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Growth Promotion in Pea Stem Sections. III. By Alkyl Nitriles, Alkyl Acetylenes and Insect Juvenile Hormones[1]

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Abstract. C_{14}, C_{15}, and C_{16} alkyl nitriles, and C_{16} and C_{18} alkyl acetylenes at 10 to 105 micromolar concentrations promote the growth of stem sections from red-light-exposed seedlings of dwarf peas (*Pisum sativum* L. cv. Progress No. 9). Similar results were obtained with substances active as insect juvenile hormones, namely farnesol, the racemic ethyl ester of 1 of the natural hormones, and a "synthetic juvenile hormone" mixture, the latter 2 having as high an activity in the pea assay as any lipid reported previously. A sterically nearly identical compound, methyl-RS-10,11-epoxyfarnesoate, is a weak insect hormone and did not promote plant growth. Thus activity in peas and in insects is in some cases parallel. Other similarities and some differences are discussed. Peas appear to require molecules longer than 20Å, while insect activity is maximal at that length. All active molecules are ineffective in promoting pea stem elongation by themselves, indole acetic acid must also be present. The lipid effect in plants and the juvenile hormone response in insects have much in common and the evidence suggests they could have a similar locus of action in a membrane controlling respiratory function.

The elongation of pea stem sections cut from seedlings which have been exposed to weak red light during development is promoted by certain lipids in the presence of sugars and auxins. Often the response is twice that to auxin alone.

The physiologically effective lipids are a diverse group but have the following characteristics in common. All are simple alkyl or isoprenoid hydrocarbons with a chain length of at least 12 carbon atoms. All are nonionizable, but contain a polar group such as an ester or hydroxyl moiety. Natural compounds which are physiologically active include such substances as triglycerides, α-tocopherol, vitamin K_1, and phytol (32). Representative synthetic substances which also are active in the bioassay include methyl oleate, some Tween detergents, and monoolein (31).

Small changes in the active molecules can completely abolish biological effectiveness. Any freely ionizable substituent, such as a carboxyl, sulfate, phosphate, quaternary ammonium or amino group destroys activity. More space filling lipid molecules like steroids and carotenoids have so far also proved ineffective, so some size limitation seems to be operative. Surface action does not explain the activity since among detergents only anionics of the fatty acid ester type (such as the Tweens) promote hormone action.

In this paper, our previous report that palmitonitrile is active in the bioassay is extended to other simple nitriles, and alkyl acetylenes also are shown to be active. In addition, the racemate of the ethyl ester of one of the natural insect juvenile hormones, as well as a synthetic mixture with juvenile hormone activity, are shown to be highly effective in promoting growth of pea stem sections.

Materials and Methods

The bioassay used has been described previously (32). Briefly, 10 mm sections cut from just below the apex of 7 day old seedlings of dwarf peas (*Pisum sativum* L. cv. Progress No. 9) which were grown at 25° under continuous extremely weak red light, are randomized and floated, 10 to a Petri dish, on 20 ml of 1.5 % sucrose solution buffered with 5 mM KH_2PO_4 (pH 5.5) and containing 50 μM $CoCl_2$.

Auxin, usually as indoleacetic acid at 1.8 μM, and gibberellic acid at 0.3 μM are added. They produce maximal elongation at these concentrations. The red light treatment and the sugar are essential for a response to the lipids. Equally crucial are the emulsifying technique for the lipids which has been described elsewhere (31), and an emulsion stabilizer, usually Pluronic F-68 (Wyandotte Chemical Company), at a final concentration of 0.004 %. Treatment of the prepared emulsion with ultrasound from a Branson sonifier Model LS-75 further improves its stability. Length of the stem sections is normally measured after 24 hr of gentle rotation on a shaker in a dark room at 25°.

