Phosphatases and Nucleic Acids in Silk Glands:
Cytochemical Aspects of Fibrillar Protein Secretion

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With two plates

SUMMARY
Silk glands of several spiders and caterpillars contain powerful cytoplasmic phosphatase activity localized along the inner border of the cells next to the gland lumen. Phosphatase activity is also high in the nuclei, particularly the nucleoli. Reservoir regions which secrete no silk lack the phosphatase border.

Although it is most easily demonstrated histochemically at pH 9, phosphatase activity in sections or homogenates of the glands is high over a considerable range of pH, from at least pH 5.5 to pH 9.8. It appears to be inhibited both by sulphydryl inactivators and by an abundance of sulphydryl groups. In view of the latter point, it is interesting to note that no sulphydryl groups can be detected in the phosphatase border zone (in the more insoluble constituents which remain in formalin-fixed frozen sections), although the main bulk of the cytoplasm is rich in sulphydryl groups.

Except in the non-secretory reservoir regions, silk glands of all the species examined are rich in cytoplasmic ribonucleic acid. Although the latter is abundant in the main body of the cell, it is, however, absent from the phosphatase border zone, so that in the cytoplasm nucleic acid ends where phosphatase begins.

This suggests that the phosphatase may be part of a system of enzymes involved in liberating the finished protein from a complex with nucleic acids (which have been concerned either in the synthesis of the protein, or in protecting it from proteases, or both). This possibility is discussed in the light of previous studies of phosphatase-nucleic acid relations in other tissues.

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INTRODUCTION

The application of the methods for the localization of alkaline and acid phosphatases described by Gomori (1939, 1941) has revealed many previously unfamiliar sites of phosphatase activity in animal tissues. In [Quarterly Journal Microscopical Science, Vol. 92, part 1, March, 1950.]
addition to various miscellaneous situations, there are two main tissue environments where phosphatases have been located. First are sites of particularly active solute transfer such as brush borders of kidney tubules and duodenal mucosa; second are sites where mineral phosphates are being deposited in structures such as bones or teeth. The experiments of Fell and Danielli (1943), which revealed a marked increase in alkaline phosphatase activity accompanying collagen formation in healing wounds, together with several reports of phosphatase activity in nuclei (Moog, 1946; Bradfield, 1950), suggested other aspects of cell metabolism, such as fibrillar protein secretion and nucleoprotein metabolism in general, where phosphatase activity might be important.

In order to investigate further the possible connexion between phosphatase activity and protein secretion, it seemed worth turning to invertebrate silk glands which possess many advantages for this purpose. In organs such as mammalian liver the existence of innumerable metabolic paths makes it difficult to unravel the exact role of any enzyme which may be detected there. Silk glands, on the other hand, show a remarkable singleness of function and it seems safe to assume that any enzyme which they contain is in some way concerned in the production of the silk thread.

When examined by the Gomori technique, the silk glands of several species of spiders and caterpillars were found to possess powerful phosphomonoesterase activity and subsequent investigations suggested that this enzyme is concerned in the nucleoprotein metabolism of the glands (Bradfield, 1946, 1950). These observations will be presented in three sections, which describe (1) the characteristic localization of the phosphatase in silk glands, (2) some of the factors affecting its activity, (3) other cytological features of the glands, particularly the distribution within them of nucleic acids, which may throw light on the function of the phosphatase. Brief comments on interpretation and validity are given in each of these sections so that the main discussion deals only with the subject of phosphatase—nucleic acid relationships in silk glands and in other tissues.

MATERIALS

A variety of caterpillars and spiders has been used. Well-grown larvae of Cosmus cossus (the goatmoth) were found to be capable of spinning at most times of the year and would surround themselves with a new web whenever transferred to a new jar. Silkworms (larvae of Bombyx mori) were studied during the period of active secretion which precedes the spinning of the cocoon. The spider most thoroughly investigated was Tegenaria domestica, found in buildings of all kinds. Specimens of Agelena labyrinthica and Epeira diadema were also examined.

Lepidopteran larvae have a single pair of long thin silk glands (Lesperon, 1937; Wigglesworth, 1942) which run the length of the body and are thrown into numerous folds before opening anteriorly just below the mouth. In Bombyx the glands consist of three distinct parts—secretory region, reservoir
region, and duct—but in Cossus glands there is little difference between the first two of these, both being actively secretory. It has been suggested that fibroin is secreted in the upper regions and receives a coat of sericin lower down the gland, but this is difficult to establish cytochemically. The wall of the secretory region in each gland consists of a single layer of very large, almost cubical cells, of side 100–200 μ, which are bounded externally by a well-developed basement membrane enclosing numerous tracheae. Internally there is a remarkable membrane, the chitinous intima, which, though hardly visible in the upper regions of the glands, becomes very thick (about 100 μ) in the lower regions, where masses of secretion globules may be seen passing through it. In the secretory cells are enormous ramifying nuclei which can be well demonstrated when freshly dissected, unfixed glands are stained in Unna’s methyl-green and pyronine (Pl. I, fig. 1). They contain a considerable number of small nucleoli. For histological study the glands, after rapid removal from the larvae, were coiled on a coverslip and either examined fresh or else fixed, sectioned, and stained.

Spiders, in contrast to caterpillars, have a large number of silk glands (as many as 200), which are of at least seven kinds. Their anatomy and distribution have been described by Apstein (1889), Warburton (1890), and many other investigators (Millot, 1926; Savory, 1928). In Tegenaria domestica only the four common types of gland are present. Three of these, the tubular, pyriform, and aciniform glands, consist solely of secretory region and duct, but in the ampullaceal glands (Pl. I, fig. 4) there is a large reservoir region into which open the ramifying secretory tubes and out of which leads the single narrow duct.

The cytology of the silk glands is, however, not so diverse as their variations in gross structure might lead one to expect. The walls of the glands consist of a single layer of cells, exactly as in the silk glands of caterpillars, but the detailed cytology in the two cases is profoundly different. The individual secretory cells of the spider silk glands are tall and narrow with a spherical, more or less basal, nucleus containing a single large nucleolus. The nucleus is surrounded by a small body of clear cytoplasm and during active secretion, which is continuous from a week or two after hatching, the whole of the rest of each cell—the inner half in fact—is filled with silk globules waiting to pass into the lumen of the gland (Pl. I, fig. 2, Pl. II, figs. 9, 12). By ordinary methods it is impossible to detect any distinct membrane along the inner borders of the cells, though presumably such exists. Externally there is a thin, but quite distinct, nucleated membrane (Pl. I, fig. 2). The length of the duct is in most cases greatly increased by the presence of several complex coils, which are strengthened internally by a stout lining membrane, as in the ducts of caterpillar glands. For histological purposes the abdomen was sectioned whole, after removal of the cuticle, so that the silk glands remained embedded in, and protected by, the digestive gland.
Methods

Several standard staining methods have been used including the Mallory and Azan triple stains, appropriate staining times being determined experimentally in each case.

Phosphatases. The methods used for demonstrating the cytological localization of phosphatases were similar to those described by Gomori (1939, 1941), except that β-glycerophosphate was used as substrate at acid as well as alkaline pH (Wolf, Kabat, and Newman, 1943). The effects of various conditions of fixation and incubation are discussed later, but in general ice-cold 80 per cent ethanol was the most satisfactory fixative. Fixation time was not longer than 2 hours and the tissues were left in absolute ethanol for not longer than 6 hours and in Waterman's wax at 53°C for not longer than 2 hours. Dehydration and removal of alcohol (overnight in benzene) were both carried out in a refrigerator at about 5°C.

Nucleic acids. For the localization of nucleic acids the Feulgen technique, basic stains, and ultra-violet microscopy were all used in conjunction with digestion by ribo- and desoxyribonucleases.

