ALKALINE PHOSPHATASE OF THE DEVELOPING DOWN FEATHER:

SUBSTRATES, ACTIVATORS, AND INHIBITORS

by

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1957
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Because of the diversity of phosphatase activity in the chick, it is important to understand the control and regulation of this enzyme. The presence of phosphatase in the uppermost region of the embryo has been found in the developing embryonic structure as teeth, beak, and feet. In the second and fourth day of development, and again at 10 days, it has been observed that there is a decrease in phosphatase activity in the uppermost region of the embryo.

In the development and growth of aliphatic phosphatase between the chick, the same author (1967) proposed the presence of the uppermost region 86 and 87.

In bone (1967) and in chromosomes (Korn, 1966), the presence of phosphatase (deoxyribonuclease, 1967) is found to change the phosphate concentration of the embryo. The role of the developing embryo in the development of the embryo is still under debate and is not yet fully understood. The presence of the uppermost region of the embryo has been found to be important in the development of the enzyme, aliphatic phosphatase, as well as in the development of the embryo.
presence of more than one enzyme has been suspected. Bodansky (1937) reported inhibition of bone and kidney phosphatase but not of intestinal phosphatase by bile salts. Schmidt and Thannhauser (1943) also reported that intestinal phosphatase was unaffected by bile salts. Emmel (1946) reported a difference between kidney and intestinal phosphatases on the basis of inhibition by KCN. The phosphatases in milk and intestinal mucosa are discrete enzymes although they have similar specificities (Morton, 1955). Dempsey and Deane (1946) reported evidence of substrate specificity within tissues which exhibit reactions with one or several substrates.

The activating effect of Mg$^{2+}$ upon alkaline phosphatase was first reported by Erdfman (1928) and has since served as a basis for differentiating enzymes by some workers. Cloetens (1939) identified two alkaline phosphatases, one which was inactive in the absence of Mg$^{2+}$ and one which was active without Mg$^{2+}$. Alkaline phosphatase of the intestine consists of three fractions, two of which become inactivated in the absence of Mg$^{2+}$ (Gomori, 1952). Emery and Dounce (1955a, b) have found two forms of alkaline phosphatase, one not activated by Mg$^{2+}$ and firmly bound in the nuclear fraction, and the other, a soluble enzyme, which is activated by Mg$^{2+}$.

Newman, Feigen, Wolf, and Kabat (1950) demonstrated three groups of enzymes which could be distinguished by their substrate specificity, sites of action, and response to various activators and inhibitors. Maengwyn-Davies and Friedenwald (1950) and Maengwyn-Davies, Friedenwald, and White (1952) differentiated phosphatase systems on the same basis. Gryder, Friedenwald, and Carlson (1955) identified two alkaline glycerophosphatases in the kidney of the rat. One, enzyme A, was inhibited by iodoacetate and
activated by zinc and glycine while the other, B, was not inhibited by
iodoacetate and was activated by magnesium and inhibited by those con-
centrations of glycine and zinc that activated enzyme A.

Gomori (1949a), using 19 different substrates, found that all except
p-chloranilidophosphonate gave a constant and independent reaction in any
given organ and concluded that there was no indication for enzymes other
than the common non-specific enzyme. Bourne (1954), using sugar phosphates,
α-naphthoquinone diphosphate and two chalcone diphosphates, suggested
that there exists within tissues a spectrum of phosphatases with over-
lapping substrate specificities. Burgos, Deane, and Karnovsky (1955) found
that separate enzymes hydrolyze glycerophosphate and naphthylphosphate at
alkaline pH.

Further evidence for the presence of more than one phosphatase and
the specificity of single enzymes within tissues has been reported. Moog
and Steinbach (1955) reported the presence of adenylylphosphatase in the
chick and Padykula and Herman (1955) reported three types of adenosine tri-
phosphatases. Mounter, Dien, and Hanutin (1955) presented evidence of a
number of dialkyl fluorophosphatases.

The distribution of alkaline hexose diphosphatase and neutral mono-
esterases appears to be universal (Deane, 1971). Hexose diphosphatase
exhibited a strict substrate specificity (Gomori, 1943; DeHaas, 1955) for
hexose diphosphate. Hexose diphosphate used as a substrate for phosphatase
showed differences in localisation and time of activation (Zorzoli and
Stowell, 1947; Allen and Bourne, 1943) when compared with alkaline glycero-
phosphatase.

A 5'-nucleotidase has been found in several animal tissues (Reis, 1937)
and is present in the nuclei of cells (Wachstein and Meisel, '52, '54).
The 5-nucleotidase does not attack other monophosphoric esters (Gullard
and Jackson, '38) and has a pattern different from that of other substrates
although there is overlapping in some regions (Gomori, '49b).

Cori and his associates have found that the mobilization of glucose in
the liver from glycogen takes place through a series of enzyme-catalyzed
reactions. One of these reactions involves the hydrolysis of glucose-6-
phosphate to glucose by the enzyme glucose-6-phosphatase. This enzyme has
been partially purified and is without action on a variety of phosphate
esters (Swanson, '50). It has been demonstrated histochemically in the
liver and kidney of the mouse (Chiquoine, '53, '55).

Alkaline phosphatase has been found in a positive core of mesoderm
in undifferentiated feather germs on the hind limb of the 10-day chick
(Mooz, '45). The localization of alkaline phosphatase and glycogen in the
developing down feather from the ninth day of incubation to hatching was
studied by Johnson and Bevelander ('47a). They reported its presence in
the mesoderm of the early feather anlage and its retention in the mesoderm
throughout the development of the feather. It also appeared in the ecto-
derm somewhat later. Koning and Hamilton ('54) described the occurrence
and localization of nucleic acids, polysaccharides and several enzymes,
including alkaline phosphatase, during the growth and morphogenesis of
the down feather. They found that phosphatase was active throughout the
mesenchymal pulp during early stages and that this activity became
localized in the basal pulp in the older feathers.

