Plant Physiol. (1971) 48, 559–565

Probing a Membrane Matrix Regulating Hormone Action

I. THE MOLECULAR LENGTH OF EFFECTIVE LIPIDS[1]

Received for publication April 21, 1971

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ABSTRACT

Auxin-induced pea (*Pisum sativum*) stem section elongation is enhanced at levels of 3 to 40 micromolar by six new classes of alkane derivatives additional to those described earlier, providing that length of their molecules exceeds 20 Å. Increasingly longer homologous series of alkyl chlorides, bromides and iodides, alkyl benzenes, dialkyl ethers, and sulfides show a logarithmically linear increase in specific activity above this length, reaching an optimum near 28 to 30 Å. Longer dialkyl ethers and sulfides are less effective, while steroids, or alkanes with substituents at both ends, are ineffective.

Neither common metabolism nor common physical properties, other than over-all length of active molecules, seem to explain these results. However, the dimensions of the most abundant phospholipid of etiolated peas, 1-palmitoyl-2-linoleoyl-3-sn-phosphatidyl choline are such that a monolayer of this lecithin would contain cavities 20 Å long. It is postulated that lipids of this length or longer are active in the pea assay by forcing apart lecithin molecules, changing the charge distribution or chelating properties of a regulatory membrane.

Steroid dimensions also match the cavity in lecithin monolayers and steroids would be maintained within such a cavity by hydrogen bonds. The name lipometrin for lipids whose physiological activity varies with length is proposed, among these, substances active in the pea bioassay could be termed oleanimins.

Small quantities of various lipids can double the growth-promoting effect of auxins on pea stem sections, if the plants from which they have been cut receive some red light during their development, and a metabolizable sugar is supplied with the hormone. Although we discovered this effect some years ago, at that time the only features in common of the effective lipids appeared to be their neutral nonpolar nature, and the fact that they were all hydrocarbons with a chain of at least 12 carbon atoms (20 and references therein). Such substances were shown to enter both chemically and centrifugally defined membrane fractions, and in all cases investigated the lipids stimulated respiration in addition to augmenting cell elongation (11).

More recently, we have noted that for three kinds of molecules, namely alkyl acetylenes, alkyl nitriles, and insect juvenile hormone analogs, an apparent minimum length of the lipid molecule was required for effect in our bioassay (19). In this paper we shall show that length is indeed the most important molecular characteristic in common among lipids which influence the pea test, and that it can be correlated with the dimensions of the major membrane constituent of seedling peas.

MATERIALS AND METHODS

Bioassay. Progress No. 9 dwarf pea (*Pisum sativum*) seeds, graded but not pesticide treated, obtained from the Asgrow Seed Company (New Haven, Conn.), were soaked in a thin layer of water with a small amount of Phygon fungicide (Naugatuck Chemical Co.) at 32 C for about 7 hr until they were completely turgid. Soaked seeds were planted in washed No. 3 vermiculite (Zonolite Corp., Trenton, N. J.) in plastic basins and placed in a 25 C dark room below very weak continuous red light sources. These were 6 w incandescent or NE-45 neon bulbs, behind a one-eighth inch No. 2444 red Rohm and Haas Plexiglas filter. The light intensity at the level of the plants was reduced to approximately 0.1 erg/cm^2·sec by means of a variable transformer. A value of approximately 80% relative humidity is required to grow plants whose sections are to respond maximally to applied lipids.

On the 7th day after soaking, 10-mm sections were cut from the third stem internode immediately below apical hooks which had just reached a 90° angle. Such sections were randomized by floating on water in a large Petri dish and 10 were later added to 20 ml of solution in a 10-cm Petri dish for each test. The sections were incubated in a basal medium composed of 1.5% sucrose, 5 mM KH_2PO_4, pH 5.5, and 50 μM $CoCl_2$ with emulsion stabilizer as noted below. When 1.8 μM IAA and 0.3 μM GA are added, this medium supports maximal extension of pea stem sections under our conditions. After 20 to 24 hr of slow rotation on a shaker at 25 C in the dark room, the length of the sections was measured to the nearest tenth of a millimeter. Average lengths and standard deviations obtained for each dish were later correctly combined via addition of sum of the squares to obtain the average values in the tables which were usually based on three assays of each substance.

Emulsification. For reproducible tests it is essential that lipids be applied as a stable aqueous emulsion. The technique we have used successfully for some years consists of dissolving equal weights of the lipid and an emulsion stabilizer, namely the high molecular weight polyoxy ethylene and propylene derived Pluronic F68 (Wyandotte Chemical Corp., Wyandotte, Mich.), in a minimum volume of acetonitrile. The resulting solution was injected by syringe via a relatively large bore No. 18 needle into a volume of water in which another equal weight of Pluronic F68 had been previously dissolved. The dispersion of the emulsion was further improved by approximately 1 min of treatment with 22 KC ultrasound from a Branson Model

[1] This work was supported in part by a grant from the National Institutes of Health (GM-06921) to B. B. Stowe.