With the Feulgen stain a range of hydrolysis times from 5 to 20 minutes was always employed and the usual precautions of staining unhydrolysed controls and washing in SO₂-water observed. Particularly in view of recent evidence that all somatic nuclei of one species may contain approximately the same amount of desoxyribonucleic acid (Boivin, Vendrely, and Vendrely, 1948; Mirsky and Ris, 1949), it must be remembered that valid deductions regarding relative contents of this substance can be drawn from variations in Feulgen staining only when attention is also given to the volumes of the structures stained.

As a basic stain Unna's pyronine and methyl green was found very satisfactory. After staining for 15 minutes, slides were washed in water, blotted, dehydrated in two jars of absolute acetone (½ minute each), cleared in xylene, and mounted in balsam. Digested and undigested slides were, both here and in the Feulgen technique, stained and mounted together under identical conditions. For the nuclease digestion it was found quite satisfactory to dissolve a trace of the enzyme in suitable buffers, adding magnesium chloride to a concentration of 0.01 M. in the case of desoxyribonuclease (salt-free ribonuclease, Kunitz, 1941; desoxyribonuclease prepared according to McCarty, 1946). Precise weighing of the fraction of a milligram of enzyme required for one set of digestions is both time-consuming and non-essential, since in any case it is desirable to follow the course of each digestion by staining slides withdrawn at intervals from the enzyme solutions. The ribonuclease solutions were heated at 80°C for 15 minutes before use to reduce the activity of any proteolytic impurities. Both nucleases have also been used in the presence of 0.005 M. cysteine without altering the effects described below, which suggests that these are due to nucleases rather than proteases, since at least some of the latter are inhibited by cysteine. Sections were covered with
several drops of enzyme solution and incubated in a moist chamber for 1 hour at 60°C. or 2–3 hours at 37°C., control slides being treated under similar conditions with buffer solutions alone.

Ultra-violet photographs were taken at 2,536 Å, i.e. in the region near the nucleic acid absorption peak at 2,600 Å, where the ratio of nucleic acid absorption to the absorption of other substances in a section is at a maximum. Hence strong absorption in a particular cell region is more likely to be due to the presence there of nucleic acid than of any other substance, and this can be confirmed by taking further photographs of the same section after nuclease digestion which will markedly diminish the absorption if it is mainly due to nucleic acid (cf. Pl. II, figs. 16 and 17). In the quantitative ultra-violet absorption studies the absorptions at several places in a cell at 2,536 Å and 2,650 Å (both near the nucleic acid absorption peak) are compared with the absorptions in the same places at 2,800 Å (at or near the protein absorption peak), in order to gain an idea of the ratio of nucleic acid to protein in various parts of the cell.

The apparatus used for ultra-violet photography was that described by Mitchell (1942). A low-pressure mercury-neon discharge tube (emitting strongly at 2,536 Å.) is used in conjunction with a filter containing 9 cm. chlorine and 2 cm. bromine vapour, which gives almost monochromatic light because transmission is high at 2,536 Å., but falls off very sharply on either side of this wave-length. There is transmission again above 4,500 Å., but the sensitivity of the photographic plate used (Ilford Zenith) falls off sharply above this wave-length. The sections, mounted on quartz coverslips, were dewaxed in xylene and photographed in liquid paraffin before and after nuclease digestion. The differences in absorption between certain parts of the cells were such as to make it possible to draw useful qualitative conclusions from direct visual examination of the negatives. For the quantitative ultra-violet absorption measurements a photomultiplier tube mounted over the eyepiece of the quartz microscope and recording via a moving mirror galvanometer was used to compare the light intensity emerging from a small region of the section (down to 1 μ in diameter) with that from an adjacent clear space in the preparation, in a manner similar to that described by Gersh and Baker (1943). A water-cooled, high-pressure mercury discharge tube was used as source, in conjunction with a quartz monochromator. A small fraction of the beam was reflected into a second photomultiplier tube to serve as a check on fluctuations in intensity of the source between measurements. From the intensity measurements the absorption or extinction of particular areas of the cell could be calculated, and these values were reproducible to within less than 0.5 per cent.

Sulphydryl groups. The prussian-blue method of Chèvremont and Frederic (1943) was found to give a more intense and permanent colour than any of the nitroprusside tests. Sulphydryl (SH) groups in formalin-fixed frozen sections reduce ferricyanide to ferrocyanide, which gives a prussian-blue deposit with ferric chloride. The section is then compared with a similarly stained control
which has been previously incubated for 10 minutes in a 0.01 M mercuric chloride solution to destroy SH groups. A positive reaction in the test slide, with a negative control, suggests that SH groups are either present in the living cell or have been produced by fixation. A negative reaction suggests that SH groups are absent from the living cell, or present only in very small quantities.

**The Localization of Phosphatases in Silk Glands**

Examination of the silk glands of the spiders and caterpillars mentioned earlier showed that, in each case, in addition to strong nuclear alkaline phosphatase there was a region of strong cytoplasmic alkaline phosphatase activity along the inner borders of the cells next to the lumen of the gland. The two groups of species require separate description as the cytology of spider glands is so different from that of caterpillar glands, but for this very reason the marked general resemblance is all the more striking.

*Tegenaria domestica.* When sections of a spider abdomen which has been fixed in ice-cold 80 per cent. ethanol are subjected to the Gomori alkaline phosphatase technique, a black deposit indicative of phosphatase activity is produced in the small basal nuclei and all along the inner margin of the cells, in the region packed with silk globules (Pl. I, fig. 2). Controls show no blackening whatever. The nuclear deposit is mainly restricted to the nucleolus. The exact width of the black border in the cytoplasm is very dependent on the length of incubation. The earliest reaction is along the innermost margin, but with longer incubation this region becomes more deeply stained and the reaction spreads into the cell until, after 24 hours' incubation, the whole inner half of the cell, i.e. the whole region packed with silk globules, gives a positive reaction (Pl. I, fig. 3). However, the black deposit never spreads farther than this and there is always the substantial zone of cytoplasm round the nucleus which is devoid of globules and of cobalt sulphide. Similarly the nucleus may, after prolonged incubation, show a solid black deposit, but this is very probably due to the very intense nucleolar reaction which gradually fills the rest of the small nucleus. If this localization of cobalt phosphate can be interpreted at its face value as a direct indication of the distribution of phosphatase activity, it would appear that there is high nucleolar phosphatase activity and that in the cytoplasm activity is high along the inner margins of the cells, but decreases rapidly nearer their centre so that at least the basal half of each is phosphatase negative. The studies of Danielli (1946) showed that calcium phosphate does not diffuse freely in kidney sections during its enzymatic precipitation. There have, however, been reports for some tissues of the diffusion of calcium phosphate from sites of high enzyme activity to nuclei which were themselves almost inactive (e.g. Jacoby and Martin, 1949). There is abundant evidence from other tissues that high phosphatase activity often occurs in nucleoli, but in these silk glands it is difficult to exclude the possibility that the nucleolar reaction is due to diffusion from the phosphatase border (though evidence will be presented below showing that this is certainly not the case in
caterpillar silk glands). In any case it is the cytoplasmic phosphatase border which is the main point of interest, and there is no reason to doubt that the black deposit along the inner cell margin represents the site of phosphatase activity in the cytoplasm. The possibility of some diffusion makes it impossible to specify the exact width of this border, but the most powerful activity is along the inner third of each cell, and it seems very likely that there is a real gradient of phosphatase activity passing into the cytoplasm until, at a point near the centre of the cell, phosphatase can no longer be detected by the Gomori method. If the observations which suggest the existence of a gradient were entirely due to diffusion it might be expected that in time the reaction would spread across the whole cell, but this is not the case; the basal region of cytoplasm round the nucleus always remains negative.

