Riboflavin, Light, and the Growth of Plants[1]

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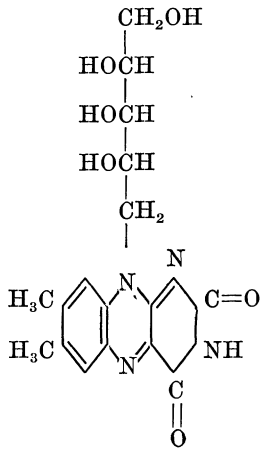
RIBOFLAVIN, also known as lactoflavin and vitamin B_2, has been known since 1932, when it was discovered almost simultaneously in the laboratories of Warburg and Szent-Györgyi. Chemically, it is 6,7-dimethyl-9-(1′-D-ribityl)-isoalloxazine). In aqueous solution it is



Riboflavin

yellow, possessing an absorption peak at about 4450 A. It is highly fluorescent, emitting green light when exposed to ultraviolet or blue wavelengths. It is capable of reversible oxidation and reduction, the reduced compound being colorless. It is unstable in light, the type of decomposition it undergoes being dependent on the pH of the medium. In neutral or acid media, the ribityl fraction is split from the molecule, leaving lumichrome, a derivative of alloxazine. In alkaline media, only a portion of the ribityl moiety is removed, leaving an N-methyl isoalloxazine derivative known as lumiflavin.

Biologically, riboflavin is of importance in that it is a constituent of the prosthetic group of various flavoproteins, or "yellow enzymes," which are involved in electron transfers in respiration. Approximately ten yellow enzymes have now been described. These differ greatly among themselves with regard to reactivity, the oxidized form of the various enzymes being reduced by such widely divergent substrates as coenzymes I and II, amino acids, xanthine, and various aldehydes. The reduced form of these enzymes is oxidizable by oxygen, although generally more quickly by other materials, among them methylene blue, fumaric acid, and cytochrome C. The prosthetic group of these enzymes is either riboflavin phosphate or flavin-adenine dinucleotide. In many instances, it has been possible to separate the enzyme into apoenzyme and prosthetic group, with attendant loss of activity. When the two moieties are brought together the enzyme is reconstituted, and the specific activity reappears. It is generally considered that the flavoproteins occupy a position in the respiratory chain of enzymes between the pyridine nucleotide enzymes and the cytochromes.

Almost all of the information concerning riboflavin and the flavoproteins has been obtained by the use of microorganisms and various animal tissues. Although riboflavin is known to be abundant in many higher plants, investigations on its physiological role have been very scanty. In 1937, van Herk (*24*) obtained evidence that flavin enzymes are involved in the prodigious respiration of the *Sauromatum* spadix. The enzyme diaphorase, a flavin enzyme which catalyzes the oxidation of reduced coenzyme I and II by methylene blue, has been found in legume seedlings, potato tubers, and pollen (*16, 18*). Flavoproteins have also been found to stimulate the activity of various dehydrogenases of the *Avena* coleoptile (*1*). These disconnected bits of evidence have not yet been incorporated into a generalized picture of plant respiration.

The fact that riboflavin is a fluorescent pigment has led certain investigators to speculate about its possible role as a light receptor in various organisms. For instance, Euler and Adler (*5*) showed that the retina of the codfish has up to 4175 μg of riboflavin per gram dry weight, a value approximately one hundred times greater than that of tissues like liver and kidney, ordinarily considered flavin-rich. The retinal flavin is mainly dialyzable, and in this free form is highly fluorescent, leading Euler and Adler to attribute to it the role of converting the difficultly

[1] Much of the experimental work herein described has been performed with the aid of Miss Rosamond S. Baker, to whom the author wishes to express great appreciation. The action spectrum herein reported was worked out at the Beltsville, Maryland, laboratory of the U. S. Department of Agriculture, with the cooperation of Drs. S. B. Hendricks, M. W. Parker, and H. A. Borthwick. The carnosine was obtained from Dr. Emil L. Smith and the methylol riboflavin from Dr. Samuel Gordon of Endo Products, Inc.