It is clear from a review of the literature that alkaline phosphatase
occurs at highly active developmental sites in the embryo and in areas of
high synthetic activity (e.g., glands) or regions of transfer (epithelia of intestine, kidney) in the adult. There is also considerable evidence from the use of metallic activators and inhibitors that several enzymes are active in splitting phosphate linkages in an alkaline environment. The present study was undertaken to determine whether one or more alkaline phosphatases may be active within a single developing organ. Sections of developing down feathers were tested for their ability to utilize the phosphate present in several substrates. To further characterize the enzyme system, the activating or inhibiting effects of various chemical substances were studied in connection with the various substrate mixtures.
MATERIALS AND METHODS

Feathers from wings of White Leghorn chick embryos of 11-12 days
(Stages 37- to 38+, Hamburger and Hamilton, '51) were fixed for 24 hours
in cold absolute alcohol containing 5 percent glacial acetic acid (Welman
and Behar, '52). The tissues were dealcoholized in isopentane (Lillie,
'54), embedded in paraffin (m.p. 56-58°C.) containing 10 percent bayberry
wax, and stored in the refrigerator until used. Sections were cut at 8 μ
and mounted on albumenized slides.

Alkaline phosphatase was demonstrated by a modification of the tech-
nique of Gomori (139). The slides were deparaffinized in xylene, coated
with celloidin, and taken to water through a graded series of alcohols.
They were incubated at 37°C. for 1½ hours in a substrate mixture of 2
percent solutions as follows: 20 ml. phosphate source, 20 ml. sodium
barbital, 8 ml. calcium chloride, 2 ml. magnesium sulfate, and 50 ml.
distilled water. Subsequent steps consisted of the replacement of calcium
phosphate with cobalt and conversion of the cobalt phosphate to the sulfide
with ammonium sulfide. The slides were then dehydrated in a graded series
of alcohols, cleared in xylene, and mounted in HSR mounting medium.
Control slides were run in the same manner but with distilled water sub-
stituted for the phosphate source. Langeron's Alizarin Red S (Lillie,
'54) was used to test for the presence of calcium in the tissue.

Fructose-6-phosphate, hexose diphosphate, glucose-6-phosphate, glucose-
1-phosphate, disodium p-nitrophenyl phosphate, yeast adenlylic acid,
muscle adenlylic acid, and sodium glycerophosphate were used as substrates
for phosphatase. The reaction obtained with sodium glycerophosphate was used as a basis by which the reactions obtained with the other substrates might be compared. The pH of the substrate solutions was adjusted to 9.0-9.4 with KOH using a Leeds & Northrup pH indicator. The effect of various activators and inhibitors was tested by adding them in varying concentrations to the aliquot of water in the substrate mixture. The following were tested: Mg++, Mn++, Fe++, Zn++, Fe+++; HAsO₄⁻, HAsO₃⁻, CN⁻, semicarbazide, iodoacetate, Versene, glycine, alanine, cysteine, histidine, lysine, methionine, proline, serine, valine and arginine.

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1Disodium p-nitrophenyl phosphate was obtained from Sigma Chemical Co.; sodium glycerophosphate from Fisher Sci. Co.; glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, hexose diphosphate, yeast adenyl acid and muscle adenyl acid from Nutritional Biochemicals Corp.
RESULTS

Substrates

The following descriptions are based on results obtained when sections of wings from chick embryos of stages 37- to 38+ (11-12 days) were incubated in various substrate solutions. All reactions have been compared with those obtained by using sodium glycerophosphate as a substrate.

Sodium glycerophosphate

With sodium glycerophosphate as a substrate, there was a characteristic pattern of phosphatase activity within the feather. The mesodermal pulp was most strongly reactive. Evidence of bilateral symmetry within the feather was seen in the dorsal and ventral concentrations of phosphatase in the basal area of the pulp. There was some spreading of phosphatase activity into the dermal area beneath the feathers (Figs. 1, 6, 11, and 17).

The cytological distribution of the phosphatase depended on the amount of activity. In areas showing slight activity, phosphatase was found only in the nucleoli. With an increase in activity, the nuclear membranes and chromatin granules also gave a positive reaction. In areas of still greater activity the entire nucleus was solidly black, the cytoplasm became deeply colored, and in some areas the precipitate was so dense as to obscure the identity of individual cells and cellular parts. At no time was cytoplasmic activity greater than that in the nuclei.

The epidermis showed phosphatase activity although always in lesser amount than in the pulp. The nucleoli of the cells gave a positive
reaction. Nuclear and cytoplasmic activity were greatest in the epidermal cells adjacent to the highly-active dorsal and ventral areas of the pulp. In cross-sections of feathers, phosphatase was found in the basement membrane of the barbs, the cylinder cells next to the basement membrane and the pulp (Fig. 14). The cylinder cells showed prominent nuclear activity and some darkening of the cytoplasm.

**Hexose diphosphate**

A comparison of the phosphatase reactions obtained with hexose diphosphate and sodium glycerophosphate showed the distribution of the enzyme to be the same although there was a decrease in the intensity of the reaction with hexose diphosphate (Fig. 12). This lessened activity was apparent in the pulp where it was possible to distinguish the nucleoli and chromatin granules in the nuclei and in the epidermis where the nucleolar activity was most prominent. The cylinder cells showed some nuclear activity and less cytoplasmic color than was found with glycerophosphate.