LS-75 Sonifier at maximum output from a one-eighth inch microtip. If necessary, a bubbling flow of nitrogen can remove most of the acetonitrile within a few minutes, as can be determined by disappearance of its smell from the solution, but since at the concentrations finally attained it has no detectable effect on the sections, this step was usually omitted.

The resulting emulsion was used as a stock solution for dilutions to the concentrations under test. The final concentration of Pluronic F68 in all test dishes, including controls, was fixed at 0.004% throughout these experiments.

It should be noted that emulsions of this dilution are probably microemulsions as defined by Schulman (14), with droplet diameters of 100 to 600 Å. This is supported by results with a 50 μM dioctyl ether preparation, which, when examined with a Coulter counter equipped with a 30 μ gate and a Nuclear Chicago particle size analyzer, showed most counts were initiated by droplets less than 1 μ in diameter, below the efficient counting range of the instrument. Thus, although for convenience lipid levels are cited as micromolar "concentrations," true solutions were not involved. Accretion of droplets on the pea stem sections seems the most likely means of lipid entry to the tissue.

Sources of Lipids. Lipids tested were of the highest purity obtainable, in most cases indicated by the supplier to be 97% or better. This contention was checked by gas chromatography on either a 3% SE-30 liquid phase or its OV-9 equivalent, but in some cases a polyethylene glycol succinate column was used at an appropriate temperature and loading. Impurities were detected in a number of compounds examined. In nearly every instance the retention volumes of the major impurity corresponded to the value expected for a compound of two methylene groups shorter length, which, as will be seen later, is in most cases logarithmically less effective biologically. This result, coupled with the fact that the bioassay responds in a linear fashion to the concentration of lipid, means that a few per cent impurity would have to be far more effective than the major component to influence the result of the bioassay. For these reasons, and particularly since the data below are consistent with the assumption that the major component is the one which is effective in the bioassay, we shall not expend the space that would be necessary here to detail the analysis of these compounds. Sources of compounds were Lachat Chemicals Inc. (Chicago Heights, Ill.) 1-chloro(do, tetra, and hexa) decane, 1-bromo (do and tetra) decane, di (hexyl, heptyl, and decyl) ethers; Analabs, Inc. (North Haven, Conn.), 1-bromo (hen. tri, and penta) decane, and 1-iodo (hen, tri, and penta) decane; Eastman Organic Chemicals Co. (Rochester, N. Y.), 1- (chloro, bromo, and iodo) decane, and 1-iodo (do, hexa, and octa) decane; Chemical Samples Co. (Columbus, Ohio), 1-bromo hexadecane, di (octyl and dodecyl) ethers, and all alkylbenzenes; J. T. Baker Chemical Co. (Phillipsburg, N. J.) di (hexyl and heptyl) sulfide; K and K Laboratories (Plainview, N. Y.), di (nonyl, decyl, and undecyl) sulfides; Aldrich Chemical Co. (Milwaukee, Wisc.), di (octyl and dodecyl) sulfides; Mann Research Laboratories (Orangeburg, N. Y.), estradiol, testosterone, and β-sitosterol; E. Merck AG (Darmstadt, Germany), estrone, stigmasterol, diosgenin, and tigogenin. Gifts of other substances are acknowledged in the text.

Dimethyl dodecandioate and octadecandioate were prepared by the BF_3 catalyzed esterification technique of Metcalfe and Schmitz (9) from the free acids (Lachat Chemicals, Inc.) and recrystallized. Dimethyl traumatate was similarly prepared from traumatic acid donated by Merck and Company (Rahway, N. J.).

Molecular Lengths. For calculations of the length of the lipid molecules, the techniques and assumptions of Vandenheuvel were employed (23). The dimensions cited are between the maximum limits set by the van der Waals' radii of the terminal atoms. In some cases, models constructed from Framework Molecular Models (Prentice-Hall, Inc., Englewood Cliffs, N. J.) and polystyrene balls of scaled radius appropiate to H atoms were used to assign values to structures, but it was usually more convenient to plot the molecule on graph paper assuming maximum linearity. The technique can be assessed by examining Figures 1 and 3 below. Reasons for the belief that maximum linearity is a reasonable assumption, particularly if placement in a membrane of these substances is involved, are provided by Vandenheuvel (23, but see also 24). Nonetheless, although our calculations were made to the nearest 0.1 Å, it seems from experience with these methods that a 2 to 4% difference from the values reported here could easily be introduced by any slight modifying effect on the molecule. Other, staggered configurations are likely in the liquid-crystalline membrane matrix (24), but even so relative lengths of these simple hydrocarbons should fall in the same order as truly linear lengths.

RESULTS

Halogenated Alkanes. Three homologous series of alkyl halides were available to us and were tested over a wide range of concentrations from which the roughly logarithmically increasing series assembled in Table I were selected. Although longer alkyl halides were available, emulsions of them could not be stabilized by our techniques, and thus reliable assays were not obtained. The longest substance tested, 1-iodooctadecane, was successfully assayed in only one instance; other attempts failed.