visible short wavelengths to longer, more easily visible wavelengths. Theorell (*23*) has also speculated on the possible role of riboflavin in color vision. Heiman (*11*) has gathered convincing clinical evidence from various sources to strengthen his belief that riboflavin is involved in vision. He cites papers by Spies and co-workers (*20*), by Pock-Steen (*19*), and by Sydenstricker *et al.* (*21*), in all of which there are reports of effective riboflavin therapy of visual disorders. In addition, photodynamic action of riboflavin *in vivo* has been noted by Heiman and Brandt (*12*) and by Blum (*2*). The photooxidation of ascorbic acid *in vitro* in the presence of riboflavin has been reported by Martini (*17*), Hand, Guthrie and Sharp (*10*), and Hopkins (*13*).

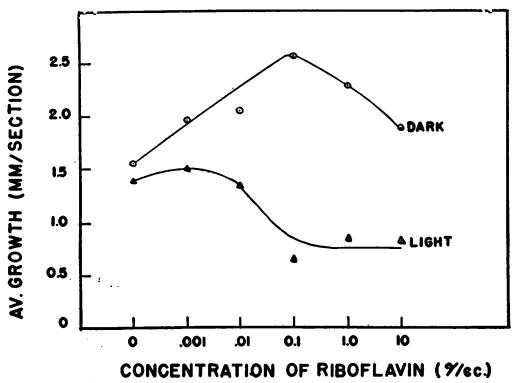


FIG. 1. The effect of riboflavin on the growth of etiolated pea epicotyl sections in darkness and light.

About two years ago, we noticed that the addition of small quantities of riboflavin to media in which plant tissues were being grown results in a slight enhancement of growth if the tissues are kept in the dark, but results in a marked inhibition of growth if they are exposed to light (Fig. 1). Our investigations since that time have led us to the belief that both free riboflavin and flavoproteins may be light-activated, and that such light-activation within the plant may lead to reactions of physiological significance. The evidence for such beliefs is presented below.

Riboflavin-sensitized photooxidations in vitro

The growth in length of plant cells is dependent upon the presence of small quantities of growth hormone such as 3-indoleacetic acid (IAA), generally believed to be metabolically derived from the amino acid tryptophan. The sections of pea stem employed in our original experiments were dependent for their optimal growth upon the presence of 0.1–1.0 μg/ml of IAA in the medium (Fig. 2). We were able to ascertain that the inhibitory effect of riboflavin upon growth of these sections in the light was dependent

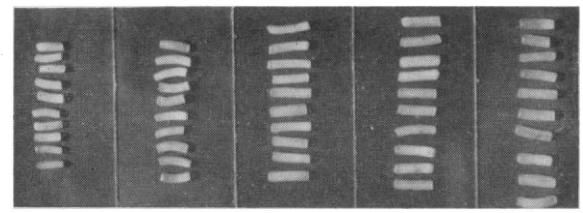


FIG. 2. The effect of indoleacetic acid (IAA) on the growth of etiolated pea epicotyl sections. From left to right, the IAA concentrations were 0 ; 0.01 ; 0.1 ; 1.0 ; and 10 μg/ml.

on the IAA level of the medium. Further investigation of this interrelation (*6*) revealed that riboflavin exerts its effect by causing the photooxidation of IAA in the nutrient medium. Since the growth of the sections is dependent on IAA concentration, such photoinactivation of IAA leads to a marked inhibition of growth of the sections. Thus, we have the paradox of a vitamin's inhibiting growth by destruction of a hormone in the presence of light.

Subsequent investigations of the riboflavin-IAA photoreaction revealed it to be a first order reaction, dependent on the presence of oxygen, and sensitive to pH and temperature. The pH data suggested that the undissociated IAA molecule reacts preferentially. The fact that the riboflavin becomes decolorized under anaerobic conditions made it possible to formulate the following series of stepwise reactions:

$$\text{Rbf} + h\nu \longrightarrow \text{Rbf}^*$$
$$\text{IAA} + \text{Rbf}^* \longrightarrow \text{oxidation product} + \text{Rbf} \cdot \text{H}_2$$
$$\text{Rbf} \cdot \text{H}_2 + 1/2\,\text{O}_2 \longrightarrow \text{Rbf} + \text{H}_2\text{O}$$

This indicates that the light-activated riboflavin (Rbf*) acts as a hydrogen carrier between IAA and oxygen, and thus is in a role here analogous to the one it plays in respiration. Manometric data indicated that the over-all reaction could be written:

$$\text{IAA} + \text{O}_2 \xrightarrow[\text{light}]{\text{riboflavin}} \text{oxidation product} + \text{CO}_2$$

The nature of the oxidation product is still unknown, but present evidence indicates that it is a condensation product of several cleaved indole rings.