The alkyl nitriles were obtained from Lachat

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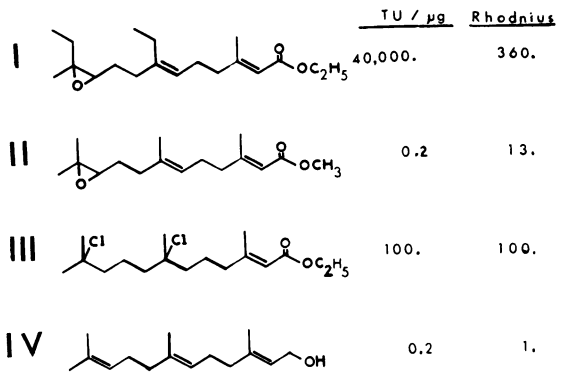

FIG. 1. Molecular structures of the insect juvenile hormones tested for growth promoting activity on pea stem sections. I. Ethyl ester of authentic juvenile hormone. II. Methyl-10,11-epoxyfarnesoate. III. Major component of "synthetic juvenile hormone" mixture. IV. Farnesol. Relative insect bioassay activities are cited in a) *Tenebrio* units per microgram from data of Röller and Dahm(2) and b) relative activity per unit weight to give score of 10 in *Rhodinus* topical application assay calculated from data of Wigglesworth (35). In latter paper, data listed for III was actually obtained with the methyl ester, but in this assay methyl and ethyl esters usually test similarly.

Chemicals Incorporated, Chicago, Illinois and were represented as 99.5 % pure, except for arachidonitrile of 99.0 % purity. The alkyl acetylenes were obtained from Farchan Research Laboratories, Willoughby, Ohio. Conventional gas chromatography did not disclose any gross impurities in either group of substances.

Four substances, chemically similar but with widely varying activity as insect juvenile hormones, were obtained. Their structures are illustrated in Fig. 1. Farnesol (IV of Fig. 1) supplied by Mann Research Laboratories, New York was vacuum redistilled before use. The synthetic mixture with insect juvenile hormone activity was obtained from Calbiochem, Los Angeles, California. It was stated to be prepared from farnesenic acid by the procedure of Vinson and Williams (34). At least 6 substances active as insect juvenile hormone exist in this mixture, but ethyl-6,7,10,11-tetrahydro-7,11-dichlorofarnesoate (III) is believed to be the major component (23). The authentic insect juvenile hormone was the racemate of the ethyl ester of one of the natural substances namely ethyl-RS-10,11-epoxy-7-*trans*-ethyl-3,11-*trans,cis*-dimethyl-2,6-tridecadienoate (I). This is about 8 times more active in the *Tenebrio* insect assay than is the naturally occurring methyl ester, and currently is the most potent juvenile hormone known (22). It was the synthetic preparation No. 1330-114A of Professor H. A. Roller of Texas A & M University (8). The methyl-RS-*trans,trans*-10,11-epoxyfarnesoate (II) was synthesized by Dr. W. S. Bowers of the Agricultural Research Service, Beltsville, Maryland (2). Both substances were kindly made available to us by Professor G. R. Wyatt of this department.

The Pluronics and Tetronics compounds used as emulsion stabilizing surface active agents were a gift of the Wyandotte Chemical Corporation, Wyandotte, Michigan.

Results

Alkyl Nitriles. The data of table I indicates that our brief report (20) that palmitonitrile can promote pea stem section elongation can be extended to some other alkyl nitriles. Several tests of myristonitrile at these and higher and lower concentrations showed no statistically valid growth promotion by this compound and the single assay cited in table I in which 2 values barely exceed significance at the 5 % level

Table I. *Effect of Alkyl Nitriles on Pea Stem Section Elongation*

The data represent the percentage elongations with standard deviations of 10 mm stem sections from Progress No. 9 dwarf pea seedlings. The basal medium is given in the text.

Test	Basal medium	1.8 µM IAA +0.3 µM GA_3	25 µM Triolein (+IAA +GA_3)	µM Alkyl Nitrile (+IAA+GA_3) 15	30	60	75	90	105
						Myristonitrile			
I	25.8±4.1	61.4±5.6	87.8±11.0	75.2±7.6	72.2±7.7	72.3±6.0	69.0± 7.2	74.8±5.4	78.1±11.1
						Pentadecanonitrile			
1	28.8±6.3	78.3±6.0	90.7± 9.0	89.7±5.3	93.8±6.7	87.9±5.4	99.0± 8.0	80.4±8.4	83.9± 8.0
II	28.1±3.9	63.2±5.6	98.0± 8.9	94.6±7.0	93.7±4.2	94.6±5.4	103.5± 4.2	88.0±6.9	89.8± 4.8
						Palmitonitrile			
1	36.2±4.1	76.5±6.0	81.8±12.9	...	80.9±8.2	89.3±9.4	93.4±12.2	109.5±6.0	85.2±11.5
II	54.6±3.6	68.6±6.8	**110.1± 8.0**	...	...	...	85.4± 8.8[1]	103.6±7.3[2]	102.0± 5.2[3]

[1] 80 µM.