The data obtained confirm the conclusion of our earlier work that a minimum molecular length of approximately 20 Å is necessary for a response of the pea bioassay to lipids. At these or higher concentrations, no substance showed activity if its length was less than 19.8 Å. That it is not the number of carbon atoms in the chain which is important can be seen from the fact that the 13 carbon atom bromine derivative is the shortest active molecule in the bromide series, whereas in the iodide series it is the 12 carbon atom compound. Since the iodine atom has larger dimensions, the over-all length of the latter molecule exceeds the crucial limit even though the carbon chain is shorter. The chlorine derivative nearest the critical length was not available, but in all three alkyl halide series the molecules show increasing effectiveness as their length is increased up to the emulsification limit.

Dialkyl Ethers and Sulfides. The activity of some ethers and sulfides as insect juvenile hormone mimics (13) prompted us to test such homologous series of these compounds as were available commercially. The results are summarized in Table II. In the case of the ethers, the shortest active compound was dioctyl ether whose length of 24.2 Å exceeds that of the other minimum chains. However, in a few bioassay dishes the shorter diheptyl ether showed significant activity, although averages of several tests always were not significant statistically. The smell of the darkroom during these tests leads us to believe that the smaller compound was in part lost from the test dishes by evaporation, and that the statistically negative results for this substance do not necessarily invalidate the generality that hydrocarbons will begin to be active in this assay when their length nears 20 Å.

Higher homologs of these series reveal a new phenomenon, namely an optimum length beyond which the effectiveness of still longer molecules begins to decline. This is quite comparable to the situation with mimics of the insect juvenile hor-

Table I. *Relation of Length and "Concentration" of Alkyl Halides to Their Growth Promotion of Pea Stem Sections*
Differences significant at 5% level from hormones controls are in bold face.

Compound	Length	Controls			Basal + IAA + GA_3 + Alkyl halide (μM)				No. Tests
		Basal medium	+ IAA + GA_3	Do. + 20 μM triolein	5	10	20	40	
	Å	*average % increase*							
1-Chloro-									
decane	16.7	35 ± 5	69 ± 7	90 ± 15	69 ± 10	63 ± 8	68 ± 8	73 ± 9	2
dodecane	19.2	35 ± 4	68 ± 6	**98 ± 11**	65 ± 8	69 ± 9	71 ± 7	68 ± 6	3
tetradecane	21.7	34 ± 5	67 ± 6	**99 ± 11**	66 ± 7	75 ± 9	**90 ± 9**	**91 ± 10**	3
hexadecane	24.2	34 ± 5	69 ± 7	**93 ± 12**	74 ± 6	**94 ± 11**	**96 ± 10**	**95 ± 11**	3
1-Bromo-									
decane	17.0	33 ± 4	71 ± 6	**98 ± 11**	72 ± 7	69 ± 8	66 ± 8	79 ± 8	2
hendecane	18.2	32 ± 6	73 ± 8	**95 ± 9**	75 ± 9	70 ± 8	73 ± 10	73 ± 9	2
dodecane	19.5	31 ± 5	71 ± 9	**93 ± 10**	72 ± 9	77 ± 8	78 ± 9	83 ± 10	4
tridecane	20.7	34 ± 6	72 ± 7	**93 ± 12**	73 ± 10	73 ± 8	85 ± 11	**92 ± 10**	3
tetradecane	22.0	28 ± 5	72 ± 10	**94 ± 8**	78 ± 10	89 ± 12	**97 ± 9**	**94 ± 11**	3
pentadecane	23.2	35 ± 6	74 ± 8	84 ± 8	79 ± 11	91 ± 16	**96 ± 9**	**88 ± 9**	4
hexadecane	24.5	35 ± 6	72 ± 5	**95 ± 12**	86 ± 13	**101 ± 9**	**101 ± 12**	**92 ± 12**	3
1-Iodo									
decane	17.3	36 ± 6	69 ± 8	**94 ± 10**	67 ± 5	68 ± 7	71 ± 9	73 ± 14	3
hendecane	18.6	36 ± 7	71 ± 8	**98 ± 12**	71 ± 8	69 ± 9	72 ± 9	81 ± 14	3
dodecane	19.8	34 ± 7	69 ± 6	**100 ± 12**	69 ± 7	72 ± 11	**84 ± 8**	**101 ± 12**	3
tridecane	21.1	34 ± 7	69 ± 6	**100 ± 12**	69 ± 6	**95 ± 16**	**93 ± 6**	**105 ± 10**	3
pentadecane	23.6	35 ± 6	74 ± 8	84 ± 8	89 ± 10	**92 ± 8**	**96 ± 10**	89 ± 8	4
hexadecane	24.8	35 ± 6	68 ± 5	**95 ± 12**	**103 ± 9**	99 ± 9[1]	**98 ± 12**[1]	**91 ± 11**[1]	3
octadecane	27.3	35 ± 5	67 ± 6	**86 ± 14**	**107 ± 12**	**99 ± 12**	**97 ± 11**	**92 ± 13**	1

[1] Average of one less test than other data in row.