Although such reactions may be studied unambiguously *in vitro,* it is dangerous to extrapolate from the test tube to the living cell. Even if riboflavin, when fed to plant cells, were to produce marked photodynamic effects, these effects might be due to oxidation of substances other than IAA. We therefore undertook a systematic survey of possible biological substrates for such photoreactions.

Using O_2 uptake in an illuminated Warburg apparatus as measurement of the reaction, we found that the amino acid histidine is rapidly photooxidized in the presence of riboflavin, and that the amino acids

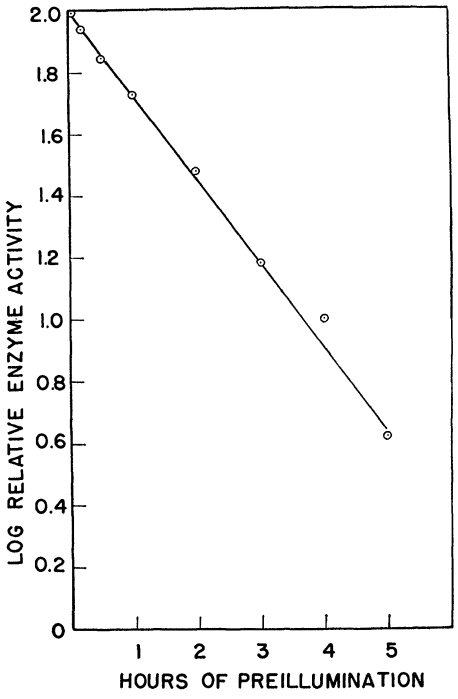


FIG. 3. The time course of photoinactivation of α-amylase sensitized by riboflavin.

tryptophan and methionine, as well as indole-containing compounds, are less rapidly acted upon. Since the amino acids are mainly found in peptide chains *in vivo,* we investigated the influence of their incorporation into peptides on their susceptibility to riboflavin-sensitized photooxidation. In general, we found them to react as well in peptides as in the free state. A good example of this is the dipeptide carnosine, which chemically is β-alanyl histidine. We knew from our experiments on free amino acids that β-alanine does not react in our system, but that histidine does. We found β-alanyl histidine to react, and the manometric data indicated that only the histidine of the dipeptide reacted.

Carrying on investigations into the realm of proteins, we found that all enzymes studied, including α-amylase, tyrosinase, and crystalline urease, are rapidly photoinactivated in the presence of traces of sensitizing riboflavin (*8*). Such photoinactivation also followed simple first order kinetics (Fig. 3). Going to still more complicated protein particles, we investigated, with the aid of Max Delbrück, the photoinactivation of a bacteriophage, T6r. Such virus particles, known to be at least mainly nucleoprotein in nature, were also rapidly inactivated in the presence of riboflavin, but the kinetics of the reaction were indicative of a more complicated "multiple hit" type of phenomenon (Fig. 4).

In view of the current interest in the phenomenon of photoreactivation of ultraviolet-inactivated bacteriophage particles, we tested these riboflavin-photoinactivated bacteriophages for photoreactivatability by incubating duplicate plates in dark and light. No evidence of photoreactivation could be found in our preliminary experiments. This photoinactivation of a bacteriophage is also of great interest because of the chemical similarity between viruses and genes. It seems not beyond the realm of possibility that

