue. Note the heavy concentration of basophilia (RNA) in the epidermis, especially in the cells which lie adjacent to the pulp. X270.
- Fig. 16. Sagittal section of a culture from the corresponding bilateral half of the piece of skin shown in Figure 15, grown in the presence of TRB (20 $\mu\text{g/ml}$). Note the nodular aggregation of epidermal cells above the pulp. X270.
- Fig. 17. Sagittal section of a control feather from an embryo of stage 32⁻. Sectioned at 7 μ and stained with toluidine blue. The epidermis is very heavily stained. X270.
- Fig. 18. Sagittal section of a control feather from the same piece of skin shown in Figure 17. Sectioned at 7 μ and stained with toluidine blue after prior treatment with ribonuclease. Note the removal of the intense basophilia in the epidermis shown in Figure 17, especially that next to the basement membrane. X270.

Plate 2 (Continued)

- Fig. 19. Sagittal section of a piece of tissue from the corresponding bilateral half of the explant shown in Figure 17, grown in the presence of TRB (20 μ g/ml). Sectioned at 7 μ and stained with toluidine blue. Note the absence of the intense basophilia normally seen in the epidermis. There is very little basophilia in the cytoplasm. X270.
- Fig. 20. Sagittal section of a piece of tissue from the same explant shown in Figure 19. Stained with toluidine blue after treatment with ribonuclease. There is no basophilic material in the epidermis and the pulp. X270.

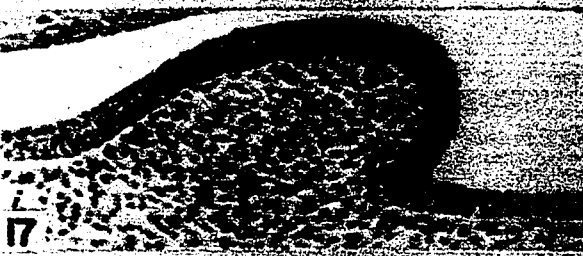
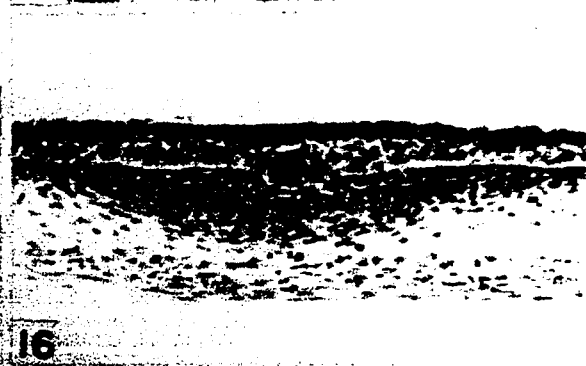
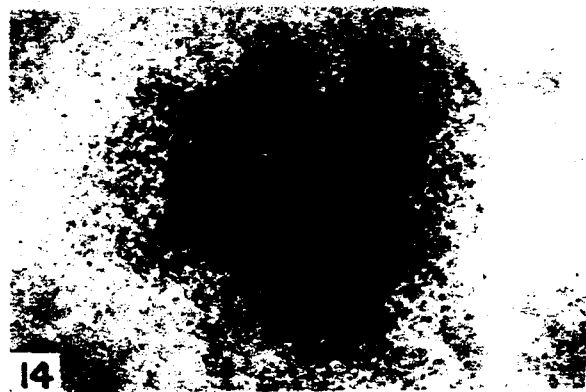
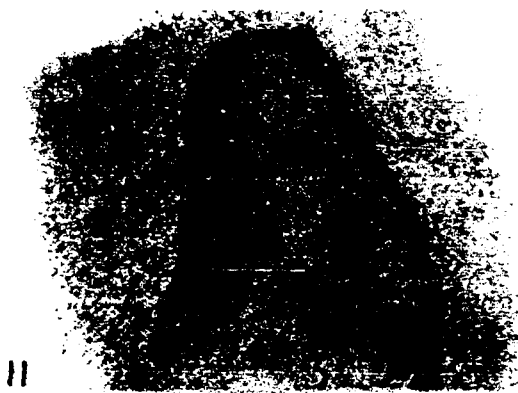