It seems unlikely that the site of phosphatase activity in the fixed tissues differs from that in the living cell because, with these spider silk glands, a variety of fixatives gave identical results. This would hardly be true if the distribution were due to fixation artifacts, which would surely vary with fixatives as different as those used: 80 per cent. ethanol, ice-cold and at room temperature; acetone; dioxane; pyridine and alcohol; 4 per cent. formaldehyde and 1 per cent. acetic acid. The only things which varied were the standard of fixation and the level of phosphatase activity, more being destroyed by some fixatives than by others, but the distribution was the same in every case.

All four main types of glands—tubular, pyriform, aciniform, and ampullaceal—show both nucleolar and border phosphatase, throughout those regions in which silk globules are being produced. In the narrow twisted ducts the powerful phosphatase border lies immediately inside the cuticular lining which has been described previously. This means that a phosphatase border is present throughout the length of the first three gland types, which consist solely of secretory region and duct. However, in the more complex ampullaceal glands there is a large reservoir region (Pl. I, fig. 4) which does not secrete silk, has only slight nucleolar phosphatase, and no border phosphatase. The cell type containing many silk globules, which is characteristic of the secretory diverticula, extends some way over the reservoir from the point at which the latter is joined by the diverticula. The border phosphatase extends to an exactly similar distance and the distinct junction between secretory and non-secretory cells coincides exactly with the junction between phosphatase-positive and phosphatase-negative cells (Pl. I, figs. 3, 4).

The other species of spiders examined—*Agelena labyrinthica* and *Epeira diadema*—resemble *Tegenaria* exactly in the distribution of phosphatase within the cell, but the distribution of enzyme along the length of the more complex glands has not been followed in sufficient detail to make comparison on this point possible.

*Cossus cossus*. In the silk glands of the goatmoth caterpillar, exactly as in the very different glands of *Tegenaria*, staining by the Gomori method indicates that high phosphatase activity is located in two sites—the nuclei and a narrow border along the inner edge of the cells (Pl. I, fig. 5). The nuclear phosphatase
reaction first appears and is subsequently strongest in the nucleoli (Pl. I, fig. 5); but in so small a volume as one nucleus filled with phosphatase-positive nucleoli, diffusion of calcium phosphate into the chromatin between nucleoli would undoubtedly occur, whether or not such chromatin possessed real phosphatase activity. The cytoplasmic phosphatase border is situated at the extreme inner edge of the cytoplasm and is separated from the silk in the lumen by the chitinous intima which, though thin in the upper region, becomes very thick in the lower regions (just as in the section of Bombyx glands shown in Pl. I, fig. 7) and extends into the ducts as a broad cuticular lining inside which there is a phosphatase border (Pl. I, fig. 6).

Of these two local concentrations of phosphatase, that in the cytoplasmic border is the most active, for with freshly prepared material a heavy deposit is obtained after only an hour's incubation, whereas the nuclei are distinct only after 2 hours. In view of this the possibility arises (as in spider glands) that the nuclear reaction is due to diffusion of calcium phosphate from the border phosphatase. The following observations strongly suggest that this is not the case, however, and that the nuclear reaction is due to phosphatase activity present in the nucleus. (1) The border reaction never shows any tendency to widen by diffusion, unlike kidney brush border and many similar sites of phosphatase activity; prolonged incubation intensifies the deposit in the border, but leads to no broadening of the zone of reaction. (2) In a section of the glands some of the elaborate nuclei extend almost from the outer margin to the inner margin of the cell (Pl. I, fig. 5). If diffusion from the phosphatase border accounted for the nuclear reaction, one would expect to see a positive reaction first in that part of the nucleus nearest the inner margin of the cells, but instead the reaction appears uniformly over the whole nucleus (allowing for variations in thickness of the slices of the ramifying nuclei included in a particular section).

The results concerning the distribution of nuclear and cytoplasmic phosphatase activity along the glands are summarized in Text-fig. 1 (together with studies on pH effects). When the operations of fixing in 80 per cent. ethanol, dehydrating, and clearing are carried out at room temperature, a positive reaction occurs only in the broad lower regions (and to a small extent in the duct), but when the glands are kept in the refrigerator throughout these stages, phosphatase activity appears at all levels of the gland, including the narrow uppermost secretory regions (Pl. I, fig. 5), though it is always restricted to nuclei and inner cell borders. It is known that during the preparation of tissues at room temperature a large fraction (up to 75 per cent.) of the phosphatase activity is destroyed (Stafford and Atkinson, 1948; Doyle, 1948). Hence the explanation of the above observations seems to be that, although phosphatase is present throughout the length of the gland, it is most active in the broad lower secretory regions, where there is most protoplasm per unit length. Accordingly, after fixation in unfavourable temperature conditions there may be so much inactivation of phosphatase that in the delicate upper secretory regions with less phosphatase per unit length, insufficient enzyme
activity remains to give a positive Gomori test. It is noteworthy that in spider glands, where the secretory cells are of uniform character, no such differences appear if glands fixed in a refrigerator are compared with glands fixed at room temperature.

*Bombyx mori*. In the silk glands of the familiar larvae of *Bombyx mori* (silkworms) the phosphatase distribution (Pl. I, fig. 7) is almost exactly the same as in the silk glands of *Cossus* larvae. Only the very broad lower region of each gland, lying in the form of a U with arms pointing anteriorly, has the thin walls and wide lumen of a reservoir. Here there is a very thick intima and comparatively sparse protoplasm. Secretory activity and the intensity of the phosphatase reaction are much reduced, though not completely absent.

Silk glands of all the species studied showed activity with a variety of substrates including β-glycerophosphate, glucose-1-phosphate, and sodium phenyl phosphate.

**CONDITIONS AFFECTING THE ACTIVITY OF SILK-GLAND PHOSPHATASE**

In considering the localization of phosphatases—or any other enzymes—in cells, it is interesting to inquire both what is responsible for the segregation observed and what factors govern the activity of the phosphatase in those places where it does occur. Knowledge and techniques are so limited in this field at the moment that such general questions remain quite unanswered, but it has been possible to study such specific points as whether the enzyme is likely to be active at physiological pH, how its activity is affected by various substances, particularly sulphydryl reagents, and finally how sulphydryl groups are distributed in the silk-gland cells.

**Alterations of pH in the incubation mixture.** The normal alkaline phosphatase test is run at pH 9 with veronal buffer and calcium ions; the acid phosphatase mixture includes lead ions and acetate buffer to give a final pH of about 5. This latter procedure can easily be adapted for use at pH 7 if the acetate buffer is replaced by water and the remaining mixture adjusted to pH 7 with 0.2 M sodium acetate solution.

The effect of such variations on the phosphatase activity of *Cossus* glands is summarized in Text-fig. 1, to which reference has already been made in describing differences between glands fixed at 0°–2° C. and at room temperature. Points where the reaction falls off to zero can be accurately determined and when a positive reaction occurs it is fairly constant throughout any one of the three main divisions of the gland. It is impossible to make anything approaching a quantitative determination with the cytochemical method, however, so that only limited significance can be attached to the relative heights; these were estimated both from the intensity of reaction after a certain period of incubation and from the time required to reach a given intensity. The results for cytoplasmic phosphatase were identical with those for nuclear phosphatase everywhere except in the ducts; here the small round nuclei showed slight activity under optimal conditions, but were negative at all other times.
In *Tegenaria* glands there was slight, but detectable, activity at pH 7, but at pH 5 the test was quite negative.