Table II. *Relation of Length and "Concentration" of Dialkyl Ethers and Sulfides to Their Growth Promotion of Pea Stem Sections*
Differences significant at 5% level from hormones control are in bold face.

Compound	Length	Controls			Basal + IAA + GA_3 + Dialkyl Compound (μM)						No. Tests
		Basal medium	+ IAA + GA_3	Do. + 20 μM triolein	3	5	10	20	30	40	
	Å	*average % increase*									
Ether											
Dihexyl	19.2	37 ± 5	69 ± 7	**91 ± 10**	...	69 ± 11[1]	68 ± 9	65 ± 10	64 ± 9	62 ± 8	4
Diheptyl	21.7	35 ± 4	68 ± 8	**92 ± 11**	...	68 ± 9[1]	71 ± 9	79 ± 10	69 ± 8	78 ± 9	8
Dioctyl	24.2	39 ± 6	68 ± 8	**97 ± 12**	...	70 ± 9	83 ± 14	**94 ± 11**	**97 ± 12**[1]	**98 ± 10**	4
Didecyl	29.2	40 ± 6	66 ± 8	**95 ± 13**	...	**99 ± 12**	**101 ± 9**	**95 ± 13**	...	**99 ± 10**	3
Didodecyl	34.2	38 ± 6	67 ± 8	**97 ± 12**	...	69 ± 8	**89 ± 9**	**94 ± 11**	**86 ± 11**[1]	**101 ± 9**	3
Sulfide											
Dihexyl	19.8	33 ± 6	68 ± 10	90 ± 14	...	...	76 ± 10	75 ± 9	82 ± 9	80 ± 10	5
Diheptyl	22.3	33 ± 6	68 ± 10	90 ± 14	...	...	77 ± 9	82 ± 12	88 ± 14	**92 ± 11**	5
Dioctyl	24.8	35 ± 5	67 ± 6	**83 ± 8**	...	71 ± 7	**87 ± 9**	**88 ± 8**	**82 ± 7**	73 ± 9	4
Dinonyl	27.3	35 ± 5	67 ± 6	**83 ± 8**	...	**92 ± 12**	**92 ± 9**	**86 ± 10**	78 ± 9[1]	78 ± 8	4
Didecyl	29.8	34 ± 4	64 ± 9	77 ± 8	82 ± 12	**90 ± 13**	**88 ± 9**	**81 ± 8**	**85 ± 9**	74 ± 9	3
Diundecyl	32.3	34 ± 4	64 ± 9	77 ± 8	70 ± 9	80 ± 9	**84 ± 9**	**83 ± 10**	79 ± 11	73 ± 7	3
Didodecyl	34.8	33 ± 6	68 ± 9	82 ± 9	...	68 ± 8	74 ± 8	77 ± 11	**89 ± 11**	83 ± 9	5
Ditetradecyl	39.8	33 ± 6	68 ± 9	82 ± 9	...	71 ± 10	73 ± 12	81 ± 11	**91 ± 10**	**89 ± 12**	5

[1] Average of one less test than other data in row.

mone, but in them the optimum is near 21 Å, whereas in the pea it appears to be between 28 and 30 Å (18). In the insect derived data a maximal length, beyond which the molecule is biologically inactive, is evident. If this is true for peas, in our assays the limit of emulsifiability was reached before biological effectiveness had completely declined.

Alkyl Benzenes. A third probe of linear dimension was provided by a homologous series of alkyl substituted benzene derivatives whose test results are summarized in Table III. Nonadecyl benzene, the longest alkyl benzene available, is one of the most active molecules tested, and it too has a length near that noted as optimum for the dialkyl ethers and sulfides, namely close to 30 Å. The activity in this series of molecules again commences at the point where the overall dimensions of the molecule exceeds 20 Å, and thus well before the length of the thinner hydrocarbon chain matches that value. Hence the

Table III. *Relation of Length and "Concentration" of Alkyl Benzenes to Their Growth Promotion of Pea Stem Sections*
Differences significant at 5% level from hormones control are in bold face.