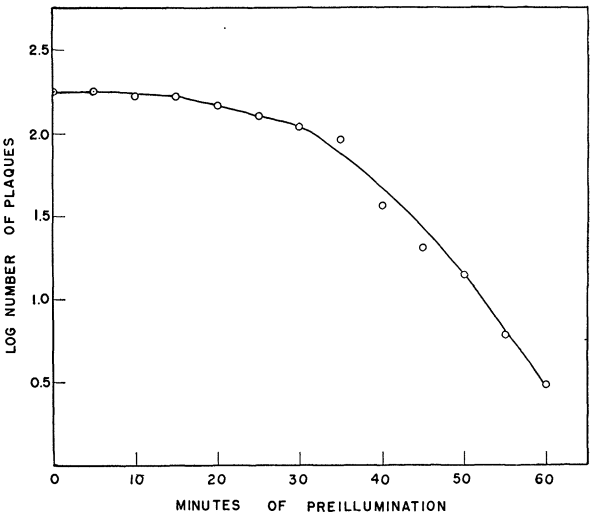


FIG. 4. The time course of photoinactivation of bacteriophage T6r sensitized by riboflavin.

riboflavin may sensitize the genic material of cells to alteration by visible light, and so produce mutations. It should be added, however, that preliminary attempts to produce such a result in the laboratory have been unsuccessful.

Thus it is clear that riboflavin may cause the photochemical alteration of many different kinds of molecules, both large and small. It should also be pointed out that other fluorescent pigments, some of a nonbiological nature, are also effective in such reactions. Among such pigments are lumichrome, methylol riboflavins, fluorescein, and eosin.

Light activation of a flavoprotein enzyme

Thus far, we have considered only those photoreactions involving free riboflavin. Since, in most cells, almost all of the riboflavin occurs in flavoproteins, it seemed desirable to investigate the possibility that flavoproteins could similarly be light-activated.

Our first experiments in this field involved the D-amino acid oxidase of hog kidney, and DL-alanine as a substrate. The experiments were conducted in a Warburg apparatus, measurements of the oxygen

consumed by identical systems being made in darkness and in light. We could find no effect of light on the oxygen uptake, and thus had to conclude that light is without effect upon the activity of the enzyme.

We turned next to an enzyme from a plant source, which was not known to be a flavoprotein, but which had the properties of an indoleacetic acid oxidase. This enzyme, described as a heme protein by Tang and Bonner (*22*), may be conveniently studied in breis of etiolated pea epicotyls. We prepared the crude enzyme from this source and, by measuring its activity in darkness and in light, were able to demonstrate a distinct light stimulation of IAA destruction by the enzyme (*9*). The action spectrum for the light stimulation (Fig. 5) is clearly that of a flavin, showing a maximum at 4400 A, a minimum at 4100 A, and a subsequent rise toward the region of the ultraviolet.

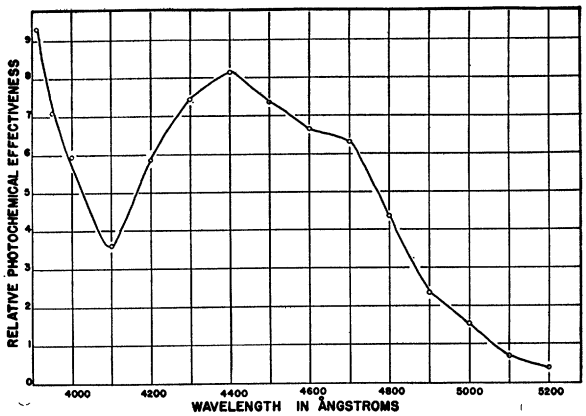


FIG. 5. Action spectrum for the photoinactivation of IAA by etiolated pea brei.

We thought at first that this action spectrum might be due to a light destruction of IAA mediated by free riboflavin, not by a flavoprotein. However, we were subsequently able to demonstrate that the photoreceptor giving the flavin spectrum is largely heat labile, and will not dialyze through a cellophane membrane. Since free riboflavin, riboflavin phosphate, and flavin adenine dinucleotide are all heat stable and dialyzable, the flavin receptor must be a flavoprotein. We therefore concluded that we had demonstrated the light activation of a flavoprotein enzyme. Such a phenomenon has previously been reported for another flavoprotein, xanthine oxidase, by Bernheim and Dixon (*1a*).

