mum activity of honey bees was seldom shown.

The number of colonies per acre necessary to maintain the desired floral visitation is not known and will doubtless vary according to the acres of cotton involved and the competition by other plants for the bees' attention. Ten colonies per acre may not be enough for small fields surrounded by competing plants, whereas one colony per acre may be sufficient in fields of several hundred acres. The extent of floral visitation by the bees is a good indicator of the number of colonies required (*3*).

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References and Notes

1. F. M. Eaton, *Science* 126, 1174 (1957).
2. The assistance of Henry Tucker, statistician, College of Agriculture, University of Arizona, is gratefully acknowledged.
3. This study was carried out in cooperation with the University of Arizona Agricultural Experiment Station, the U.S. Department of Agriculture Cotton Field Station (Sacaton), the Arizona Cotton Planting Seed Distributors, Inc., and the Arizona State Beekeepers' Association.

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Phospholipase-Induced Release of Cytochrome *c* from the Electron Transport Particle

Abstract. Digestion of the electron transport particle with phospholipase A results in the loss of its oxidative capacity. Evidence presented indicates that this is primarily due to the cleavage of the phospholipid-cytochrome *c* complex within the mitochondria.

In recent years there has been considerable discussion of the discrepancy between the activities of exogenous (free) and endogenous (bound) cytochrome *c* (*1–4*). It has been shown in this (*5*) and other (*2, 4*) laboratories that bound cytochrome *c* is only in small part extractable with salt solutions from particles derived from heart mitochondria. Thus, the bound cytochrome *c* of the electron transport particle is not extractable at all by 0.1*M* phosphate buffer, *p*H 7.4 (*6*), although the existence of a hemoprotein with an α band at 550 mμ can be confirmed by low-temperature spectroscopy (*7*). After the electron transport particle has been exposed to deoxycholate, an extract can be obtained which contains a hemoprotein with the α band characteristic of cytochrome *c*. When this extract is thoroughly dialyzed to remove bile salts, the hemoprotein precipitates out of solution and the precipitate can be redissolved only in deoxycholate solution. In an analogous fashion an insoluble hemoprotein with the spectral characteristics of cytochrome *c* can be extracted from the electron transport particle after treatment with isooctane. This particulate hemoprotein can also be dissolved in deoxycholate solutions (*6*).

We now find that cytochrome *c* is also extractable from the electron transport particle when the latter is exposed to the action of phospholipase A in the presence of calcium ions, but in this instance the hemoprotein is in the soluble, classical form. The hemoprotein is taken up on an IRC-50 column and eluted, under conditions described by Margoliash (*8*), for cytochrome *c*, and the spectral characteristics of the column-purified hemoprotein are indistinguishable from those of authentic cytochrome *c*.

The particulate forms of the hemoprotein obtained by deoxycholate and isooctane treatment of the electron transport particle are also converted to the soluble form of cytochrome *c* by the action of phospholipase A.

When bound lipid cytochrome *c* is released from the electron transport particle by deoxycholate or isooctane, a requirement for cytochrome *c* in the oxidation of both reduced diphosphopyridine nucleotide (DPNH) and succinate by molecular oxygen emerges (*3, 6*). The same relationship has now been observed for the phospholipase-treated electron transport particle. Coincident with the release of cytochrome *c*, the succinic and DPNH activities of the electron transport particle decline, and these activities are in part restored by the addition of externally derived cytochrome *c*. There are significant differences, however, in the respective decay curves for DPNH and succinic activity. The former activity is lost almost immediately in the phospholipase-treated electron transport particle, whereas the latter activity declines more gradually (see Table 1) and reaches a maximum coincident with the maximal release of bound cytochrome *c*. Furthermore, restoration of DPNH oxidase activity by addition of cytochrome *c* is not as complete as the restoration of succinoxidase activity. A similar pattern of decline and restoration of the two activities has also been observed with the isooctane-treated electron transport particle. These observations suggest (i) that phospholipase may release components other than bound cytochrome *c* which are required in the DPNH oxidase chain exclusively; (ii) that more than one form of bound cytochrome *c* is present in the electron transport particle; or (iii) that the bound cytochrome *c* of the succinic and DPNH chains may be different.

As Edwards and Ball (*9*) have shown, the addition of fatty acids such as oleate, which would be released by phospholipase action, to the mitochondrial electron transport system causes inhibition of succinoxidase. We find that the addition of beef heart mitochondrial phospholipid or of beef serum albumin will completely reverse the inhibition of both succinic and DPNH oxidase activities caused by the addition of fatty acids to the untreated electron transport particle. Cytochrome *c*, however, does not help reverse this inhibition. After long-term digestion of the electron transport particle with phospholipase, addition of beef serum albumin or phospholipid along with cytochrome *c* becomes necessary for the maximum restoration of oxidase activities. This phospholipid or beef serum albumin requirement can thus be related

Table 1. Effect of phospholipase treatment on DPNH and succinate oxidation by the electron transport particle. PL, phospholipid, Cyt. *c*, cytochrome *c*.