**Fructose-6-phosphate**

The phosphatase activity with fructose-6-phosphate was much reduced when compared with that obtained with hexose diphosphate and sodium glycerophosphate (Fig. 13). Activity was found in longitudinal sections of the long flight feathers on the edge of the wing and in a smaller number of cross-sections. Activity was both nuclear and cytoplasmic in the pulp, but the epidermal reaction consisted only of slight activity in the nucleoli. The dorsal andventral areas of the base of the pulp showed the greatest phosphatase activity. With fructose-6-phosphate the activity did not extend down to the base of the feather or into the
In substrate mixture lacking a phosphate source to check on the possible
matter which the source of phosphate (CaPO_4, 19) controls it's use in
cartilage cells and in the perichondral region of the developing bone no
strong reaction for alizarine phosphate was always obtained in
A control with no substrate

sodium 2-fluorophosphate (FPO_4, 7, 9, and 18)

the above substrates gave phosphate reaction which were similar

phosphate
Glucose-6-phosphate, glucose-1-phosphate, and sodium-p-nitrophenyl

phenolphthalein

In the epidermis the dark reaction in the nuchal region was particulary
put up and occasionally extended into the dermal area beneath the epidermis

muscle above the spinal cord (Fige. 71) the reaction, both nuchal and cytoplasmic
was found particularly in the ventral and dorsal areas at the base of the

The reaction with yeast adenyllic acid was less intense than that with

Yeast adenyllic acid

esters

research and underhydrated dermal areas while in the experimental group both nuchal and cytoplasmic in the put of the

distillation did not gather than that observed with sodium 2-fluorophosphate

muscle adenyllic acid gave a phosphate reaction which was similar in

muscle adenyllic acid

dermal area beneath the organ.
presence of preformed phosphates. These gave a negative reaction for phosphatase in feathers and bone except for a black ring within the bone (Fig. 10). When control tissues were stained with Alizarin Red S, which is specific for calcium, a scarlet reaction was obtained which indicated that calcium was present in this same area (Fig. 21). Thus, what appeared to be a positive reaction for phosphatase in bone was in part an artifact caused by calcium phosphate deposited in vivo. Moog ('44) also reported such a false positive reaction in control slides and attributed it to the presence of preformed phosphate deposited during the development of the bone.

Activators and Inhibitors

The distribution of the activity of phosphatase in the presence of the various substrates was so similar that it gave little evidence for differential activity, with the possible exception of the activity in the nucleoli of the epidermis with yeast adenylic acid. In order to test further the capacity of the feather phosphatase (or phosphatases) to utilize these substrates, several metallic ions and other substances which were known activators or inhibitors of phosphatase were added to the substrate solution as an aid to further characterizing the enzyme system. Table 1 summarizes the substances used, their molar concentrations, and the substrates with which each was employed.

There was a range of concentrations with each substance which would produce an orderly series of changes in the pattern of phosphatase reactivity. At higher concentrations of the chemicals, there was complete inhibition (c). In some cases slight activity (c-) was found in the pulp
Table 1. Molar concentrations of chemicals affecting the alkaline phosphatase reaction in the developing down feather.

<table>
<thead>
<tr>
<th>Activators and inhibitors</th>
<th>Sodium glycero-phosphate</th>
<th>Fructose 1,6-</th>
<th>Fructose 6-</th>
<th>Muscle adenylc acid</th>
<th>Yeast adenylc acid</th>
<th>Glucose 6-</th>
<th>Glucose 1-</th>
<th>p-nitrophenyl phosphate</th>
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<td>with Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>without Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<td>(0.0016)</td>
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Zn<sup>2+</sup>

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<td>.0001 n</td>
<td>.00001 n-</td>
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CN<sup>-</sup>

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<td>.025 c</td>
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<td>.01 p</td>
<td>.001 p</td>
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<td>.001 n-</td>
<td>.0001 n</td>
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HAsO<sub>4</sub><sup>-</sup>

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<td>.01 c</td>
<td>.001 c</td>
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<tr>
<td>.005 p</td>
<td>.001 p</td>
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<td>.001 n-</td>
<td>.0001 n</td>
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CH<sub>2</sub>COO<sup>-</sup>

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<td>.15 c</td>
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<tr>
<td>.1 n-</td>
<td>.1 n-</td>
</tr>
<tr>
<td>.05 n-</td>
<td>.05 n-</td>
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Semi-carbazide

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<td>.05 p</td>
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<td>.02 n-</td>
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<td>.05 n-</td>
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### Table 1. (Continued)

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<th>Substrates</th>
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<th>Fructose 1,6 phosphate</th>
<th>Fructose 6 phosphate</th>
<th>Muscle adenylie acid</th>
<th>Yeast adenylie acid</th>
<th>Glucose 6 phosphate</th>
<th>Glucose 1 phosphate</th>
<th>p-nitrophenyl phosphate</th>
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<td>Cysteine</td>
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- positive phosphatase reaction
- negative phosphatase reaction
- complete inhibition
- slight traces of activity
- partial inhibition
- near normal
- normal
- slight activation
of a few feathers. With intermediate concentrations there was partial inhibition (p) involving a reduction in epidermal phosphatase and in the nuclear and cytoplasmic phosphatase of the pulp. Nearly normal (n−) and normal (n) reactions were obtained at low concentrations. In a few instances, reactions darker than controls (a) indicated that there was activation of phosphatase.

Mg^{2+}

Magnesium sulfate was normally present in the substrate mixture in controls in a final concentration of 0.0016 M. The activating influence of Mg^{2+} was tested by omitting it from the substrate mixture. It was found that sodium glycerophosphate, glucose-6-phosphate, glucose-1-phosphate, disodium p-nitrophenyl phosphate, and yeast adenylic acid showed no difference in distribution of the phosphatase and only a slight difference in intensity of the reaction when Mg^{2+} was not present in the substrate mixture (Figs. 5, 8, and 10). Its absence in the substrate mixtures of fructose-6-phosphate, hexose diphosphate, and muscle adenylic acid produced complete or almost complete inhibition of phosphatase activity with these substrates (Fig. 3).