Benzene	Over-all Length	Alkyl Length	Controls			Basal + IAA + GA_3 + Alkyl Benzene (μM)							No. Tests
			Basal medium	+ IAA + GA_3	Do. + 20 μM triolein	1	3	5	10	20	30	40	
	Å		average % increase										
Decyl	19.2	14.7	33 ± 7	76 ± 10	86 ± 10	...	...	72 ± 7	79 ± 12	80 ± 13	76 ± 11	78 ± 8	3
Undecyl	20.5	15.9	33 ± 6	69 ± 9	87 ± 10	...	...	70 ± 6	81 ± 13	81 ± 10	**91 ± 9**	**84 ± 7**	3
Dodecyl	21.8	17.2	33 ± 6	69 ± 9	87 ± 10	...	...	76 ± 9	89 ± 12	**97 ± 10**	**96 ± 12**	**91 ± 7**	3
Tridecyl	23.0	18.4	32 ± 8	76 ± 10	85 ± 6	...	...	75 ± 13	84 ± 8	**99 ± 10**	**96 ± 9**	91 ± 11	3
Pentadecyl	25.5	20.9	30 ± 6	70 ± 7	**88 ± 11**	...	...	82 ± 7	**100 ± 9**	**95 ± 11**	...	...	3
Heptadecyl	28.0	23.5	34 ± 6	70 ± 8	**93 ± 10**	73 ± 7[1]	75 ± 9[2]	**98 ± 12**	**106 ± 12**[2]	**104 ± 14**[1]	...	...	4
Nonadecyl	30.5	26.0	35 ± 6	70 ± 7	**93 ± 11**	69 ± 7[2]	**92 ± 11**[3]	**94 ± 11**	**101 ± 10**[3]	**95 ± 9**[3]	...	...	4

[1] One test only. Significance assessed from controls of that assay.
[2] Average of two tests. Significance assessed from controls of those assays.
[3] Average of three tests. Significance assessed from controls of those assays.

relatively bulky benzene ring seems to be accepted at the active site.

Bifunctional Lipids. Molecular chains with two substituents, one at each end of the molecule, appear to be ineffective. Tetradecadinitrile, dimethyl dodecandioate, dimethyl octadecandioate, and dimethyl traumatate (2-trans-dodecendioate) were not growth stimulating over a wide range of concentrations, even though all exceed 20 Å in length and singly substituted esters and nitriles of similar lengths are highly effective (19, 20). Thus, it appears that one end of the molecule must be relatively unencumbered to be functional at the active site.

Steroids. These are known to be membrane constituents, include well known animal hormones, and some have linear dimensions exceeding 20 Å. Accordingly, three groups selected to represent major chemical types (4) and lengths (25) were tested. Estrone and estradiol had no influence on the pea test up to 40 μM, but are only 12.7 Å in length. Testosterone, with nearly the same dimensions, at even 0.1 μM was significantly inhibitory, but no growth stimulation was noted at any concentration. Stigmasterol and β-sitosterol were ineffective, although their length of 21.5 Å exceeds the 20 Å limit and both occur in peas (3). Diosgenin and tigogenin which are slightly shorter than these, also failed to influence elongation.

Nature of the Chain. Both simple and isoprenoid hydrocarbon chains are biologically active. If length is the major determining factor, other, chemically dissimilar, chains might be effective. For this reason several fluorocarbon derivatives were tested. Simple fluorocarbon chains of sufficient length were not available, but as these probably assume a helical form due to repulsion of fluorine atoms on adjacent carbons (1), they might not have been suitable. Instead, Freon E-3, E-4, and E-5, which are fluorocarbon polyethers of the general formula $F(CF_3CFCF_2O)_nCHFCF_3$ where n is indicated by the digit in the E series, were kindly supplied by the E. I. DuPont de Nemours Co., Freon Products Division, for testing. Although they should be able to maintain a linear structure and their lengths of 17.0, 20.5 and 24.1 Å respectively reach the potentially active range, all were inert up to 80 μM as far as effects on elongation are concerned. Similarly Fombline-L Fluid, a boiling point fraction of mixed perfluorethers distributed by Peninsular ChemResearch Inc. (Gainesville, Fla.), failed to stimulate growth.

DISCUSSION

Metabolic Relationships. The only consistent, common properties of active lipids that we have discerned are molecular length and low polarity. So far, no substance much smaller than 20 Å is active, and if the smallest active molecule in each homologous series is drawn to the same scale, as in Figure 1, it can be seen that their over-all dimensions fall within a narrow range.

It seems unlikely that these substances have a common metabolism which would not be shared by smaller chains. And among those tested, the ethers and alkyl benzenes ought to be particularly inert. To be sure, as has been demonstrated and summarized recently by Kolattukudy (6, 7), even paraffins can be metabolized by plants. But as we showed earlier, even readily metabolized esters of fatty acids contribute no more than a few per cent to the increased CO_2 output they initiate (11). Moreover, isoprenoids, which are supposed to enter quite different pathways of metabolism, are comparably effective to these alkane derivatives (20).

Although some elaborate metabolic schemes can be formulated that might account for these facts, it seems simpler to conclude that it is some common physical property of long molecular chains rather than common metabolism which is responsible.

Physical Properties. The difficulties we encountered testing the shorter ethers, which were attributed to their volatility, immediately suggest a trivial explanation for our results, namely that the other hydrocarbons in Figure 1 are also readily lost from solution when near 20 Å in length. However, most are low melting point solids, ranging from the −5 C of undecyl benzene to a melting point of 19 C for tetradecanitrile; only the ethers and sulfides have no recorded physical constants for the solid state. In their case, the shortest molecules with significant activity are dioctyl ether and diheptyl sulfide, whose boiling points of 292 C and 298 C do fall within the range of boiling points of the other compounds, *i.e.*, from that of methyl dodecanoate boiling point 262 C to tetradecanitrile boiling point 332 C. Extrapolation from all boiling point values to the 25 C bioassay temperature shows a 20-fold range of approximate vapor pressures with none higher than 0.1 mm Hg, indicating that this physical property is unlikely either to be important or to be the common denominator of these molecules.