It has been possible to make a few quantitative measurements of phosphatase activity on homogenates of *Bombyx* glands and the limited results shown in Table I indicate that there is phosphatase activity over a considerable range of pH. The histological studies show that at each pH tested the cell border remains by far the most active site of phosphatase activity in the cell and therefore we can conclude that it is the cell border which is mainly responsible for the activity measured in the work with homogenates. Since activity in the homogenate is high at pH 6.5 (greater in fact than at any of the more extreme hydrogen ion concentrations), there is good reason for believing that the silk gland phosphatase is active in vivo. When tested with phenol red solutions, particularly advocated by Cowdry (1943) for measurements of pH in vivo, the silk glands of both caterpillars and spiders took on a colour resembling most closely that found in a buffer at pH 7. In view of the artifacts involved in applying an indicator to such a complex system, the result is probably of
little significance, and in any case there may well be appreciable spatial variations in intra-cellular pH. However, it can at least be said that, as far as pH is concerned, it has been impossible to find any reason why the border phosphatase should not be active in vivo.

Table I. *The Phosphatase Activity of Silk-gland Homogenates* (Bombyx mori)

The incubation mixture consisted of 7.5 or 14 cu. mm. of a distilled water-homogenate, containing 0.203 mg. gland (fresh weight) per cu. mm., 23 cu. mm. buffer and 26 cu. mm. substrate in a 0.2 ml. reaction tube held at 30°C. 30 minutes after addition of substrate, aliquots of 41.5 cu. mm. were taken, the reaction arrested by addition of strong sodium carbonate solution and the phenol liberated from the disodium phenol phosphate substrate was estimated colorimetrically by the Folin reaction, using a Beckmann spectrophotometer set at 6,000 A. and comparing the optical density with a standard curve for known phenol concentration. Inhibitors were made up in the buffer to give the final concentrations shown below. The buffers used were M/5 acetate and M/5 ammonium hydroxide/chloride.

<table>
<thead>
<tr>
<th>Sample of homogenate used</th>
<th>pH</th>
<th>Inhibitors or other substances added</th>
<th>Phenol liberated</th>
<th>Phenol liberated per mg. gland (fresh weight)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 14 cu. mm. un-centrifuged homogenate.</td>
<td>5.7</td>
<td>.</td>
<td>1.90</td>
<td>0.67</td>
</tr>
<tr>
<td>9.8</td>
<td></td>
<td></td>
<td>0.53</td>
<td>0.19</td>
</tr>
<tr>
<td>B. 7.5 cu. mm. supernatant from A after 5 mins. at 1,000 g.</td>
<td>5.7</td>
<td>.</td>
<td>0.65</td>
<td>0.43</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
<td>0.82</td>
<td>0.55</td>
</tr>
<tr>
<td>9.8</td>
<td></td>
<td></td>
<td>0.23</td>
<td>0.15</td>
</tr>
<tr>
<td>C. 14 cu. mm. supernatant from A after 5 mins. at 1,000 g.</td>
<td>9.5</td>
<td>p-chloromercuribenzoate (0.005 M.)</td>
<td>0.75</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cysteine hydrochloride (0.001 M.)</td>
<td>0.00</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>magnesium chloride (0.01 M.)</td>
<td>0.55</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>.</td>
<td>1.05</td>
<td>0.37</td>
</tr>
</tbody>
</table>

* Whether the results are related to fresh weight, dry weight, or nitrogen content, they are unavoidably complicated by the presence in the glands of a considerable, but variable, amount of inert silk.

The differences between the results obtained by the histochemical method and the quantitative method are not surprising in view of the inhibitory effect of the lead ions present in the Gomori acid phosphatase incubation mixture. This results in a general weakening of the reaction at pH 5 and pH 7 to such an extent that, even when the glands are fixed and prepared in a refrigerator, the narrow upper secretory regions where there is least activity initially fail to stain and appear negative. It must, of course, be remembered that *Bombyx* glands with phenyl phosphate substrate were used in the homogenate experiments and *Cossus* glands with glycerophosphate substrate in the histochemical experiments. Since, however, these two substrates were completely interchangeable for the Gomori reaction and the upper regions of *Bombyx* glands
are hardly distinguishable from *Cossus* glands, these differences can be neglected.

In many tissues there appear to be two or more phosphomonoesterases with distinct pH optima (Perlmann and Ferry, 1942; Dempsey and Deane, 1946; Moog, 1944, 1946; Gomori, 1939, 1941, 1949). Whether or not this is the case in silk glands is uncertain, but it seems very probable that there is, rather, a single enzyme with some degree of activity over a considerable range of pH. However, this point has by no means been fully investigated.

**Addition of activators and inhibitors.** Little if any inhibition was caused by the presence of mercury, silver, copper, or zinc ions, iodoacetate (all o-001 M.) or p-chlormercuribenzoate (o-0001 M.) in the Gomori incubation mixture. For testing the more precious substances, sections mounted on coverslips were incubated in small grooved perspex boxes requiring only 2–3 ml. incubation mixture for ten coverslips. The failure of inhibition may in several cases have been due to removal of inhibitor in insoluble form by the alkaline pH and the o-02 M. glycerophosphate substrate, though only copper produced a permanent precipitate. It was not possible to check all these substances by the quantitative method, but o-0005 M. p-chlormercuribenzoate, a fairly specific sulphydryl inactivator, caused 100 per cent. inhibition by this procedure (Table I).

The most clear-cut results were obtained with cysteine which caused complete inhibition histochemically (at o-01 M.) and in homogenates (at o-001 M.). o-001 M. cysteine and o-01 M. reduced glutathione caused marked inhibition histochemically. Incubation was for not longer than 5 hours to avoid difficulties of oxidation of cysteine to cystine (though the latter was without effect in saturated solution). That the histochemical inhibition is not due to amino-acid effects on the solubility of calcium salts (Lehmann and Pollak, 1942) is suggested by the fact that o-01 M. alanine is without effect. Cysteine action can also be demonstrated by covering the sections with a few drops of o-1 M. cysteine (pH 7), standing for 1 hour in an atmosphere of nitrogen (to prevent the rapid oxidation which occurs in air), and then, after washing in running water for 15 minutes, incubating in the normal alkaline phosphatase medium with no added cysteine. Such sections show marked inhibition compared with controls treated with water in an atmosphere of nitrogen.

This inactivation by reducing agents may explain the observation that o-01 M. cyanide causes much more marked inhibition than o-01 M. azide.

It appears, therefore, that silk gland phosphatase is inhibited not only by potent inactivators of sulphydryl groups, but also by an abundance of substances containing sulphydryl groups. As the latter are often abundant in metabolically active tissues it seemed of interest to try to investigate the distribution of sulphydryl groups in the silk-gland cells.

**The distribution of sulphydryl (SH) groups in the cytoplasm of silk glands.** When freshly dissected glands of caterpillars or spiders are treated with a few drops of 5 per cent. sodium nitroprusside solution and one drop of dilute ammonia solution they show a bright-pink coloration. Evidently SH groups—
or possibly easily reduced disulphide (SS) groups—are abundant in the glands, but all attempts to apply this reaction to frozen sections were unsuccessful. The pink colour, though plainly visible in the whole gland, is quite undetectable in sections cut at 15 or even 25 μ.

The prussian-blue method of Chèvremont and Frederic (1943), on the other hand, gives a dark-blue coloration which can readily be detected in 15 μ frozen sections of silk glands fixed in 4 per cent. formaldehyde for ½ hour and cut from a gelatin block. In sections of spider glands the prussian blue appears in the basal part of the gland wall, but the inner margin next to the gland lumen is colourless or only very slightly tinged. With Cossus glands the main body of the cytoplasm is deeply stained, but a narrow inner border zone, exactly corresponding with the phosphatase border, remains unstained (Pl. I, fig. 8). It is difficult to avoid some random sedimentation on the gelatin-coated slide, but considering the nature of the frozen-section technique, the results are fairly satisfactory. Slides incubated for 10 minutes in 0.01 M. mercuric chloride solution (and washed before staining) show no blue colour in any part of the section.