Zn^{2+}

A concentration of 0.001 M ZnSO₄ in the substrate mixtures of sodium glycerophosphate, hexose diphosphate, glucose-6-phosphate, and glucose-1-phosphate was sufficient to inhibit completely all phosphatase activity. When the Zn^{2+} was diluted one hundred-fold, normal or near-normal reactions were obtained. The adenylic acids responded quite differently with
Zn\textsuperscript{++}. A level of Zn\textsuperscript{++} that permitted normal phosphatase activity with the above-mentioned substrates, almost completely inhibited the reaction of phosphatase with muscle adenylic acid and slightly activated the reaction with yeast adenylic acid.

\textit{CN}^-

All phosphatase reactions with sodium glycerophosphate, glucose-6-phosphate, glucose-1-phosphate, and disodium p-nitrophenyl phosphate were inhibited completely by KCN in concentrations of 0.025-0.05 M. Activity with fructose-6-phosphate and yeast adenylic acid was inhibited completely by 0.01 M KCN. The phosphatase reaction with all six of the above substrates was normal or inhibited partially by 0.001 M KCN, with hexose diphosphate it was inhibited completely, and with muscle adenylic acid there were only slight traces of activity in the pulp of a few feathers.

\textit{HAsO}_4^2-

Sodium arsenate, 0.01 M, completely inhibited the reaction of phosphatase with glycerophosphate but permitted a normal reaction at a level of 0.001 M. This same lower concentration completely inhibited phosphatase activity with hexose diphosphate and fructose-6-phosphate. A still lower concentration, 0.0001 M completely inhibited the phosphatase reaction with muscle adenylic acid. The phosphatase reaction obtained with yeast adenylic acid and 0.01 M arsenate differed considerably from that obtained with the other substrates. At this level the nucleoli were more active in the epidermis and pulp of the feathers on the treated slides than in the controls with yeast adenylic acid alone (Figs. 15
and 16). With 0.001 M sodium arsenate the phosphatase activity was inhibited partially although the nucleoli were dark and the epidermis showed more phosphatase activity than the controls with only yeast adenylic acid. If the period of incubation was increased to 2 hours, the nucleolar phosphatase was also inhibited by 0.01 M sodium arsenate, and at the 0.001 M level activity was inhibited more than when incubated 1½ hours. Normal reactions were obtained with 0.0001 M sodium arsenate at both times of incubation.

Iodoacetate

Iodoacetate, 0.1 M, almost completely inhibited the reaction of phosphatase with sodium glycerophosphate and hexose diphosphate. At this same level the phosphatase reaction obtained with yeast adenylic acid showed only a slight reduction in intensity. Normal phosphatase activity was obtained with all of the substrates in the presence of 0.05 M iodoacetate.

Semicarbazide hydrochloride

Semicarbazide, 0.1 M, produced partial inhibition with sodium glycerophosphate and hexose diphosphate, and almost complete inhibition with yeast adenylic acid except for traces of activity in a few feathers. The inhibition resulted in a general reduction in the intensity of the phosphatase reaction in the entire sections. Sodium glycerophosphate, hexose diphosphate, and fructose-6-phosphate continued to show partial inhibition with 0.05 M semicarbazide although they were slightly more reactive than at the 0.1 M level. The intensity of the reaction with sodium glycerophosphate and hexose diphosphate returned to normal or
near normal with 0.02 M semicarbazide.

**Glycine**

The effect of glycine upon the phosphatase reaction differed somewhat with the different substrates. It was found that 0.1 M glycine inhibited the reaction of phosphatase with fructose-6-phosphate, hexose diphosphate, and sodium glycerophosphate except for occasional traces of activity in the pulp of a few feathers in the presence of the latter two substrates. The utilization of the adenylc acids was only partially inhibited by 0.1 M glycine, for the pulp of the feathers remained reactive. With 0.05 M glycine there was partial inhibition of the reaction with all of the above substrates. A normal phosphatase reaction was obtained with 0.01 M glycine. When muscle adenylic acid was used as a substrate, phosphatase was partially inhibited with 0.1 M glycine, a normal reaction was obtained with 0.05 M amounts, and 0.01 M glycine produced a reaction slightly darker than normal.

**L-cysteine**

With sodium glycerophosphate as a substrate, 0.01 M cysteine completely inhibited phosphatase. The pulp of a few feathers was reactive at the 0.005 M level; this activity included the pulp of more feathers with 0.001 M cysteine; and with 0.0005 M amounts the phosphatase reaction was slightly lighter than the control but similar in distribution. The fructose sugars, hexose diphosphate and fructose-6-phosphate, were completely inhibited by 0.005 M cysteine, but regained almost normal activity with 0.0005 M amounts.
Cysteine inhibited the utilization of yeast adenylic acid more effectively than the utilization of muscle adenylic acid as substrates. The use of muscle adenylic acid by phosphatase was completely inhibited by 0.05 M cysteine; traces of activity appeared at the 0.005 M level; and with 0.0005 M cysteine the reaction was still much reduced. With yeast adenylic acid, 0.005 M amounts of cysteine completely inhibited phosphatase; the reaction reappeared at the 0.0005 M level in the form of occasional traces of activity within the pulp area where the nucleoli were more darkly stained than the cytoplasm.

L-histidine monochloride

The phosphatase reaction obtained with sodium glycerophosphate was completely inhibited by 0.01 M histidine, was slightly reduced by a 0.001 M concentration, and normal at the 0.0005 M level. With muscle adenylic acid, it was completely inhibited by 0.01 M histidine, and partially inhibited by a 0.001 M concentration. The intensity of the reaction was only slightly diminished by 0.0001 M histidine. When yeast adenylic acid was used as a substrate, a nearly complete inhibition was obtained with 0.001 M histidine; the only indication of a phosphatase reaction was seen in the nucleoli of the pulp of the long flight feathers. At the 0.0001 M level there was still a reduction in the intensity of the reaction.

The following substances were used only in conjunction with sodium glycerophosphate as a substrate.