Also difficult to reconcile on a metabolite basis is the increasing effectiveness of longer chains. Figure 2 is a plot of the logarithm of minimum "concentration" at which significant growth promotion was noted *versus* molecular length for five of the homologous series. Their effectiveness increases essentially log linearly and with similar slopes toward the optimum, which is to say that they similarly become more effective per unit of length as they become longer. Gross utilization as

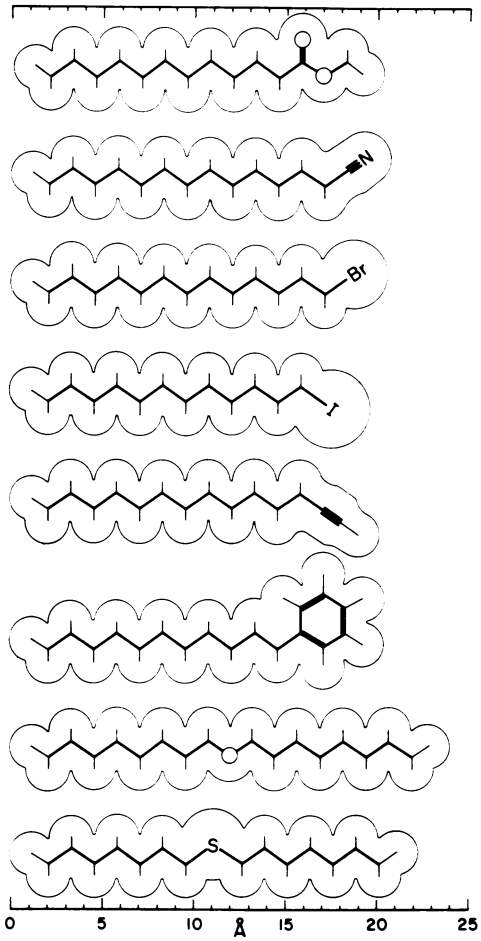

FIG. 1. Scale drawings by method of Vandenheuvel (23) of alkyl derivative molecules at minimum length found to be active in pea stem section elongation bioassay in this or earlier work (20). Outer envelope represents van der Waal's radii of atoms. Carbon atoms are at unlabeled intersections, lighter lines are bonds to hydrogen atoms. Maximum linearity is assumed. From top: methyl laurate, tetradecanitrile, 1-bromotridecane, 1-iodododecane, tetradeca-1-yne, undecyl benzene, dioctyl ether, and diheptyl sulfide.

a substrate would be more likely to be a simple linear function. Optimal effectiveness appears to be reached between 28 and 30 Å; interestingly, the lengths of two of our most effective isoprenoid compounds (20), vitamins E and K_1, fall in this range too.

Although the slopes of these plots appear to be the same within the limits of error, there do seem to be differences in the relative effectiveness of the alkyl derivatives. At any given chain length iodo is nearly always the most effective, followed by the other halogens, alkyl benzenes, and sulfides, and lastly the ethers. The data is not sufficiently precise to discriminate further, but these three groups are the most consistently separable along vertical axes on Figure 2.

Another property, polarity, comes to mind as a possible explanation of relative activities, but among these substituents, chlorides should be the most polar, whereas ethers and benzenes would be expected to be the least (8, 12). Nor do their volumes, or other dimensions, seem sufficiently different to fully explain the ranking observed.

However, even though all of these substances are "nonpolar" in common parlance, their relative polarities (which would be accentuated in a nonpolar medium such as a biological membrane) could influence their placement in that membrane. In this respect, it would be expected that compounds penetrating a biological membrane would interdigitate between the hydrocarbons there, with their more polar end being forced towards the lipid surface which is in contact with the aqueous phase. This could explain our negative results with the disubstituted linear molecules with two slightly polar groups, one at each end. These molecules might not penetrate the lipophilic medium to begin with, or if they did, would be subject to forces tending to fold them over so that both relatively polar groups would be at the more hydrophilic surface. In either case, the length of the molecule could not be measured by the membrane matrix.

Relationship to Lipid Monolayers. Why should molecular chain length modulate hormone action and respiration? Although several physical factors such as viscosity, solubility, rotation, and others (12, 17) are influenced by chain length and do have pertinence to effects on a membrane, we would like to suggest a hypothesis which includes an explanation of the minimum and optimum chain lengths observed. This is based on the work of Schulman and Montagne (14) with lipid monolayers.