It is impossible to draw conclusions about the soluble SH compounds in the glands, but it can at least be said that although, like many active tissues, the main bulk of the silk glands gives a strong SH reaction, free SH groups are almost or completely absent from the less soluble constituents of the phosphatase border region. This is in accordance with the observations, described above, that various SH compounds inhibit silk gland phosphatase. Such factors as these may be important in the cellular segregation of phosphatases from synthetic phosphorylating enzyme systems, the activities of the two sets of enzymes being clearly opposed to each other, although they may all participate in a particular metabolic process.

THE LOCALIZATION OF NUCLEIC ACIDS IN SILK GLANDS

The process of silk formation can be divided into three main stages: (1) the synthesis of fibroin and sericin within the cells; (2) the passage of these substances from cytoplasm to lumen; (3) the spinning of a silk thread from the silk in the lumen of the gland. The question naturally arises of where and how the phosphatase is involved in this sequence of events.

Several investigations have shown that in Bombyx the last of these stages is purely mechanical in nature and quite independent of enzyme action (Brill, 1930; Ho, Shen, Tan, and Yu, 1944). Threads can easily be spun artificially from the duct or from the reservoir and these have an X-ray diffraction pattern closely similar to that of the natural fibres. The muscular press and glands of Lyonet are, therefore, not essential for the formation of a fibre. The press probably serves to unite the fibres into a single thread. The small pair of interesting glands named after Lyonet, who described them in Cossus in the long memoir he wrote (1762) on ‘La chenille, qui ronge le bois de saule’, probably produce some sticky secretion binding the threads together. In some species of Lepidoptera the silk produced by the larva is dark brown in colour.
and insoluble in cupri-ethylene diamine solutions; these properties suggest the existence of phenolic tanning similar to that found by Pryor (1940) in the cockroach ootheca and subsequently discovered in many other places. There is, however, no evidence of phenolic tanning in the silk of Cossus or Bombyx. As soon as they are orientated by squeezing and stretching during flow through the duct, the protein micelles seem to snap into their position in the silk fibre (the glycine-alanine-serine polypeptide chains being particularly important here) and only reagents like cupriethylene diamine solutions will disrupt this stable configuration (Meyer, 1942; Coleman and Howitt, 1949; Drucker and Smith, 1950). If this is also true in spiders (where the silk is slightly different in amino-acid composition from that in caterpillars, but very similar in X-ray diffraction properties) one can appreciate the significance of the existence in both cases of the well-defined lining cuticle or intima, which will confer a high degree of structural rigidity on the long thin ducts, the length of which is often increased by considerable coiling in spiders. It is clear, however, that the alkaline phosphatase is not primarily concerned in this stage of the process, though it is possible that the duct phosphatase removes from the protein any remaining phosphate groups which would hinder the orientated micelles from taking up the silk fibre configuration.

Concerning the release of silk from the cells we have no information. In spiders the conspicuous silk globules pass bodily out of the cell. In caterpillars the silk first forms well-defined globules in the intima immediately outside the protoplasm, but nothing like pore canals has been detected in the intima.

For the present, therefore, attention has been concentrated on the first aspect of the whole process—the production of the protein within the cells. Several investigators have observed that protein-synthesizing cells possess a much higher content of cytoplasmic ribonucleic acid than cells engaged in other metabolic processes; the evidence has been reviewed by Brachet (1947), Caspersson (1947), and Davidson (1949). Consequently it seemed desirable to study the distribution of nucleic acids in these silk glands.

The methods used for this study have already been outlined—the Feulgen technique, pyronine and methyl green staining and ultra-violet microscopy, all used in conjunction with ribo- and desoxyribo-nuclease. These methods have, in all cases where they overlap, given results which are in complete agreement with each other.

As might be expected, the nuclei contain considerable amounts of desoxyribonucleic acids, as indicated by all three methods. The strong ultra-violet absorption in the nuclei at 2,536 Å which resists ribonuclease digestion (Pl. I, fig. 10) is abolished by digestion with desoxyribonuclease (Pl. I, fig. 11).

In accordance with the findings of Caspersson and Brachet in other tissues the cytoplasm of the silk glands of both Cossus and Tegenaria contains much greater quantities of ribonucleic acids than the cytoplasm of most other tissues in these species. In the reservoir regions of spider glands and in the ducts of both animals there was, however, much lower nucleic acid content.
Pl. II, fig. 9 shows an ultra-violet photograph of a secretory region and a reservoir; the difference in absorption is apparent. Ribonuclease removes much of the absorbing material from the cytoplasm of the secretory cells leaving it (Pl. II, fig. 10) with roughly the same absorption as the reservoir, which is unaffected by this treatment. The further effect of desoxyribonuclease has already been mentioned. Pl. II, fig. 12, shows the pyronine-methyl green picture of one wall of a secretory region and one wall of a reservoir region; the contrast in basiphilia is apparent and the pyronine staining material of the secretory cells is completely removed by ribonuclease. Pl. II, fig. 14, shows the junction between secretory and reservoir cells, where again a contrast in basiphilia can be distinguished. The glands of Cosus and Bombyx also have intense cytoplasmic ultra-violet absorption and basiphilia, which is greatly diminished by ribonuclease digestion (Pl. II, figs. 16, 17, 18).

However, the ribonucleotides are not uniformly distributed in the cytoplasm of the secretory cells, for in most cases there is a region just along the inner margin of the cells which has markedly lowered ultra-violet absorption and basiphilia, indicating that in this region—exactly where the phosphatase is situated—there is a lower concentration of ribonucleic acid than in the main body of the cytoplasm. The experimental evidence is shown in several photographs. In Pl. II, fig. 12, the strong basiphilia of the secretory region is restricted to the basal half of the cell and is absent from a broad region on the inner side of the cell where the phosphatase activity is highest, as shown by Pl. II, fig. 13, a Gomori preparation of a section close to that seen in Pl. II, fig. 12. Basal ribonucleic acid and inner border phosphatase in adjacent sections are also shown in Pl. II, figs. 14 and 15. The cytoplasm of the reservoir region in these sections lacks both ribonucleic acid and phosphatase. The lower ultra-violet absorption near the inner margin appears despite the fact that this region is packed with silk globules containing some 10 per cent. tyrosine, which has a strong absorption peak at a wave-length only some 200 Å. higher than the wave-length near the nucleic acid absorption peak which is used for photography. In Tegenaria glands this region, poor in nucleic acids, is broad and diffuse like the phosphatase zone with which it coincides. In caterpillar glands, however, this region is only a few μ wide—again corresponding with the phosphatase border, which is here very narrow and well defined (Pl. I, fig. 7, and Pl. II, fig. 18, Bombyx; Pl. I, fig. 5, and Pl. II, figs. 16 and 17, Cosus).

To confirm the existence of this inner border zone, poor in nucleic acids, a few precise measurements were made of the optical density near the nucleic acid absorption peak at several points across the silk-gland cell. Sections cut at 2 μ from frozen-dried Bombyx glands were dewaxed in petroleum ether and mounted, via ethanol, in glycerol. By means of the apparatus described earlier, the transmission was measured (a) at each of three points equally spaced across the gland cell, point 1 being nearest the periphery, (b) at a point 3-4 μ inside the inner margin of the cell, i.e. in the phosphatase border zone suspected of having a low ribonucleic acid content, (c) at a randomly selected
point in the silk in the lumen of the gland. The values for the extinctions at these points across any one cell are joined in Text-fig. 2 for convenience of inspection. Several such 'curves' were measured for each wave-length; those shown in Text-fig. 2 are typical. The extinctions at 2,650 Å (the nearest wave-length to the nucleic acid absorption peak at which measurements could be made) are high across the main part of the cell, but fall sharply in the border zone. The same is true of the values at 2,536 Å, which is also close to the nucleic acid absorption peak. However, when measurements are made at 2,800 Å, the protein absorption maximum, there is no marked fall in absorption in the border zone (Text-fig. 2). There is, therefore, no fall in protein concentration between point 3 and the border zone, so that the decreases in $E_{2650}$ and $E_{2536}$ are not due to a general decrease in the solid content of the cytoplasm. In view of this it seems most unlikely that anything else than a considerable fall in nucleic acid content can account for the marked fall in $E_{2650}$ and $E_{2536}$ between point 3 and the border zone.
(With regard to the $E_{2800}$ curve in Text-fig. 2, it can be seen that far from there being any fall in protein concentration in the border zone, there is very probably a considerable increase. Nucleic acids have an $E_{2800}$ which is about half their $E_{2650}$. If then there is a fall of 0.4 in $E_{2650}$ between point 3 and the border zone due to a fall in nucleic acid concentration, there should be a fall of about 0.2 in $E_{2800}$. In fact there is only a fall of about 0.05 in $E_{2800}$ between point 3 and the border zone, and it can be shown that to account for this there must be, approximately, a twofold increase in the concentration of other groups such as proteins absorbing at 2,800 A. This would be in accordance with the accumulation of silk at the cell border before it passes out through the intima to the lumen. No attempt has been made to deduce actual concentrations of substances and so it has not been felt essential to evaluate light-loss due to scattering.)