Mn⁺⁺

Manganese chloride was added to the substrate mixture in place of Mg⁺⁺ to determine its effect upon phosphatase. Results showed no
The reaction was reduced with 0.05 M ascorbate there was partial inhibition, but the reaction in the presence of 0.1 M ascorbate completely inhibited the phosphatase reaction. Except for occasional traces which showed traces of activity in the absence of substrate, no activity was obtained with 0.001 M ascorbate.

Phosphate reaction was obtained with 0.0001 M ascorbate in concentrations of 0.01, 0.001, and above. A normal phosphate reaction occurred when added to the substrate mixture in concentrations of 0.01, 0.001, and above. 0.0001 M ascorbate had no apparent effect upon the phosphate reaction. Perchloric acid 0.0005 M in the presence of 0.1 M ascorbate completely inhibited the enzyme. Phosphate activity in the presence of 0.05 M ascorbate was inhibited in the presence of 0.1 M ascorbate.

* then those having neither N⁺⁺⁺ or N⁺⁺⁺⁺ in the substrate solution but those having both N⁺⁺⁺⁺ and N⁺⁺⁺⁺ were in the darker with N⁺⁺⁺⁺ and the difference in the distribution of the enzyme in the tissues. The results
in intensity and limited primarily to the nuclei of the pulp and nucleoli of the epidermis.

**L-arginine monochloride**

Complete inhibition was obtained with 0.1 M arginine. There was partial inhibition and a decrease in the intensity of the reaction with 0.05 M amounts. At the 0.01 M level the activity was slightly less than in the controls.

**DL-lysine monochloride**

Lysine, 0.1 M, almost completely inhibited the phosphatase, with only the basal areas of the pulp showing any activity. The epidermal phosphatase was inhibited. An almost normal reaction was obtained with 0.05 M lysine, the reaction being only slightly lighter than in controls.

**L-methionine**

A partial inhibition of alkaline phosphatase was obtained with 0.1 M methionine, with some reduction in the intensity of the reaction. A normal reaction was obtained with 0.05 M methionine, and at the 0.01 M level there was some indication of slight activation.

**L-proline**

Proline, 0.1-0.01 M, produced a slightly darker reaction than that normally obtained.

**DL-serine**

With 0.1 M serine there was a lessening of the intensity of the reaction, notably in the epidermis.
DL-valine

Partial inhibition, with considerable reduction in the intensity of the reaction, was obtained with 0.1 M valine. A normal reaction was obtained with 0.05 M amounts, and at the 0.01 M level the reaction was slightly darker than normal.
DISCUSSION

Origin of Phosphatase within the Cell

On rational grounds, one would expect that phosphatase is formed within the cell as a result of genic action and that it might thus occur both within the nucleus and the cytoplasm. The literature reveals considerable difference of opinion on this matter, based in part on questions of the reliability of the techniques for demonstrating phosphatase.

Two methods are commonly employed for demonstrating alkaline phosphatase: the method of Gomori which involves the fixation of tissues in alcohol or acetone or by the freeze-drying method and the visualization of the site of enzymatic action by the initial precipitation of calcium phosphate, its conversion to cobalt phosphate, and finally the precipitation of cobalt as its sulfide. A second method involves the reaction of a phenol and diazonium hydroxide to form an azo dye. Nuclear phosphatase activity has been reported after the use of Gomori's technique (Danielli, '46) but not after the diazonium reaction (Gössner, '55). In comparing the two methods, Yokoyama, Stowell, and Mathews ('51) urged caution in the interpretation of the Gomori technique. Danielli ('53) reported that it was not possible to determine details of distribution of phosphatase in the nuclei of mammalian somatic cells with the azo dye method.

There have been many reports regarding diffusion of either the enzyme or its activator or of calcium phosphate with the technique of Gomori. Danielli ('53) has reported that prolonged periods of incubation enhance diffusion, the diffusing substance being either the enzyme or an
activator. Martin and Jacoby (149) and Newman, Feigin, Wolf, and Kabat (150) reported evidence of diffusion of the enzyme or its products during long periods of incubation and the precipitation of calcium phosphate in structures having no inherent alkaline phosphatase. Buyter and Neumann (149) considered nuclear phosphatase to be an artifact and a result of diffusion due to the solubility of the enzyme in low concentrations of alcohol. Barter (154) and Leduc and Dempsey (151) reported that nuclear phosphatase was the result of diffusion of the enzyme or of an activator.

Artifacts with the Gomori technique have also been reported to be caused by the diffusion of calcium phosphate and its adsorption by the nuclei of cells. Cleland (150b) reported that the enzyme is bound in the tissue by fixation and that the nucleus shows activity because of the adsorption of calcium phosphate. Gomori (150) could find no evidence of diffusion effects at pH 9.2 but only at pH 7.0-8.5. However, Novikoff (151) reported that calcium phosphate does diffuse at pH levels as high as 9.7. Gomori (151) suggested that artifactual nuclear reactions were caused by the adsorption of calcium, and suggested that they might be lessened by using high pH, high concentrations of calcium, and short periods of incubation. Gomori and Benditt (153) stated that diffusion is not an important source of artifacts if the incubation period is not too long. Danielli (153) reported no evidence of diffusion of calcium phosphate in sections. Thus, there seems to be no general agreement about the presence of diffusion artifacts or their cause, except that it is generally recognized that short periods of incubation are desirable.

In this study sections were incubated in sodium glycerophosphate for 30, 60, 90, and 120 minutes in order to select a suitable period of
incubation for comparing the activity obtained with the various substrates, activators, and inhibitors. The distribution of the activity was the same at all periods of incubation although the intensity of the reaction increased with time. Ninety minutes was selected as the optimal period, because it gave a reasonably dark reaction in controls and was still short enough to avoid artifacts from diffusion.