Subsequent investigations of this light activation have revealed some interesting features of the mechanism of the light effect. We noticed, as had Tang and Bonner, that dialysis of the crude enzyme greatly increases its activity in the dark, presumably by removal of some dialyzable inhibitor of the reaction. Such dialysis-purified enzymes show very little light activation, as compared with the crude enzyme (Fig. 6). Yet if a colorless solution of inhibitor is added back to the dialyzed enzyme, the inhibition in the dark is reimposed, and this inhibition is once again reversible by blue light, the degree of reversal being dependent on the amount of light relative to the amount of inhibitor present. This led us to the belief that dialysis and light are accomplishing the same effect, i.e., "removal" of an inhibitor. Thus *light activation* of *this flavoprotein enzyme occurs through reversal of a naturally occurring inhibition,* similar in principle to the light reversal of the CO inhibition of heme enzymes.

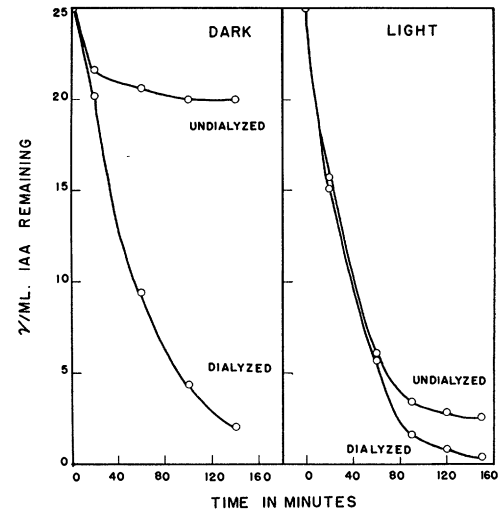
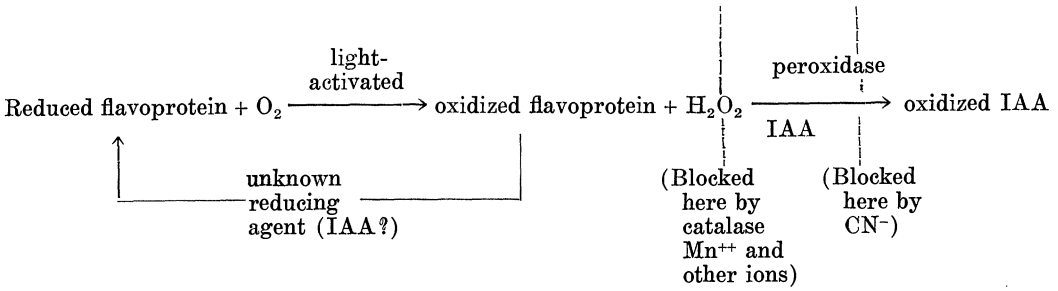


FIG. 6. Relative light activation of undialyzed and dialyzed IAA-oxidase from etiolated peas.

The role of the flavoprotein in this enzyme complex has since been shown to be that of producing H_2O_2, which is then utilized by an IAA-peroxidase (the cyanide-inhibited enzyme of Tang and Bonner). Thus, the reaction destroying IAA is inhibitable by catalase and by various metallic ions which react with H_2O_2. In the absence of inhibitor, H_2O_2 is not limiting to the reaction, and therefore the system is not light-activated. However, in the presence of inhibitor, H_2O_2 is limiting to the reaction and under these conditions, blue light accelerates the reaction, presumably by stimulating the production of H_2O_2 by the flavoprotein system. Our best evidence indicates that the substrate for the flavoprotein is IAA itself; thus IAA oxidation by oxidized flavoprotein appears to lead to the production of H_2O_2, which then is instrumental in the oxidation of more IAA through the peroxidase. The reaction scheme can probably be represented as follows:



The significance of riboflavin-sensitized photoreactions in vivo

Our attention was directed to the possibility that a riboflavin-sensitized photoreaction might be involved in the phototropic curvature of various plant organs. This phenomenon, first studied systematically by Charles Darwin (*4*), is of great historical interest because it led to Went's discovery in 1928 (*25*) of the plant growth hormones. It is now believed that phototropic curvature is the result of a differential growth hormone distribution, leading to a differential rate of growth on the two sides of a unilaterally illuminated plant organ. It can be demonstrated, for instance, that unilateral illumination of the coleoptile (leaf sheath) of a dark-grown grass seedling, such as that of the oat (*Avena sativa*) leads to a lower auxin content on the illuminated side than on the darkened side. In general, it is believed that only the topographic distribution of auxin is affected by light, and not the total amount formed in the tip. This has led to the view that light causes the migration of auxin from the illuminated to the darkened side of the coleoptile, although other explanations involving photoinactivation of auxin and of an auxin-producing enzyme are possible (*7*).