The existence of differences in staining properties between the phosphatase zone and the rest of the cell had already been deduced from standard staining methods. Some techniques, e.g. for fats and glycogen, gave quite negative results. The Baker sudan black method showed, for instance, that the silk glands contain very little material stainable in this way, though the eggs of Tegenaria were richly packed with lipoids. Tests for glycogen and other polysaccharides failed to reveal appreciable amounts of these substances. However, the Mallory and Azan triple-staining methods indicated profound staining differences between the inner marginal zone and the main bulk of the cell. With Azan, for instance, the body of the cell in all species stained deep red with the azocarmine component, but the inner marginal zone stained with aniline blue only and could be clearly seen to contain masses of silk globules to the exclusion of any red-staining material. Study of the results of Azan staining in sites of known nucleic acid distribution show that the azocarmine component stains sites of high nucleic acid concentration (ribo- or desoxyribonucleic acid). Hence the restriction of azocarmine staining to the basal parts of the secretory cells is completely in accordance with the results obtained by using the more specific cytochemical techniques described above. It was also noted that there are differences between the staining properties of the silk in the upper and in the lower parts of caterpillar silk glands, but the reason for this is unknown.

Despite their special advantages for studies of cell physiology, silk glands have received little attention from previous investigators. Lesperon (1937), in a long study of insect silk secretion, did not comment particularly on the basiphil nature of silk-gland cytoplasm, but noted the low lipoid content. Millot (1926), however, in an interesting and extensive study of the histophysiology of spiders, noted that the basal cytoplasm round the nucleus of the silk-gland cell is ‘remarquablement basophile’ exactly as described above and resulting from the considerable amounts of ribonucleic acids which more specific techniques show that it contains.

Study of nucleic acid distribution has, therefore, led to two main conclusions. The cytoplasm of spider and caterpillar silk glands is rich in ribonucleic
acids (in cells of the secretory, but not of the reservoir, regions). This nucleic acid is not uniformly distributed in the cells for there is, in all species examined, a region of much lower nucleic acid content along the inner cell margin, exactly corresponding with the region of high phosphatase activity.

**Discussion of the Relation Between Phosphatases and Nucleic Acids in Silk Glands**

Although it is a matter of great importance, there is little detailed evidence concerning the mechanism of protein synthesis. The many chemical possibilities have been reviewed recently by Linderstrom-Lang (1949). It is known that adenosinetriphosphate is necessary for the acetylation of sulphanilamide (Lipmann and Kaplan, 1949) and for the formation of glutamine from glutamic acid (Elliot, 1948), both of which bear suggestive parallels to peptide bond synthesis. There is no clear evidence of whether peptidases are involved; certainly the synthesis of complex carbohydrates is very different from their hydrolysis by amylases.

The constant association of high local concentrations of nucleic acids with protein synthesis (Caspersson, 1947; Brachet, 1947; Davidson, 1949) is apparent from studies of pancreas, salivary glands, hair follicles and the basal layer of the epidermis, eggs and embryonic tissues in general, regenerating tissues, and, as shown here, the silk glands of invertebrates. To explain the mode of action of nucleic acids some sort of mould or template action has often been invoked, not from direct evidence, but chiefly by analogy with Astbury and Bell’s (1938) well-known X-ray crystallographic demonstration of the close similarity in the spacing of groups along the desoxyribonucleotide chain and along the fully extended polypeptide chain. No satisfactory corresponding picture has been obtained for ribonucleoprotein, which, in bulk at least, is more important. The only sort of information available here is that ribonucleoprotein complexes can readily be obtained from protein synthesizing tissues such as pancreas (Mirsky and Pollister, 1946) and that the simplest self-reproducing units such as tobacco mosaic virus, consist solely of ribonucleoprotein.

If in fact some sort of combination occurs between nucleic acid and protein during the synthesis of the latter, then it is possible that the phosphatase of silk glands is concerned in some stage of the process of liberating the finished protein from the nucleic acid in association with which it has been synthesized. The distribution of substances in the silk-gland cells certainly suggests this, in that as one follows the course taken by secretory products across the cytoplasm towards the lumen, the high concentration of nucleic acid ends where the phosphatase begins. By itself a phosphomonoesterase of the type described here would be unlikely to bring about the breakdown of a nucleoprotein, but it might well be one of a system of enzymes involved in this process. Until more direct evidence is available, however, this suggestion must remain very tentative, for the enzyme may promote the transfer of secretory products across the cell boundary in some other way not involving direct action on
nucleic acid or protein phosphate groups. It is of interest briefly to review related chemical and cytological work, which adds considerable evidence in favour of the view that phosphomonoesterases are important in nucleoprotein metabolism (Bradfield, 1950).

From the chemical aspect it is known that ribonucleoprotein complexes occur in tissues (Mirsky and Pollister, 1946). In order to obtain more direct evidence about whether proteins are synthesized in actual combination with nucleic acids it would be interesting to separate ribonucleoproteins from silk glands and discover whether any of their protein components correspond with silk fibroin in amino-acid composition, or whether the incorporation of radioactive glycine into silk-gland proteins \textit{in vitro} (Zamecnik, Loftfield, Stephenson, and Williams, 1949) occurs preferentially in proteins associated with ribonucleic acids.

Posternak found that four synthetic peptides containing phosphotyrosine (phosphate on the para-hydroxyl) were completely unaffected by peptidases. The protective action extends to a peptide bond separated from the phosphotyrosine by a glycine residue; larger peptides were not studied. As soon as the phosphate group was removed, the peptides were split quantitatively to amino-acids. It appears, therefore, that combination of inorganic or organic phosphate with serine and tyrosine hydroxyl groups might be a means of preventing proteolytic breakdown of the fibroin—both of the amorphous and the crystalline segments of the polypeptide described by Meyer (1942) and by Drucker and Smith (1950). Hence it may be that the role of the phosphatase is only to remove such protective phosphate groups during the final release of the protein from the cell.

Various combinations of snake venom phosphatases will dephosphorylate yeast ribonucleic acid to an extent of 75 per cent. (Gulland and Jackson, 1938), and Schmidt and Tannhauser (1943) state that gut phosphatase almost completely dephosphorylates this substance, though it is without effect after heating at 80° C. for 15 minutes, which seems to rule out ribonuclease activity.

From the cytological aspect phosphatase activity has been correlated with nucleoprotein metabolism in both nucleus and cytoplasm.