Alkaline phosphatase has been reported in many intracellular structures. It is more active in embryonic tissues than in adult tissues (Chèvremont and Firket, '53), and is reported by Moog (‘44) to be quite active in the nuclei and less active in the cytoplasm during growth and differentiation. It is present also in the nuclei of adult tissues. Dounce (‘43) found that the nuclei of rat liver cells were richer in phosphatase than the tissue as a whole. Krugelis (‘46) reported the presence of phosphatase in the cytoplasm, nucleoli, and chromosomes of the salivary glands of Drosophila. Bradfield (‘50) also reported the presence of alkaline phosphatase in the nucleolus. The nucleoli, chromatin particles, and nuclear membrane are active with 5-nucleotidase (Wachstein and Meisel, ‘52). Phosphatase is present in the nuclear membranes of cells of the guinea pig and rat liver (Baud and Fulleringer, ‘48).

Alkaline phosphatase has been found in intracellular locations other than the nuclei. Emery and Dounce (‘55a, b) studied phosphatase in mitochondria, microsomes, and the soluble fraction of cytoplasm as well as in the nuclei. They reported high activity in the isolated nuclei, the presence of very little alkaline phosphatase in mitochondria, small quantities in microsomes, and large quantities in the supernatant fluid from centrifuged cells. Microsomes have been reported rich in alkaline
phosphatase (Albert, '55), glucose-6-phosphatase (Hers, Berthet, Berthet, and deDuve, '51; Beaufay and deDuve, '54), and glucose-1-phosphatase (Sonnenschein and Kopac, '55). Hers et al. reported the presence of hexose diphosphatase in the soluble fraction of liver cells. Both acid and alkaline phosphatase (Moog and Steinbach, '46) as well as adenylylpyrophosphatase (Steinbach and Moog, '45) have been found in cytoplasmic granules of embryonic extracts. Alkaline phosphatase has also been found in the Golgi apparatus of the epithelial cells of the liver, kidney, uterus, epididymis, and intestine (Deane and Dempsey, '45).

In the developing down feather alkaline phosphatase is present in the nucleoli, chromatin granules, nuclear membrane, and cytoplasm of the cells of the mesodermal pulp. In the epidermis, it is present in the nucleoli of the cells of the barbs and in the nucleus and cytoplasm of the cylinder cells. In isolated sections through the epidermis alone, phosphatase is found only in the nucleoli. At no time is cytoplasmic activity greater than that in the nucleoli. Furthermore, the action of inhibitors was that of removing the phosphatase by degrees from the cytoplasm and nucleus before noticeably affecting that in the nucleolus. This suggests that the nucleus, and specifically the nucleolus is the ultimate source of the alkaline phosphatase.

The presence of phosphatase in the nucleoli, microsomes, and cytoplasm of cells, sites which are rich in ribonucleic acid, has led to the frequent association of phosphatase with the metabolism of nucleic acids and proteins. Caspersson and his co-workers reported that the nucleolus is the center of synthesis of nucleoproteins, and Bradfield ('50) states that the wide occurrence of alkaline phosphatase in the nucleoli is
correlated with the synthesis of proteins. The close association of RNA and alkaline phosphatase in the developing down feather has led to the view that alkaline phosphatase expedites the synthesis of RNA by splitting phosphate esters and transporting certain products across the basement membrane to the epidermis where they can be used in the construction of ribonucleoproteins (Hamilton and Koning, '56). The present study shows a clear connection between sites of phosphatase activity and places where RNA is known to accumulate, i.e., in nucleoli and in the cytoplasm of cylinder cells next to the basement membrane bounding the pulp. This observation supports the idea that the two are causally or functionally connected.

Multiplicity of Phosphatases

It was pointed out in the introduction and review of literature that several workers have claimed the existence of more than one alkaline phosphatase in tissues on the basis of their substrate specificity, sites of action, and response to various activators and inhibitors. It has been established that alkaline phosphatases from several sources are activated or inhibited by metallic ions and amino acids. The activation of alkaline phosphatase by Mg\(^{++}\) was first reported by Erdtman ('28). Many studies since then have reported similar activating effect of Mg\(^{++}\) in tissue sections, in homogenates, and in partially purified preparations of the enzyme. Feigin and Wolf ('57) reported that nuclear phosphatase in the uterus, ovary, and adrenal cortex of the guinea pig was activated by Mg\(^{++}\). As mentioned earlier phosphatase activity in the presence or absence of Mg\(^{++}\) has been used by many workers as the basis for distinguishing between
the phosphatases. In the feather it was found that Mg** was necessary for activity with muscle adenylic acid, hexose diphosphate, and fructose-6-phosphate.

Other ions have also been observed to have an activating effect on phosphatase. These include such ions as Ni**, Co**, Mn** (Cloetens, '41), Ca**, Fe**, and Zn** (Theai, Roche, and Roger, '47). Bamann and Neumüller ('40) found that Mn** activated phosphatase to a greater extent than Mg**, while Cleland ('50a) found evidence to the contrary. Phosphatase in the feather is only slightly influenced by Mn**.

Zinc has been reported to increase the activity of crude alkaline phosphatase of the intestine, to decrease that of kidney and bone, and to inhibit completely all three after dialysis (Hove, Elvehjem, and Hart, '40). Hoare and Delory ('55) reported that Zn** reactivated KCN-inactivated phosphatase more effectively than did Mg**. Zinc has been reported to inhibit 5-nucleotidase (Kaye, '55). In the down feather high concentrations of Zn** inhibited activity with all substrates except fructose-6-phosphate, and in low concentrations activated the reaction with yeast adenylic acid. At the concentration that stimulated activity with yeast adenylic acid there was almost complete inhibition of activity with muscle adenylic acid.

The inhibition of alkaline phosphatase by Be** in crude and purified preparations of the enzyme has been extensively investigated (Dubois, Cochran, and Masur, '49; Klemperer, Miller, and Hill, '49; Orier, Hood, and Hoagland, '51; Schubert and Lindenbaum, '51; Morton, '55). Chévremon and Firket ('51) studied the inhibitory effect of Be** in tissue cultures and the reversal of this inhibition by Mn** and Mg**. Hamilton and Koning ('56) found that Be** inhibited feather development in vitro by completely
inhibiting alkaline phosphatase in the mesodermal pulp. Alkaline phosphatase in sections of feathers was completely inhibited by levels above 0.001 M Be**.