It has been known for many years that blue light is most effective phototropically. In etiolated plants, wavelengths longer than 5200 A are generally entirely ineffective, but in green plants red light may also produce some curvature. As the result of action spectrum determinations for phototropism, such as those of Johnston (*14*), the belief has developed that the receptor pigment for phototropism is a carotenoid, such as β-carotene. In support of this view it has been pointed out that β-carotene is found in abundance in typical photoreceptor organs, both in plants and in animals, and that various carotenoids can sensitize the photoinactivation of auxin-*a* by visible light (*15*). Despite this evidence, we feel that riboflavin or a flavoprotein is a more likely photoreceptor.

In the first place, the absorption spectra for riboflavin and β-carotene are very similar in the visible portion of the spectrum (*9*). Because of the approximate nature of action spectra *in vivo,* it would seem that either pigment could fit the action spectra now available. In fact, most of the action spectra for fungal phototropism (*3*) resemble the flavin spectrum more than the β-carotene spectrum. Second, although β-carotene will sensitize the photoinactivation of auxin *a,* it will apparently not so act toward IAA. The recent evidence of Wildman and Bonner (*26*) seems to indicate that the native auxin of the *Avena* coeoptile is largely, if not entirely IAA. We have in addition found (*9*) that riboflavin can sensitize the photoinactivation of the native diffusible auxin of the *Avena* coleoptile, the time course of the disappearance closely resembling that for pure IAA (Fig. 7). Third, riboflavin is abundant throughout the *Avena* coleoptile, including the most light-sensitive apical 0.25 mm, which is relatively free of carotenoids. Fourth, our best evidence indicates that riboflavin, like auxin, is present in the cytoplasm, whereas β-carotene is known to be localized in plastids. It is a little difficult to see how the small quantities of light that suffice to produce phototropic curvatures could be effective if the photoreceptor were

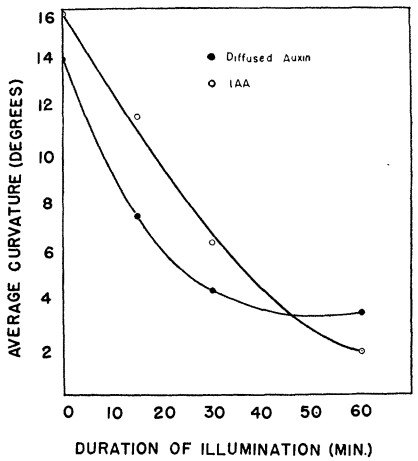


FIG. 7. Relative rates of destruction of pure IAA and of native diffusible *Avena* coleoptile auxin sensitized by riboflavin.

spatially removed from the substrate of the reaction.

Finally, we have the recent experiments of Bandurski and Galston (unpublished) with albino corn. A mutant strain of corn was obtained in which carotenoid pigments were absent, or if present at all, were less than 0.01 the normal concentration. The coleoptiles of such corn, devoid of carotenoids but possessing the normal content of riboflavin, showed approximately normal phototropic curvature when exposed to about 600 meter-candle-seconds of blue light. This experiment does not, of course, disprove the possible participation of carotenoids in normal phototropism, but it does show that other pigments (presumably including flavins) can serve as light receptors for phototropic curvature.

Whether or not a flavin pigment is actually involved in phototropism, it is clear that the reactivity of free riboflavin and of flavoproteins toward certain substrates is markedly affected by light. This fact compels us to examine further the possibility that the ubiquitous flavins may be important photoreceptors in biological systems other than higher plants.