These workers noted that introduction of neutral hydrocarbons into a monolayer expands it, spreading further apart any ionizable amphipathic molecules already present, and thus changing the charge density of the monolayer. In another system, using a phosopholipid monolayer, Shah and Schulman (16) noted that similarly expanding the lipid surface by increasing chain unsaturation changed the chelation of metal ions by the membrane, modified enzymic attack on the monolayer (presumably because spacing of the charged groups at the water-lipid interface had been changed) and created cavities into which steroids could be fitted. Any one

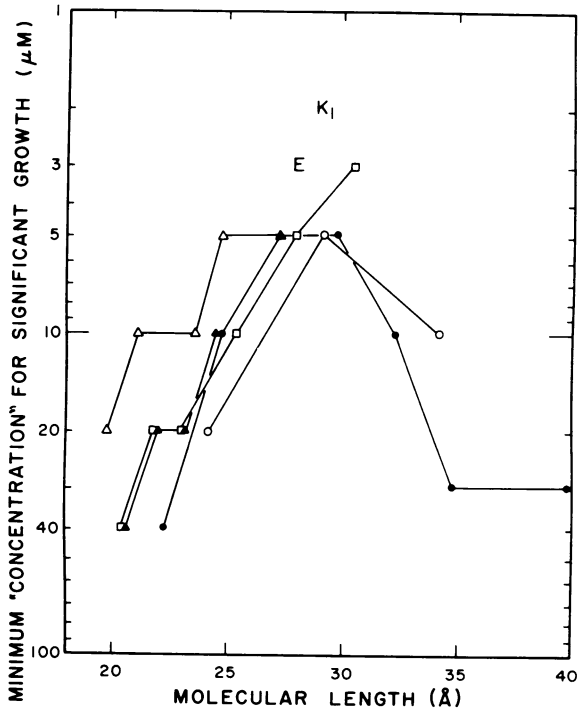

FIG. 2. Relation of molecular length to "concentration" (log scale) at which first significant growth promotion was noted in pea stem section bioassay for five homologous series of alkyl derivatives and two isoprenoid vitamins. As lipid emulsions were employed the term "concentration" indicates relative quantities available for absorbtion by the sections. △: 1-iodoalkanes; ▲: 1-bromoalkanes; □: alkyl benzenes; ○: dialkyl ethers; ●: dialkyl sulfides; E: α-tocopherol; K_1: phylloquinone.

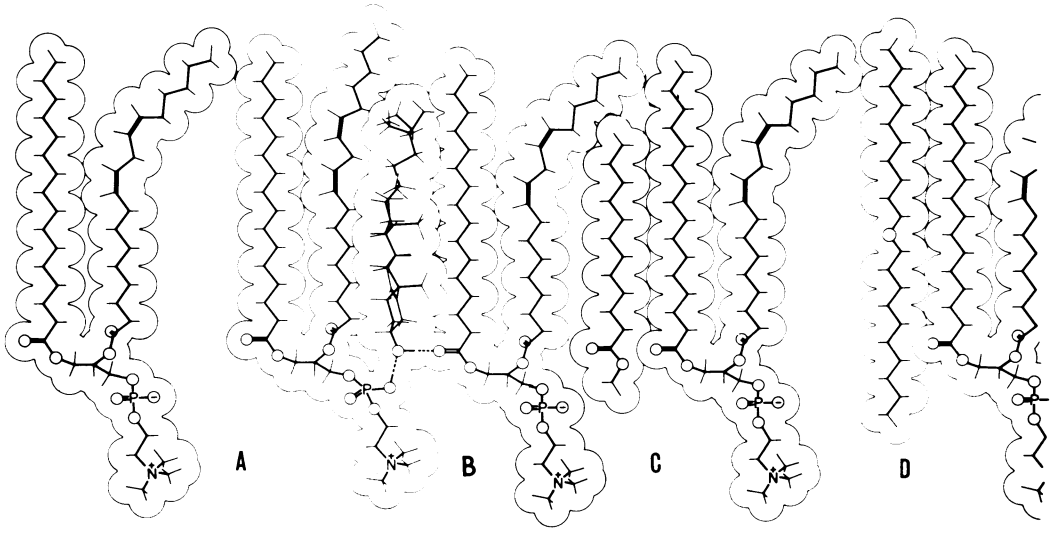

FIG. 3. Scale diagram of membrane monolayer formed by 1-palmitoyl-2-linoleoyl-3-sn-phosphatidyl choline drawn by method of Vandenheuvel (23), conventions as in Figure 1. A: The cavity between closely packed unit molecules, due to the nonlinearity of the linoleoyl chain and the liquid state of these molecules as postulated by Schulman (14, 16), is illustrated. B: The most abundant steroid of green peas, β-sitosterol, is inserted in such a cavity. The projection is from the side of the plane of the molecule as conventionally drawn, and is adapted from the mirror image of Vandenheuvel's projection of cholesterol (23) through carbon 23. It should be noted that the angular methyl groups are above the plane of the lecithin molecules. Space-filling molecular models support this representation as drawn, but since the linoleoyl chain is forced back it may be that the relative positions of the choline groups are maintained by hydrogen bonds as indicated by dotted lines in the figure. C: A molecule of methyl laurate is inserted in the cavity to illustrate that it just begins to interfere with the linoleoyl chain at its length of 20.4 Å. D: A molecule of didecyl ether is sufficiently long enough to completely push the lecithin molecules apart, leading to maximal charge separation between phosphatidyl choline groups.

of these four effects could easily provide a molecular basis for regulatory action at a biological membrane.