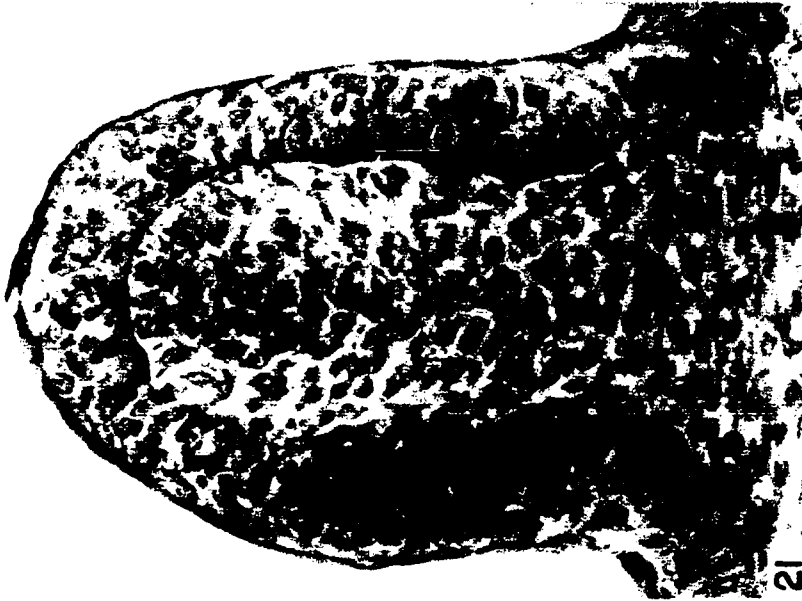
Plate 3

Explanation of Figures

- Fig. 21. Sagittal section of a control feather from an embryo of stage 32. Sectioned at $7\ \mu$ and stained with toluidine blue. Note the large amount of basophilia (RNA) in the epidermis, especially in the cells bordering the basement membrane. X600.
- Fig. 22. Sagittal section of a piece of skin from the back of an embryo of stage 33⁻, grown in the presence of puromycin (1 μ g/ml). The epidermis is thickened, but there is no outgrowth of feathers. Compare with a control, Figure 21. X600.
- Fig. 23. Sagittal section of a beginning feather from an embryo of stage 32⁺. The epidermis, which is thickened and has begun to elevate from the skin, is strongly basophilic. X600.
- Fig. 24. Sagittal section of a piece of skin from an embryo of stage 32, grown in the presence of puromycin (1 μ g/ml). There is a slight protrusion of the epidermis, but a lack of the strong basophilia seen in Figure 23. X600.
- Fig. 25. Control explant of skin from the back of an embryo of stage 32, showing a normal reaction for phosphatase and normal feather growth. X70.
- Fig. 26. The corresponding bilateral half of the piece of skin shown in Figure 25, grown in the presence of puromycin (1 μ g/ml). The reaction for alkaline phosphatase is diffuse. X70.
- Fig. 27. Part of a cross section of the feather shown in Figure 21. The nucleoli are small and usually number two per nucleus. X1890.
- Fig. 28. A higher magnification of Figure 22, showing a single nucleolus in each nuclei. X1890.

Plate 3 (Continued)

- Fig. 29. Part of a cross section of skin from the corresponding bilateral half of the explant shown in Figure 21, grown in the presence of puromycin (1 μ g/ml). The nucleoli are multiple. X1890.
- Fig. 30. Part of a cross section of the feather shown in Figure 15, showing two nucleoli. X1890.
- Fig. 31. A higher magnification of Figure 16 showing a single nucleolus in each nucleus. X1890.
- Fig. 32. A higher magnification of Figure 19, showing multiple nucleoli within the nucleus. X1890.



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