Several investigators (Gersh and Bodian, 1943; Hyden, 1943) have shown that the Nissl bodies in the cytoplasm of motor neurones contain large quantities of ribonucleoprotein, which is in accordance with their well-known affinity for basic stains such as toluidine blue. Consequently, the marked chromatolysis, or loss of basiphilia and the subsequent recovery of Nissl substance which follows axon section, is a period of intense nucleoprotein metabolism, and Bodian and Mellors (1944) have shown that there is during this period a marked rise in the acid phosphatase activity of the cell-body cytoplasm. Immediately after axon section there follows a regressive period during which both protein and nucleic acid components of the Nissl bodies diminish greatly; the recovery of Nissl substance commences about 15 days after operation, when the major part of axon replacement has yet to be accomplished. It is approximately at this period that the rise in acid phosphatase
activity becomes appreciable; it persists throughout the recovery phase, but cannot be detected biochemically because these neurones constitute only 5 per cent. of the substance of the cord. It is extremely probable that the cell-body cytoplasm is, during this period, synthesizing new protein for the relatively enormous bulk of the regenerating axon, which is constantly supplied with materials from the cell-body (Weiss, 1943; Young, 1945). Much remains to be discovered about the metabolism of neurones, but there is a distinct correlation between phosphatase activity and nucleoprotein metabolism in the period of intense protein formation during axon regeneration.

As mentioned earlier, collagen formation in healing skin wounds of rats and guinea-pigs is associated with a marked rise in phosphatase activity in fibroblast nuclei, cell processes, and the fibres themselves (Fell and Danielli, 1943). Wounds in scorbutic guinea-pigs show no increased phosphatase activity and fail to form proper collagen fibres (Danielli, Fell, and Kodicek, 1945). In regenerating rabbit nerves Marchant (1949) found that high phosphatase activity coincided with collagen formation in the degenerating peripheral stumps, but only appeared late during collagen formation in the scar tissue surrounding the cut ends of the nerves. This emphasizes that many different cases must be studied before any valid generalizations can be made, but on the other hand it must be noted that the rabbit tissues in these experiments were fixed in 80 per cent. ethanol for 20 hours at room temperature, which may well have inactivated all but the most powerful sites of phosphatase activity. Accompanying the secretion of fibrillar proteins in osteoblasts and odontoblasts is considerable alkaline phosphatase activity and a high content of cytoplasmic ribonucleotides (Thorell and Wilton, 1945; Lorch, 1947; Greep, Fischer, and Morse, 1948). Phosphatase and nucleic acids are commonly found in close association in placental membranes, but the precise details of their relationships vary greatly from one species to another (Wislocki and Dempsey, 1946). Acid phosphatase can be detected in the keratohyalin granules of mammalian skin, but whether phosphatase participates in keratin formation in hair follicles is uncertain; activity is very high in the dermal papilla (as in the blood capillaries of many tissues), slight in the root sheath and low elsewhere (Bourne, 1943; Johnson, Butcher, and Bevelander, 1945). Jeener (1948) observed interesting changes in the phosphatase activity and nucleic acid content of the mouse vagina as a result of treatment with oestrogens. The injection of 10 mgm. of oestradiol into an ovariectomized mouse caused, in 24 hours, a marked rise in alkaline phosphatase activity in the proliferating vaginal epithelium (quantitative estimates showed a threefold rise in activity) and a marked accompanying rise in cytoplasmic ribonucleic acid content. Very similar increases appear in the circular muscle layer. In the epithelium there is strong evidence that these changes are related to the secretion of keratin, of which oestradiol causes a rapid synthesis, while there is as yet less complete evidence that the changes in muscle may be related to the synthesis of myosin. This correlation with keratin formation constitutes a very interesting parallel with events in silk glands where, as shown here,
high phosphatase activity and high ribonucleic acid content are associated with formation of the fibrillar protein of silk.

Jeener (1946) has also shown that cytoplasmic particles rich in ribonucleic acid can be divided into a nucleic acid-containing fraction soluble in 0.6 M KCl and an insoluble residue rich in phosphatase.

Turning to the nucleus, it is interesting to note that similar conclusions have been reached regarding the so-called 'residual chromosomes'. By centrifugation of various tissue suspensions Mirsky (1947) obtained nuclear material from which desoxyribonucleohistone was removed by treatment with molar NaCl solution, leaving what have been described as residual chromosomes. The latter contain most of the ribonucleic acid and phosphatase activity present in the original nuclei, together with tryptophane-containing proteins probably related to the 'chromosomin' of Stedman and Stedman (1947).

Mirsky's preparations certainly contained nucleoli but it is clear from his and other work (Brachet, 1947; Ris, 1947; Price, Miller, Miller, and Weber, 1949) that these do not account for all the nuclear ribonucleic acid, much of which occurs in the chromosomes (of interphase as well as dividing nuclei). Ribonucleic acid can be removed from these 'residual chromosomes' leaving a residue rich in phosphatase just as for Jeener's cytoplasmic particles described above. The chemical aspect of these findings is interesting and significant, whether this material is closely related to the chromosome proper or whether, as some have suggested, it is merely distorted fragments of nuclear material (Lamb, 1949). The topographical relation between phosphatase and nucleic acid is on a much smaller scale within these nuclear and cytoplasmic particles (0.1–2 μ in diameter) than in the silk-gland cells (cubes of side 200 μ), so that associations of these substances are found at many levels of organization within the cell.

For massed liver nuclei isolated by bulk centrifugation, Dounce (1943) has shown that the concentration of alkaline phosphatase is twice that in the cytoplasm. Brachet (1947), using a cytochemical method, found in various organs of the mouse a definite parallel between the intensity of nuclear phosphatase and the rate of turn-over of thymonucleic acid phosphorus (known from studies of Hevesy and Otteson, 1943). 'Organs with a high turn-over of thymonucleic acid P give a strong reaction (intestinal mucosa, testis and spleen), while the reaction is very faint in the nuclei of organs where the turn-over is low (muscle, kidney, brain). The intensity of the nuclear alkaline phosphatase reaction is increased in rat's liver after partial hepatectomy; so is also the thymonucleic acid P turn-over, as shown by Brues, Tracy, and Cohn (1944).’ In the mitotic fibroblast Willmer (1942) found phosphatase markedly concentrated on the chromosomes, and in Drosophila salivary chromosomes Danielli and Catcheside (1945) and Krugelis (1946) showed that phosphatase is actually restricted to the Feulgen-positive bands where the desoxyribonucleic acid is concentrated.

Finally, phosphatase is of widespread occurrence in the nucleolus, which is of considerable interest, since in the view of Caspersson (1947) and his
collaborators the nucleolus is an important site from which protein migrates to the nuclear membrane, there to induce, or participate in, the formation of ribonucleo protein in the cytoplasm. Indeed, the vast literature surveyed by E. B. Wilson is full of observations on nucleolar physiology which foreshadow these conclusions, and numerous subsequent workers (Ludford, 1925; Gresson, 1929, 1932; Raven, 1949) have shown, from studies of living and fixed tissues, that nucleolar secretion products pass into the cytoplasm—the process being particularly conspicuous during active cell metabolism, as in oogenesis and embryonic development. Clearly it is very significant to find abundant phosphatase in a structure rich in ribonucleic acid, which is manufacturing protein and passing it (or-ribonucleoprotein) to the cytoplasm.

The exact role of phosphatase in these sites is unknown at present, but in all of them—regenerating Nissl bodies, chromosome bands and especially the nucleolus—it may well be concerned in the liberation of protein from a nucleoprotein complex, so that viewed in this light the suggested role of phosphatase in silk glands may well be only a specially conspicuous case of a general phenomenon in nucleoprotein metabolism.