[2] 100 µM.

[3] 120 µM.

was the only time this was observed. Pentadecanonitrile, however, was repeatedly and convincingly active.

Attempts were also made to test stearonitrile and arachidonitrile, but the emulsions of these compounds were not stable and small specks of the substances were floating on the surface of the bioassay medium by the following morning. Sporadic growth promotion observed in a few Petri dishes make it likely that these compounds too would show convincing activity if the emulsion could be maintained. Other emulsion stabilizers, namely Pluronics F-38, P-66, F-108 and Tetronic 908 were tested in various ratios and concentrations with these nitriles but proved no more successful in maintaining an emulsion when left overnight than the Pluronic F-68 used routinely. Pluronics F-77 and F-98 failed to even stabilize the initial emulsion.

Alkyl Acetylenes. Table II summarizes the results with the 3 alkyl acetylenes available to us. All 3 were as active in the assay as triolein, the lipid used for a standard, and at comparable concentrations. Recent tests have shown 1-tetradecyne and 1-pentadecyne also to be active, while 1-tridecyne was inactive.

Insect Juvenile Hormones. Four materials active as juvenile hormones in some insects were tested and are compared in table III.

In agreement with our earlier results (32), farnesol causes less elongation and at a higher optimal concentration than the triolein standard. The more active insect hormones I and III, however, cause elongation of pea stem sections as great as any we have observed previously, comparable to that obtained with methyl linoleate (31), vitamin E or vitamin K_1 (32). Since the synthetic hormone mixture contains 6 compounds known to be active on insects (34), it cannot strictly be compared on a molar basis. However, both it and the racemic ethyl ester of the natural juvenile hormone (I) show optimal activity at about the same concentrations by weight as the triolein standard. Since emulsions are employed, a true concentration comparison is uncertain in any case. It is probably the material which

Table II. *Effect of Alkyl Acetylenes on Pea Stem Section Elongation*[1]

The data represent percentage elongation with standard deviations of 10 mm stem sections from Progress No. 9 dwarf pea seedlings. The basal medium is given in the text.

Test	Basal medium	1.8 μM IAA +0.3 μM GA_3	25 μM Triolein (+IAA +GA_3)	μM Alkyl acetylene (+IAA+GA_3) 10	15	20	30	45	60
						1-Hexadecyne			
I	36.7±7.1	65.7±10.0	79.0± 5.9[1]	...	...	...	99.7± 6.9	100.4± 7.1	...
II	33.3±4.5	77.2± 9.1	104.9± 7.9	78.7±11.1	...	108.9± 8.8	99.2± 4.7	110.8± 6.9[1]	...
						8-Hexadecyne			
I	30.9±2.2	61.7± 9.7	97.8± 9.1	...	88.7± 4.0	...	88.5± 5.8	82.7± 6.7	91.7± 8.1
II	28.2±2.9	70.7± 5.6	101.7± 8.6	78.8± 9.8	84.7± 6.8	89.9±10.5	101.3± 5.5	...	90.6± 6.6
						1-Octadecyne			
I	32.3±4.6	69.0±12.3	100.9± 6.3	78.4± 8.6	96.9±11.5	97.7± 6.9	94.4± 6.9	100.4± 7.5[1]	98.3± 7.9
II	27.0±3.9	66.1± 6.5	91.7±11.9	85.5±11.4	90.6± 4.9	87.3± 6.9	88.1± 2.6	86.5± 3.0[1]	79.0± 8.7

[1] 40 μM.

Table III. *Effect of Substances Active as Insect Juvenile Hormones on Pea Stem Section Elongation*

The values are percentage elongations with standard deviations of 10 mm stem sections from Progress No. 9 dwarf pea seedlings, measured at 24 hr after cutting except as noted. The basal medium is given in text. Molarity of synthetic hormone mixture is based on assumption molecular weight same as farnesol.