Phospholipase* treatment (min)	Succinic oxidase† (μmole/min × mg)			DPNH oxidase† (μmole/min × mg)			Cytochrome *c*‡ released (μmole × 10^{-3}/mg of protein)
	+ PL	+ Cyt. *c*	+ Cyt. *c* + PL	+ PL	+ Cyt. *c*	+ Cyt. *c* + PL	
0	1.24	1.2	1.2	1.8	1.9	2.0	
7				0.6	1.1	1.2	0.04
15	0.8	0.9	0.9	0.1	0.3	0.4	0.08
60	0.4	1.0	0.9	0.0	0.0	0.1	0.12
120	0.1	0.4	0.7	0.0	0.0	0.0	0.15
120 (control)	1.1	1.2	1.1	1.6	1.7	1.8	0.02

* Each tube contained 60 mg of electron transport particle protein; $3 \times 10^{-4}M$ potassium phosphate buffer at *p*H 7.4, $5 \times 10^{-5}M$ calcium chloride, and 0.5 mg of venom in a total volume of 5 ml. The tubes were incubated in a water bath at 38°C. The control was incubated under the same conditions, without venom. At the indicated intervals of time, the mixtures were chilled to 0°C, mixed with cold sucrose containing $5 \times 10^{-5}M$ Versene, and immediately centrifuged at 40,000 rev/min for 30 min. to separate the particles. The enzyme was further washed with cold sucrose and used for activity determination. The phospholipase A used in these experiments was purified from *Crotalus adamanteus* venom by heat treatment (*9*). Similar results are obtained by using untreated venom.

† The assay procedure was the same as that described previously (*6*): 50 μg of beef heart mitochondrial phospholipid (PL) and 1.0 mg of cytochrome *c* were used in the succinoxidase assay, whereas 5 μg of PL and 0.1 mg of cytochrome *c* were used in DPNH oxidase as a supplement.

‡ Cytochrome *c* was assayed spectrophotometrically by following the change in absorbance at 550 mμ with dithionite on the supernatant from the incubated mixtures; it was also assayed after adsorption on and elution from the IRC-50 column.

to the inhibition caused by fatty acids released during digestion. The effect of phospholipase A on the oxidase activities of the electron transport particle and the release of cytochrome *c* from the particle are shown in Table 1.

These results (*10*) provide evidence that cytochrome *c* exists in the electron transport particle as a phospholipid-cytochrome *c* complex similar to insoluble complexes of cytochrome *c* which have previously been described (*6*).

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References and Notes

1. E. C. Slater, *Biochem. J.* 46, 499 (1950); D. Keilin and E. F. Hartree, *Nature* 176, 200 (1955); D. Keilin and T. E. King, *ibid.* 181, 1520 (1958).
2. C. L. Tsou, *Biochem. J.* 50, 493 (1951–52).
3. B. Mackler and D. E. Green, *Biochim. et Biophys. Acta* 21, 1 (1956).
4. E. G. Ball and O. Cooper, *J. Biol. Chem.* 266, 755 (1957).
5. B. Mackler and D. E. Green, *Biochim. et Biophys. Acta* 21, 6 (1956).
6. C. Widmer and F. L. Crane, *ibid.* 27, 203 (1958).
7. R. Estabrook and B. Mackler, *J. Biol. Chem.* 224, 637 (1957).
8. E. Margoliash, *Biochem. J.* 56, 529 (1954).
9. S. W. Edwards and E. G. Ball, *J. Biol. Chem.* 209, 619 (1954).
10. We wish to thank Dr. D. E. Green for his encouragement of this investigation. The valuable technical assistance of Mrs. Wanda Fechner is gratefully acknowledged. This work was supported in part by research grants RG-5506 from the Division of Research Grants and H-458 from the National Heart Institute, both of the National Institutes of Health, and by Atomic Energy Commission contract No. AT(11-1)-64, project 4. The animal tissue was generously furnished by Oscar Mayer and Company, Madison, Wis.
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Multiple *p*H Levels in Chromatograms

Abstract. Lines of buffer solutions drawn longitudinally on chromatograms with a capillary pipette provide different levels of *p*H which affect the R_f values, color, fluorescence, and other properties of the chromatographed substances.

In chromatographic studies of the decomposition products of indole derivatives (*1*) we have observed the influence of *p*H and of the nature of buffer solutions on the following properties of the chromatographed substances: (i) R_f values; (ii) color, fluorescence, and absorption of short-wave ultraviolet light; (iii) changes in color, fluorescence, and absorption which result from exposure of the chromatograms to ultraviolet radiation; and (iv) color reactions with several reagents.