Versene inhibits alkaline phosphatase by chelating the natural metallic activator of the enzyme. The inhibition of alkaline phosphatase by Versene and the reactivation of the enzyme by various metallic ions have been studied (Hofstee, '55; Frieman, '56).

There have been numerous claims associating amino acids with the activation of phosphatase. Bodansky ('36) found that α-amino acids accelerate phosphatase activity, and that glycine and Mg** used concurrently increase the velocity of the reaction. Thoai, Roche, and Roger ('47) reported that alanine is necessary for phosphatase activity in the intestine, and Morton ('55) reported that its addition to intestinal and milk phosphatases reduced the inhibition by Zn** and Be**. Bodansky ('48) found that the phosphatases of the intestine, bone, and kidney reacted differently to the inhibitory effects of histidine, lysine, and glutamic acid. Fischer and Greep ('48) reported that amino acids acted as activators in the presence of Mg**, and that trypsin was effective in reactivating purified phosphatases.

High concentrations of cysteine, histidine, glycine, alanine, arginine, lysine, and methionine had in common the effect of inhibiting the activity of alkaline phosphatase in the developing down feather. At low concentrations, valine, proline, and methionine gave some indication of activating phosphatase with sodium glycerophosphate as substrate, and glycine promoted activity with muscle adenylc acid. Cysteine and histidine
had a greater inhibitory effect on phosphatase than the other amino acids.

It seems probable that the amino acids, like Versene, inhibit alkaline phosphatase by chelating and removing metallic ions necessary for the activation of the enzyme. This is considered to be their action in protecting sperm against metallic ions (Tyler, '55). The inhibitory action of the metallic ions tested is probably that of successfully competing with the natural activators for sites on the enzyme.

The presence of more than one phosphatase in the developing down feather could not be determined on the basis of substrate specificity alone. The pattern of phosphatase activity was identical with all substrates except yeast adenylic acid, although the intensity of the reaction varied somewhat with the different substrates. With yeast adenylic acid there was a general reduction in enzymatic activity with prominent nucleolar activity in the epidermis. The gradient of activity between feathers and evidences of bilateral symmetry in feather development reported by Koning and Hamilton ('51) were also seen in this study.

A comparison of the results obtained when the various chemicals and substrates were used concurrently shows that there were some differences in reactivity (see Table 1). The phosphatase activity with yeast adenylic acid differs from that with muscle adenylic acid in its response to magnesium and various concentrations of zinc, arsenate, glycine, and cysteine. The reaction with both of the adenylic acids differs from that obtained with sodium glycerophosphate in some respects. Generally, activity with the adenylic acids was inhibited by lower concentrations of the chemicals than were effective in inhibiting the activity with sodium glycerophosphate. However, with yeast adenylic acid there was some
activity at concentrations of zinc, arsenate, and glycine that completely inhibited activity with sodium glycerophosphate. The reaction with yeast adenylic acid was only slightly less than normal at levels of iodoacetate and semicarbazide that completely or partially inhibited phosphatase with the other substrates. The fructose sugars showed a difference in reaction and in general were inhibited by lower concentrations of chemicals than inhibited activity with sodium glycerophosphate.

These data suggest that the developing down feather contains more than one phosphatase capable of utilizing the various substrates at a pH 9.0-9.4. The phosphatase activity present in the epidermis in the form of prominent nucleolar activity, particularly with yeast adenylic acid, shows a greater sensitivity to the inhibiting agents than the phosphatase in the pulp. This increased sensitivity may be the result of a smaller quantity of enzyme within the epidermis or due to the presence of a different enzyme. However, the presence of nucleolar activity with concentrations of arsenate which inhibits phosphatase activity with the other substrates suggests the latter view. There is further supporting evidence in the observation that most inhibitors removed the activity of cytoplasmic phosphatase before they affected that of the nucleus.
SUMMARY AND CONCLUSIONS

1. Sodium glycerophosphate, disodium p-nitrophenyl phosphate, glucose-1-phosphate, glucose-6-phosphate, hexose diphosphate, fructose-6-phosphate, muscle adenylic acid, and yeast adenylic acid were used as substrates to determine whether one or more alkaline phosphatases were present in the developing down feather.

2. Phosphatase was found in the nucleoli, chromatin granules, nuclear membrane and cytoplasm of the cells in the mesodermal pulp. In the epidermis, phosphatase was present in the nucleoli of the cells of the barbs and in the nucleus and cytoplasm of the cylinder cells. The pattern of activity was the same with all substrates except yeast adenylic acid, although the intensity of the reaction varied with the various substrates. With yeast adenylic acid, epidermal activity was confined primarily to the nucleoli of the cells.

3. To determine whether the differences in reactivity were due to different phosphatases, the activating or inhibiting effects of Mg$^{++}$, Mn$^{++}$, Be$^{++}$, Zn$^{++}$, Fe$^{+++}$, HAsO$_4$$^{2-}$, HASO$_3$$^{-}$, CN$^-$, semicarbazide, iodoacetate, Versene, glycine, alanine, cysteine, histidine, methionine, proline, valine, serine, lysine, and arginine were studied in conjunction with several of the substrates.

4. Mg$^{++}$ was necessary for activity with muscle adenylic acid, hexose diphosphate, and fructose-6-phosphate. At high concentrations, Zn$^{++}$ inhibited activity with all substrates except fructose-6-phosphate, and in low concentration stimulated activity with yeast adenylic acid. Yeast adenylic acid showed strong nucleolar activity at concentrations of
arsenate which inhibited activity with all other substrates. Versene, iodoacetate, semicarbazide, Be++, and CN− in high concentrations inhibited phosphatase activity. Mn++, Fe+++., and HAsO₃− had no apparent effect on phosphatase. Cysteine and histidine had a greater inhibitory effect on phosphatase than the other amino acids. At low concentrations, glycine promoted activity with muscle adenylic acid. Valine, proline, and methionine in low concentrations gave some indication of possible stimulation of activity with sodium glycerophosphate.