Test	Basal medium	1.8 μM IAA +0.3 μM GA_3	20 μM Triolein (+IAA + GA_3)	Farnesol (IV) (+IAA + GA_3)		Synthetic insect hormone mixture (III) (+IAA + GA_3)		
				20 μM	*40 μM*	*20 "μM"*	*40 "μM"*	*60 "μM"*
1	21.8± 2.3	53.2±5.8	79.1±10.7	72.3± 9.5	77.6±10.0	63.8±10.0	90.5± 6.1	90.2±11.1
2	21.1± 1.3	75.2±7.7	90.7± 6.5	75.2± 5.3	86.3±15.2	68.3±15.2	86.2± 6.6	102.0± 5.3
3	27.9±10.0	71.1±6.1	98.8± 4.5	88.0±12.5	87.4±11.7	78.6± 7.0	113.3± 8.1	102.4±10.0
				Methyl 10-epoxy farnesoate (II) (+IAA +GA_3)				
				5 μM	*10 μM*	*20 μM*	*40 μM*	*60 μM*
4	22.6± 1.9	55.7±4.1	92.4± 8.3	65.1± 8.1	64.4± 4.4	62.1± 4.8	75.0± 8.0	72.1± 8.6
5	27.2± 4.7	70.2±4.6	93.8±10.8	66.9± 7.9	70.5±10.9	65.8± 6.5	72.9±11.0	64.8± 8.5
				Racemic ethyl insect hormone (I) (+IAA +GA_3)				
6 (24 hr)	28.7± 4.6	62.1±5.4	76.7± 5.2	63.9± 4.5	69.7± 3.0	108.8±14.7	100.7± 8.2	67.9± 4.8
6 (40 hr)	35.2± 6.1	70.8±8.3	81.1± 6.7	70.7± 6.4	78.9±10.0	117.0±16.0	112.6±10.5	86.0± 8.3

is swept out of solution and deposited on the cuticle which becomes biologically effective.

The virtually complete inactivity of the structurally so similar II is equally interesting. Only 1 just "significant" datum can be noted and can be dismissed as due to chance, since another test at 80 and 120 μM was completely negative. These 3 tests exhausted our limited supply of II.

Measuring the sections treated with I again after 16 hr more incubation revealed only a uniform growth increment in all treatments. The juvenile hormones thus do not restore the stem growth to that of the intact plant (see Fig. 1 in 21). The small quantity of material available to us was exhausted in this and 1 preliminary experiment, so replicate tests were not possible.

Need for Auxin. An important characteristic of the pea bioassay is the inability of the previously tested lipids to induce stem section elongation in the absence of auxin (30). Two experiments simultaneously comparing 1-octadecyne, palmitonitrile and the synthetic insect hormone mixture with the triolein standard are summarized in table IV. Similar results were observed when compounds were tested separately in smaller experiments. In no case was any significant effect of the lipids noted, other than occasional growth inhibition, unless auxin is present. Interestingly, the morphogenetic effects in insects of juvenile hormone are believed to be expressed only in the presence of ecdysone (22).

Discussion

Molecular Requirements for Activity. Among alkyl nitriles, the 14 carbon chain was appreciably less active than the 15, reminiscent of our results with fatty acid esters whose activity waned at a 12 carbon chain (31). But since the methyl ester moiety must be added, the actual length of both myristonitrile and methyl laurate is nearly the same, namely about 20 Å (33). This then, appears to be the minimum chain length necessary for activity in the pea section bioassay. This could also explain the striking difference between insect hormones I and II. Although the former is a C_{13} chain, geometrical isomerism gives it an effective length of C_{12}. It is the ethyl ester moiety which makes it one methylene group longer than II, just exceeding 20 Å. The natural insect hormone, being a methyl ester, is probably ineffective in the plant assay. In the acetylene series, the shortest effective compound, 1-tetradecyne, is also just 20 Å long.

8-Octadecyne is the first substance tested with a centrally located polar group. A polar, but nonionizable, group has been present in all molecules found to be active so far, but the fact that this need not be located at the end of the chain may prove difficult to reconcile with any steric fit hypothesis to explain the action of these substances. The insect hormones tested here have 1 to 3 polar groups, but only 1 appears necessary for activity (27, 35). A polar group could help stabilize the emulsion, but emulsions are ineffective in insects (35).

The relatively high activity of the alkyl acetylenes tested demonstrates once again that comparatively inert substances can activate the response to auxin. Since even the readily metabolizable triolein and methyl oleate do not contribute directly to more than a fraction of the respiratory stimulus observed with the active lipids (20), it continues to be likely that the activity of all these diverse compounds is not a direct result of their being metabolized. Instead, the size and polarity requirements, and the fact that fatty acid esters (21) and vitamin K_1 (9) enter cell fractions rich in membranes, point strongly at the possibility that active lipids match some steric matrix, probably in a membrane.