Many sites of conspicuous globular protein secretion lack a cytoplasmic phosphatase which can be demonstrated by the Gomori technique (pancreas, gastric mucosa, salivary gland, and albumen-secreting region of the hen's oviduct). The reversible conversion of proteins from the fibrillar to the globular state (silk fibroin) and vice versa (insulin) suggests that the differences between these groups are less fundamental than might be supposed, but they are none the less real and important for the living organism. Nucleic acids are abundant in sites of formation of proteins of both types, but at present phosphatases have been detected in numerous sites of fibrillar protein secretion (proteins both of the collagen and of the keratin-fibrinogen-myosin groups), but in very few sites of globular protein secretion. So little is known about the mode of formation of any protein that it would be idle to speculate about differences between the modes of formation of different proteins, but it is not surprising that such exist.

Finally, it may be of interest briefly to comment on the possible relation of silk-gland phosphatases to other more familiar phosphatases found in quite different tissue environments, such as the calcifying regions of bones and teeth and the free borders of kidney tubules and intestinal epithelia. With regard to bone, it is interesting to recall that during osteogenesis, in either young animals or bone wounds, high phosphatase activity appears before calcification and that during this period it is very probably concerned with the formation of the matrix of fibrillar protein upon which occurs the subsequent deposition of mineral salts. In intestinal epithelia, and very probably in other absorptive epithelia, the free borders which are so rich in phosphatase activity have a structural basis of fibrillar protein. Indeed, it almost seems as if a phosphatase concerned in the formation of a fibrillar protein (bone matrix or kidney brush border) has remained subsequently in functional association with the finished protein. Such a view is, of course, at the most tentative and
might seem quite unwarranted but for the remarkable circumstances in the excretory tubules of certain insects. In the third-stage larva of *Sisyra fuscata* (Neuroptera), for instance, the Malpighian tubes consist of two quite different segments. The upper half of each is a typical unmodified excretory tubule. The lower half secretes the silk from which the cocoon is spun and consists of very large cells with ramifying nuclei. The same phosphatase inner border is continuous throughout both. In the excretory region it is closely analogous with the brush border of a kidney tubule and in the lower half it exactly resembles the phosphatase border of a caterpillar silk gland. Moreover, it is very probable that a similar state of affairs exists in the kidney of the stickleback (*Gasterosteus*) where a substance resembling silk is secreted in the renal tubules. It would be unwise to attach too much significance to such comparisons, but they do suggest some degree of unity between the very diverse sites where phosphatases have been shown to occur.

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EXPLANATION OF PLATES

In the alkaline phosphatase preparations, sites of phosphatase activity are deep black, but only the blackening specifically referred to is due to enzyme activity—other darkening appears also in controls and may be due to preformed phosphate, counterstain, or other causes. The ultra-violet photographs are of unstained 2μ sections taken at 2,536 Å, i.e. in the region where the ratio of nucleic acid absorption to the absorption of other substances in the section is a maximum. Magnification is indicated by a scale equivalent to 50μ, except in Pl. I, fig. 3, where the scale is equivalent to 100μ. All six ultra-violet photographs (Pl. II, figs. 9, 10, 11, 16, 17, 18) are at the same magnification indicated by a single 50μ scale in Pl. II, fig. 16.

Plate I

Fig. 1. Cossus. Whole silk gland freshly dissected and stained with pyronine and methyl green. Large ramifying nuclei can be readily distinguished in the secretory region; those in the duct are smaller and discoid. The 'nozzle' effect is evident as the secretory region passes into the duct, where the micelles become orientated in spinning.

Fig. 2. Tegenaria. Alkaline phosphatase in nucleoli and along inner cell-borders of a small silk gland. Some cells are packed with globules almost throughout; others have a basal region of clear cytoplasm. Several basement membrane nuclei can be distinguished. Incubation was for 4 hours at 37° C.

Fig. 3. Tegenaria. Two large silk glands of the ampullaceal type. Diagonal shading indicates the regions containing the cytoplasmic border phosphatase, which is absent from the dilated reservoir region in both cases.

Fig. 4. Tegenaria. Phosphatase preparation of a section passing obliquely through the base of a reservoir of the kind shown in Fig. 3. It shows the well-defined junction between phosphatase positive secretory region and phosphatase negative reservoir region, lacking the mass of silk globules which can be seen passing out of the cell in the secretory region. Incubation was for 24 hours at 37° C.; hence the maximal width of the phosphatase border.

Fig. 5. Cossus. Phosphatase in nuclei and cytoplasmic border of the upper region of a silk gland (wall 125μ thick). The intensely reacting nucleoli can be distinguished in some branches of the ramifying nuclei. This upper region gives a positive reaction only when ice-cold fixative is used; the latter gives not only better enzyme preservation, but also much less distortion and shrinkage. Incubation was for 1½ hours at 37° C.

Fig. 6. Cossus. The strong phosphatase border in the duct of the silk gland. (Secretory region, left; transitional region, centre; duct, right.)

Fig. 7. Bombyx. Phosphatase in nuclei and inner cytoplasmic border of the silk glands. This section is taken through the lower secretory region where the intima, or inner lining membrane, is much thicker than in the upper regions (Fig. 5). The darkening of the intima appears also in controls. Fixative used at room-temperature (as here) gives results inferior to those with ice-cold fixative (Fig. 5). Incubation was for 2 hours at 37° C.

Fig. 8. Cossus. The distribution of the blue coloration in a frozen section of a silk gland after treatment by the sulphydryl method of Chevremont and Frederic. The phosphatase border region reacts much more weakly than the rest of the cell. The random sedimentation is difficult to remove since the slide is coated with gelatin.

Plate II

Fig. 9. Tegenaria. Ultra-violet photograph of the silk glands (unstained). Cytoplasmic absorption is high in the secretory region (right, with silk in its lumen) and low in the reservoir region (left, only one wall shown). Absorption in the phosphatase border zone of the secretory region is distinctly lower than in the main body of each cell.

Fig. 10. The same section as in Fig. 9 but after digestion with ribonuclease for 2 hours. The dense cytoplasmic absorption of the secretory region has been markedly decreased to about the level seen in the reservoir, which has been little affected.

Fig. 11. The section as photographed in Fig. 10 has now been treated with desoxyribonuclease. Most of the desoxyribonucleic acid has been removed from the cell nuclei.

Fig. 12. Tegenaria. Silk gland stained with pyronine and methyl green. There is high basiphilia in the basal regions of secretory cells, but only very slight staining in the reservoir. The basiphilia of the secretory region is completely removed by ribonuclease.
**Bradfield—Phosphatases and Nucleic Acids in Silk Glands**

**Fig. 13.** *Tegenaria.* Phosphatase preparation of a section close to that in Fig. 18. The secretory region (left) has powerful phosphatase activity along the outer cell margins, which are poor in nucleic acids; the phosphatase content of the basal regions (rich in nucleic acids) is much lower. The reservoir (right) lacks appreciable cytoplasmic phosphatase and its nuclei are much less active than those in the secretory regions.

**Fig. 14.** *Tegenaria.* Wall of silk gland stained with pyronine and methyl green, showing a secretory region (upper right) with marked basiphilic staining in the basal part of the cells, passing into a reservoir region (lower left), where the cytoplasm is devoid of basiphilic material.

**Fig. 15.** *Tegenaria.* Phosphatase preparation of a section adjacent to that in Fig. 14. The enzyme is restricted to the secretory region (upper right) and is there localized along the cell border in the zone of low nucleic acid concentration.

**Fig. 16.** *Cossus.* Ultra-violet photograph of a silk gland (only the inner edge of the cells is shown). The main body of the cytoplasm has high absorption, but along its inner edge there is markedly lower absorption (indicated by arrow), i.e. in the narrow border region with high phosphatase activity.

**Fig. 17.** As Fig. 16, but after ribonuclease digestion for 2 hours. Cytoplasmic absorption has been greatly diminished—except in the inner border where little change has occurred. Nuclei still absorb strongly.

**Fig. 18.** *Bombyx.* Ultra-violet photograph of silk gland showing the region of low absorption along the inner border of the cells. This section was taken from high up the gland where there is only a very thin intima.