Most of those effects can be conveniently observed in a single chromatogram prepared as follows: On a 7- by 15-cm sheet of Whatman No. 1 filter paper, four parallel, lengthwise lines are drawn with a straightedge and a capillary pipette filled with buffer solutions at convenient *p*H values. After the lines have dried, the solution to be chromatographed is applied with a pipette, crosswise to the strips of buffer solution, in a band 5 mm wide, over the whole width of the sheet.

Figure 1 shows a chromatogram, prepared according to this method, of a $10^{-3}M$ aqueous solution of indole-3-acetic acid, decomposed by ultraviolet radiation. Citric acid ($0.1M$)–disodium phosphate ($0.2M$) buffer solutions at *p*H 3, 5, 7, and 8 were applied along the indicated vertical lines. Ascending chromatography with acetone and water (8/2) was used. The outline of the fluorescent zones of the completed chromatogram is shown. Except for zones IV and VI, which were not affected by the buffer strips and which are presumably nonionizable substances, all the zones have more or less wavy outlines. Zones IV, V, and VI overlap, and zone V is visible as an increase of the fluorescence of zones IV and VI, according to a wavy pattern.

An example of the graphic analysis to which the zones may be submitted is shown for zone II. Closed circles mark the middle of the zone at the level of the buffer strips and crosses that at the interval between strips. At low *p*H levels the substance is in the undissociated form if it is an acid and in the cation form if it is a base. Its R_f value for zone II at the *p*H 3 level is approximately 0.1, and the R_f value of the crosses is, on the average, only slightly greater. In between the buffer strips the substance is therefore in the same form as it is at the *p*H 3 level (*2*).

At high *p*H levels the substance is in the undissociated form if it is a base and in the anion form if it is an acid. At the *p*H 5, 7, and 8 levels, zone II has an R_f value in the neighborhood of 0.28, which may be taken as that of the base or anion. It is seen that the anion (or base) is more soluble in the solvent than the acid (or cation) of the substance of zone II. Traces of acidic or basic vapors in the chromatographic tank may shift the R_f value in between strips from the undissociated to the ionic R_f value or vice-versa.

Regions of more intense fluorescence are represented by heavier hatching. They were conspicuous in zones II, III, VII, IX, and XI. The dark areas of zone X (indoleacetic acid) due to the quenching of the blue fluorescence of the filter paper under short-wave ultraviolet light were more clearly visible at the lower *p*H values.

As a result of exposure of the chromatogram to ultraviolet radiation, the outline of zone X, in part overlapping zone XI, was made clearly visible, thanks to its faint blue fluorescence at the lower *p*H levels and its strong turquoise green fluorescence at the level of the *p*H 8 strip (*3, 4*).

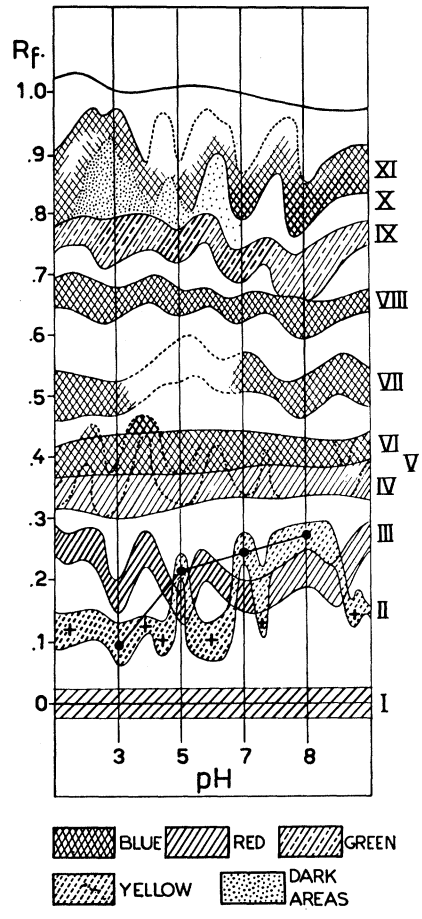

Fig. 1. Chromatogram of decomposition products (by ultraviolet radiation) of indole-3-acetic acid. Ascending chromatography. Acetone and water, 8/2. Citric acid–disodium phosphate buffer solutions (0.1 *M*) at *p*H 3, 5, 7, and 8 were applied along the vertical lines before chromatography. The decomposed solution was applied at I. The approximate fluorescence color is indicated by conventional hatching.

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References and Notes

1. A. A. Bitancourt, K. Schwarz, A. P. Nogueira, *Arquiv. inst. biol. São Paulo* 24, 169 (1957); K. Schwarz and A. A. Bitancourt, *ibid.*, p. 183.
2. Difference in solubility of citric acid and disodium phosphate changes the *p*H of the trips as the solvent ascends. In short chromatograms, however, the change is not likely to be very important or to alter greatly the effect of *p*H on the chromatographed substances.
3. A detailed analysis of this chromatogram and of other similar ones and the conclusions therefrom regarding the chemical identity of the decomposition products of indole-acetic acid is in preparation.
4. We are grateful to M. Meneghini for constructive criticism.
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