Analogy Between Plant and Insect Hormones. Carlisle *et al.* (3) have briefly reported that a locust extract containing ecdysone-λ stimulated the growth of intact dwarf pea internodes, while gibberellic acid was active in an insect development bioassay. They concluded that both the plant and insect growth hormones had "similar effects on both plants and locusts". Later, ecdysone itself and related substances were found in plants (4). Ecdysones are a

Table IV. *The Dependence Upon Auxin of the Stimulation of Pea Stem Section Elongation by Lipids and Insect Juvenile Hormone*

The values are percentage elongations with standard deviations of 10 mm stem sections from Progress No. 9 dwarf pea seedlings. The basal medium is given in text. Molarity of synthetic insect hormone mixture is based on assumption molecular weight is same as farnesol.

	Test	No lipid	20 μM Triolein	40 μM Synthetic insect hormone mixture	100 μM Palmitonitrile
Basal	I	26.4 ± 4.6	23.5 ± 3.2	27.4 ± 2.8	20.7 ± 4.4
medium	II	30.6 ± 3.7	22.0 ± 3.0	26.5 ± 2.8	18.8 ± 2.7
+0.3 μM+	I	37.7 ± 2.6	32.9 ± 3.3	25.7 ± 2.8	28.8 ± 4.0
GA_3	II	35.6 ± 6.4	39.8 ± 2.0	28.1 ± 3.9	33.0 ± 4.0
+1.8 μM	I	62.0 ± 6.6	71.5 ± 4.2	103.2 ± 8.4	73.7 ± 3.4
IAA	II	58.6 ± 8.7	79.7 ± 10.0	82.1 ± 12.0	65.8 ± 6.6
+IAA	1	67.5 ± 6.4	77.1 ± 7.2	91.8 ± 10.0	79.2 ± 10.4
$+GA_3$	II	60.1 ± 6.8	87.5 ± 9.6	98.1 ± 7.3	85.1 ± 7.5

different group of insect hormones than the juvenile hormones tested here, and being steroids have a terpenoid biogenesis in common with gibberellic acid. Other investigators (C. M. Williams, G. R. Wyatt, unpublished personal communications) have failed to find any ecdysone-like activity of gibberellic acid on brainless silkmoth pupae. Although we have not yet tested the insect juvenile hormones on intact dwarf pea plants, in many unpublished experiments (some with Mrs. C. Kuiper) using the Kende and Lang (17) gibberellic bioassay, we have failed to find any trace of growth promotion by other active lipids like vitamin E and alkyl nitriles on intact plants. Our tentative conclusion then, is that Carlisle *et al.'s* results and ours are not directly comparable.

More pertinent is the large body of data on the molecular diversity of substances active as insect juvenile hormones. Schneiderman *et al.* (25) noted juvenile hormone activity in plant extracts, but this seemed non-specific after a large range of isoprenoid substances were found to mimic the natural insect hormone. The reviews by Schneiderman and Gilbert (26) and Roller and Dahm (22) provide access to this literature. Examination of the relative activity of compounds reported by Schmialek (24), Slama (28), Schneiderman *et al.* (27), and Wigglesworth (35) reveals a number of similarities between molecules active on plants or on insects, even when they have not yet been tested in both systems. In fact, the conclusions that Schneiderman *et al.* (27) reached for the insect active substances, *i.e.* there is no obvious structural relationship between active molecules, there is a critical size required for activity, and the compounds themselves must be active since they are not likely to be precursors of any common substances, are the same conclusions we have reached (20, 21) for the materials active in the pea test.

In a detailed comparison, there are differences. In 3 homologous series, Schneiderman *et al.* (27) found a distinct molecular length for optimum activity at 20 Å. The 2 authentic hormones identified since (18) are the same length. This length is just where activity on peas begins. Farnesol, being shorter, is an exception, but it could be built up to a longer compound metabolically.

In insects, if homologous molecules are increased in length by 2 or 3 methylene units above the optimum, they become ineffective. We have not yet been able to show a distinct optimum or upper size limit to molecules effective on peas, in good part because stable emulsions of such compounds become increasingly difficult to prepare. Metabolism might also be expected to break some of these down to an effective length.

The most obvious difference between the pea and insect data is that in the pea fatty acid esters, and thus true fats, are among the most active substances. In the insect tests, olive and peanut oil are frequently used as an "inert" vehicle to introduce the juvenile hormone. Carlisle and Ellis (5) have recently criticized this practice, pointing out that minor constituents could influence the assay. In any event, it is clear that fats which are highly active in the pea test do not have more than faint activity, if any, in the insect assays.