References

1. BERGER, J. and AVERY, G. S., JR. *Amer. J. Bot.*, 1944, **31**, 11.
1a. BERNHEIM, F. and DIXON, M. *Biochem. J.*, 1928, **22**, 113.
2. BLUM, H. F. *Photodynamic action and diseases caused by light.* New York: Reinhold, 1941. P. 120.
3. CASTLE, E. S. *J. gen. Physiol.*, 1931, **14**, 701.
4. DARWIN, C. *The power of movement in plants.* London: John Murray, 1880.
5. EULER, H. and ADLER, E. *Z. physiol. Chem.*, 1934, **228**, 1.
6. GALSTON, A. W. *Proc. nat. Acad. Sci.*, Wash., 1949, **35**, 10.
7. ———. *Bot. Rev.*, in press.
8. GALSTON, A. W. and BAKER, R. S. *Science*, 1949, **109**, 485.
9. ———. *Amer. J. Bot.*, 1949, **36**, 773.
10. HAND, D. B., GUTHRIE, E. S., and SHARP, P. F. *Science*, 1938, **87**, 439.
11. HEIMAN, M. *Arch. Ophthalmol.*, 1942, **28**, 493.
12. HEIMAN, S. and BRANDT, R. Unpublished. Cited (*11*).
13. HOPKINS, F. G. *C. R. Trav. Lab. Carlsberg, Ser. Chem.*, 1938, **22**, 226.
14. JOHNSTON, E. S. *Smithsonian misc. Coll.*, 1934, **92**, 1.
15. KÖGL, F. and SCHURINGA, G. J. *Z. physiol. Chem.*, 1944, **280**, 148.
16. LOCKHART, E. E. *Biochem. J.*, 1939, **33**, 613.
17. MARTINI, E. *Bull. Soc. Ital. Biol. Sper.*, 1934, **9**, 1235.
18. OKUNUKI, K. *Acta Phytochim.*, 1940, **11**, 249.
19. POCK-STEEN, P. H. *Geneesk. tijdschr. Néerl.-Indië*, 1939, **78**, 1986.
20. SPIES, T. D., BEAN, W. B., and ASHE, W. F. *Ann. int. Med.*, 1939, **12**, 1830.
21. SYDENSTRICKER, V. P. *et al. J. A. M. A.*, 1940, **114**, 2437.
22. TANG, Y. W. and BONNER, J. *Arch. Biochem.*, 1947, **13**, 11.
23. THEORELL, H. *Biochem. Ztschr.*, 1935, **279**, 186.
24. VAN HERK, A. W. H. *Rec. Trav. Bot. Néerl.*, 1937, **34**, 69.
25. WENT, F. W. *Rec. Trav. Bot. Néerl.*, 1928, **25**, 1.
26. WILDMAN, S. G. and BONNER, J. *Amer. J. Bot.*, 1948, **35**, 740.

Technical Papers

Static Electricity Elimination During Sectioning with a Microtome

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Paraffin ribbons of tissue sections become charged with static electricity as they are cut on a microtome, and in dry weather the charge may be great enough to pull the ribbon into contact with the metal parts of the instrument, or other objects, making it difficult or impossible to obtain serial sections without loss. This widespread difficulty has vexed biologists and they have tried in a number of ways to lessen it. For example, they have tried grounding the instrument, and using high frequency generators to ionize the air near the microtome. The first procedure helps, but is inadequate, and the generators are somewhat cumbersome to use and they introduce some hazard of shock.

A simple and efficient solution of this problem is to place a surface on which polonium has been plated about an inch from the edge of the knife. The alpha radiation from the polonium ionizes the air and discharges the static electricity as it forms, leaving an easily handled, limp, uncharged ribbon. A further advantage is that the sections do not tend to stick to the knife facet during cutting, and they are less distorted and compressed when cut without the formation of frictional electricity.

A convenient unit (Fig. 1) consists of a polonium-plated, radiating surface (A) recessed into a rotatable head (B) held on a flexible tube mounted on a base (C). When in use it should be placed with the head about 1 in. from the surface of the specimen block (D) and the emitting surface turned to radiate both the surface of the paraffin block and the ribbon as it forms at the knife edge. This arrangement dissipates the charge formed on the surface of the block on its upstroke and on the ribbon from the friction of cutting. Alpha radiation has little penetrating power to damage the specimen or the sections. The useful life of the emitting surface should be somewhat more than a year from the