5. The activity of phosphatase with yeast adenylic acid differs from that with muscle adenylic acid in its response to some of the inhibitors and activators. The activity with both of the adenylic acids differs from that obtained with sodium glycerophosphate. The fructose sugars were inhibited by lower concentrations of the chemicals than inhibited activity with sodium glycerophosphate.

6. Epidermal phosphatase shows a greater sensitivity to the inhibiting agents than the phosphatase in the pulp. Cytoplasmic phosphatase is more easily inhibited than that in the nucleoli.

7. These data indicate that the nucleolus is the source of at least part of the phosphatase. They also suggest that the developing down feather contains more than one phosphatase capable of utilizing the various substrates at a pH of 9.0-9.4.
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APPENDIX
Figures 1-10 are longitudinal sections of developing down feathers that were incubated in various substrate solutions for 1½ hours at 37°C, and carried through the reaction for alkaline phosphatase. Figures 1-5 are from a series from the wing of a 12-day chick embryo (stage 38). Figures 6-10 are from a series from the wing of another embryo of the same stage.

Fig. 1 Control feather incubated in a solution of sodium glycerophosphate. Note the intense reaction in the mesodermal pulp, the spreading of activity into the dermal area beneath the feather, and the strong reaction in the epidermis. X 155

Fig. 2 Feather which was incubated with muscle adenylic acid as a substrate. Phosphatase activity in the pulp and epidermis is reduced when compared with the activity with sodium glycerophosphate. X 155

Fig. 3 Basal portion of a feather which was incubated in a solution of muscle adenylic acid lacking Mg ions. Note the absence of phosphatase activity in the pulp and epidermis. X 155

Fig. 4 Feather incubated in a solution of yeast adenylic acid. The intensity of the reaction is reduced in the pulp, and is present in the epidermis primarily in the nucleoli. X 155

Fig. 5 Feather incubated with a solution of yeast adenylic acid lacking Mg ions. Note that there is little difference in the reaction when compared with that in Figure 4. X 155

Fig. 6 Control feather incubated in a solution of sodium glycerophosphate. Note the concentrations of phosphatase in the dorsal and ventral areas at the base of the feather and its spreading into the dermal area beneath the feather. X 155

Fig. 7 Feather near the edge of the wing which was incubated with glucose-1-phosphate as a substrate. Note the similarity in the distribution and intensity of the phosphatase reaction to that obtained with sodium glycerophosphate. X 155

Fig. 8 Feather incubated in a solution of glucose-1-phosphate lacking Mg ions. The phosphatase reaction is only slightly less intense than that having Mg ions in the substrate solution. X 155

Fig. 9 Feather incubated in a solution of glucose-6-phosphate. The distribution and intensity of the reaction are similar to that in Figures 6 and 7. X 155
PLATE 1 (Continued)

Fig. 10 Feather incubated in a solution of glucose-6-phosphate lacking Mg ions. Activity is slightly reduced when compared with that in Figure 9, particularly in the epidermis but the distribution of the enzyme is the same. X 155
Feathers in Figures 11-18 were incubated in various solutions for 1½ hours at 37°C. and carried through the reaction for alkaline phosphatase.

Fig. 11 A longitudinal section of a feather from the wing of an embryo of stage 38+. Sodium glycerophosphate was used as the substrate. X 79

Fig. 12 Feathers from the same wing as above. The substrate was hexose diphosphate. Phosphatase activity is present in both the nuclei and cytoplasm of the cells in lesser quantities than with sodium glycerophosphate. X 79

Fig. 13 Feather from the same series, which was incubated in fructose-6-phosphate. Note that the phosphatase activity does not extend throughout the length of the feather or into the dermal area beneath it. The phosphatase reaction is much reduced in the pulp and epidermis when compared with that in Figure 11. X 79

Fig. 14 Cross-section of a feather from a 12-day chick embryo (stage 38+). Sodium glycerophosphate was used as the substrate. Phosphatase is present in the pulp, the basement membrane, and the cylinder cells of the barbs. Activity is greatest in the nucleoli of the other cells of the barbs. X 205

Fig. 15 Cross-section of a feather from an embryo of stage 38-. Yeast adenyllic acid was used as the substrate. Phosphatase activity in the pulp is both nuclear and cytoplasmic, while in the epidermis phosphatase is present primarily in the nucleoli. X 205

Fig. 16 Cross-section of a feather from the same wing as Figure 15. A yeast adenyllic acid solution to which 0.01 M sodium arsenate had been added was used as the substrate. All phosphatase activity was inhibited by the arsenate except for that present in the nucleoli of the epidermis and pulp. Compare with Figure 15. X 205

Fig. 17 Longitudinal section of a feather from the wing of a 12-day chick embryo (stage 38). The substrate was sodium glycerophosphate. X 155

Fig. 18 Longitudinal section of a feather from the same wing as Figure 17. Disodium p-nitrophenyl phosphate served as the substrate. Distribution and intensity of the phosphatase reaction are almost identical with those obtained with sodium glycerophosphate. X 155

Fig. 19 Cross-section of a bone in the upper wing of a chick embryo of stage 38. The section was incubated in sodium glycerophosphate. Phosphatase is present in the cells of the cartilage and in a heavy ring in the perichondrium. X 95
PLATE 2 (Continued)

Fig. 20 Cross-section of bone incubated in a substrate solution lacking any phosphate source. Black ring is calcium phosphate deposited in vivo. X 95

Fig. 21 Cross-section of bone treated with Alizarin Red S, which is specific for calcium. A scarlet reaction was obtained in the same ring as was found in Figure 20. X 95