No lipids yet tested restore more than half of the missing growth of the pea stem section, *i.e.* to the length it would attain on the intact plant (21, 31). It could be then, that all our effective lipids are relatively poor mimicks of a plant "juvenile hormone". Any effort to demonstrate such a substance will be fraught with difficulties, because of the many other substances present which are active in the bioassay. In fact, Miss Jean B. Obreiter, in 2 years of unpublished work in this laboratory, found activity in virtually all lipid fractions extractable from peas, with no indication that any one was more effective than another.

Relation to Other Plant Responses. We have cited earlier (20, 31, 32) a miscellany of responses in various plants to small quantities of lipids, which often mimicked the effect of hormones. Since then, a number of lipids effective as chemical pruning agents have been developed and described by Cathey and Steffens (6). These are different from lipids active in the pea, not only because they are primarily toxic agents used at a thousand fold higher concentrations, but because a distinctly different range of molecules are involved. In their case, free acids are active, and chain lengths close to 10 carbons are required. Pruning action virtually disappears when methyl esters of chain lengths highly active in the pea assay are reached. Their most active substances are propargyl derivatives, which contain an acetylene group, but again toxic action is at much shorter chain lengths than those we report here. Propargyl *n*-decyl sulfite and propargyl tridecyl sulfite are highly toxic in our pea bioassay, inhibiting growth even at 0.01 μM. No growth promotions were noted at any concentration.

More germane appear to be a significant series of papers by Hirai and his colleagues. Investigating the ancient practice of oleification of figs, they have noted that lipids vary in their effectiveness (11), respiration is increased (10, 12, 14), there are relationships to plant hormone changes (10, 12, 14, 16) and sugar metabolism (15) while correlations with the classic climacteric and ethylene production of ripening fruit exist (10, 12, 16). Although, unlike the pea, they find free fatty acids among the most effective substances (13, 14, 15, 16), the relationships between the lipid effect and respiration that they have exposed in the fig are in many respects analogous to those we have noted in the pea. Free fatty acids do dramatically and specifically modify development in 1 insect (28).

Mode of Action. As we noted above, and suggested earlier (32), a site of action in a moderately stereospecific membrane appears to be the only likely common role for these substances. Wigglesworth (35) has advanced a similar hypothesis from his

detailed studies of 42 farnesyl derivatives. And indeed, Baumann (1) has reported that substances active as juvenile hormones depolarize the membranes of max moth salivary glands, while inactive or weakly active substances do not. However, any membrane effect can only be the first of a train of events.

In the pea, the earliest known effects of active lipids are observed from one-half to 3 hr after application when both respiration and elongation are stimulated. Lipids will stimulate respiration in the absence of elongation. And this respiratory stimulus occurs even when protein or nucleic acid synthesis is blocked by inhibitors such as actinomycin D, puromycin, or cycloheximide (21).

The small amounts of authentic juvenile hormone available to us have not permitted an investigation of its effect on pea stem respiration. But, since in all other respects its effect is so similar to those lipids known to stimulate pea stem respiration, we would predict that it should also do so. It is noteworthy that the typical effect of juvenile hormone in insects is to change the nature of the molt to a larval or pupal form, times when drastic changes in cellular respiratory systems normally occur (19, 26). Stegwee (29) and Clarke and Baldwin (7) have briefly reported stimulation of oxygen uptake of isolated insect respiratory organelles with crude juvenile hormone extracts. Minks (19) failed to confirm these observations, although he did find marked changes in P:O ratios, but he used different isolation and incubation media.

Since in the pea, active lipids bring about the appearance of dinitrophenol sensitive respiration (21), one could hypothesize that the major physiological action of effective lipids is in some way to promote the production of energy containing phosphate compounds. Minks (19) evidence supports this idea. Perhaps in plants and insects, the effective lipids help to maintain the activity of a phosphorylating part of the electron transport system which is later abandoned during normal development.

Thus, the primary site of lipid action could be either in a membrane of a respiratory organelle, as indicated by the above papers, although our previous efforts to show this directly in pea organelles were unavailing (21), or in a membrane system regulating the flow of glycolytic intermediates to the electron transport system. In either case, the enhancement of auxin action would be secondary, resulting from the increased supply of respiratorily derived energy.

Acknowledgments

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