In our peas we have found palmitic and linoleic to be the most common saturated and unsaturated fatty acids respectively (Obreiter and Stowe, unpublished). Recently, Trémolières and Lepage (21), using essentially the same pea seedling material as ours, have provided a breakdown of all major lipid classes and report this is also true for the most abundant phospholipid, phosphatidyl choline, which is 38% of the total lipid in leaves of 7-day-old etiolated seedlings. It contains 19% palmitic and 51% linoleic acid. Almost certainly then, the most common membrane lecithin of peas is 1-palmitoyl-2-linoleoyl-3-sn-phosphatidyl choline.

If this molecule is present as a monolayer of a regulatory membrane, it could have the linear structure indicated in Figure 3, the choline being at the aqueous interface and the hydrocarbon tails backing up against the lipophilic surface of another monolayer facing the other direction. As Shah (15) points out, these tails cannot be static as they are not in the solid state; the movement they undergo as a result of being liquid and the curvature of the cis,cis unsaturated chain cause a cavity to be formed at the lower end (Fig. 3A). If long enough, such cavities will accommodate sterols without expanding the monolayer. If the cavities are too short, the monolayer will be expanded when sterols are added. The most abundant total steroids of green peas were found by Goad and Goodwin (3) to be β-sitosterol, stigmasterol, and campesterol. Gaunt (2) has studied sterols from our etiolated peas and in unpublished work noted 0.1 to 0.5 mg/g fresh weight levels of free sterols in them. One isolated sterol had melting point 143–7 C and its acetate melting point 131–2 C, but could not be conclusively identified at that time. Dr. C. Grunwald has kindly examined it by gas chromatography and identifies it as β-sitosterol. Accordingly, β-sitosterol was used to illustrate that such sterols can fill the cavity formed in pea lecithin (Fig. 3B).

Such cavities are of sufficient width to accommodate all of our biologically active lipids. In the case of our most typical pea membrane constituent, 1-palmitoyl-2-linoleoyl-3-sn-phosphatidyl choline, its cavity is 20 Å long. In Figure 3C a molecule of methyl laurate is drawn in it to show that this molecule is just long enough to begin to interfere with the flailing of the linoleoyl tail. As longer lipid molecules are introduced they will be increasingly more effective in forcing the lecithin molecules apart, and maximal separation of the charges on adjacent lecithin molecules will be brought about by molecules 28 to 30 Å long, as is illustrated by didecyl ether, 29.2 Å long (Fig. 3D). The decreasing effectiveness of still longer molecules which we have observed in four cases above could be due to interference with the molecules in the overlying adjacent monolayer, or interference with the functions of the phosphoryl choline groups.

We are well aware this picture is an oversimplified view of the membrane. Vandenheuvel (24) has presented a convincing argument for staggered configurations in liquid alkanes which would lead to shorter lengths than those calculated here. However, the shortening of molecules (and broadening of the cavity) should be relatively the same for all alkane derivatives. Tinoco and McIntosh's (22) data for the same lecithin we have pictured here indicates the maximally packed cross sectional area of this molecule in a monolayer is about 60 Å^2, more than would be predicted from either the linear or staggered molecular model. In reality, some more random configuration subject to thermal agitation must exist, especially well within the monolayer, while those parts of the chain nearest the hydrophilic surface and under the greatest surface pressures would be expected to assume a more uniform, regular array. In any case, their data does show some "disappearance" of cholesterol in lecithin mixtures, although not as much as in Shah and Schulman's experiments (16). Cholesterol is about 21 Å long and, because of its cyclic structure, could not undergo configuration chain shortening except for its tail.

Thus its dimensions are relatively invariant and conditions which tend to shorten the alkyl chains of the monolayer would not influence it.

Our viewpoint assumes cavities of the Shah and Schulman type occur in membranes, where they are likely to be normally occupied by steroids. There will also be sites normally occupied by isoprenoid vitamins and coenzymes. In this connection, it is noteworthy that our lipid effect requires pretreatment of seedlings by red light a day before harvesting bioassay material. Red light is known to modify terpenoid synthesis (5). Whereas our early gross measurements of sterols in our material did not show changes in total steroids, isoprenoid vitamin content was modified (2). It is not unreasonable to suggest that in a low lipid seedling like the pea somewhat steroid- or isoprenoid vitamin-deficient membranes might result from red light exposure at the time of their assembly, leaving a number of sites empty to be occupied by our membrane expanding active lipids.

Our model displays another potentially important molecular interaction. Sterols when placed in the cavity as illustrated in Figure 3B can easily hydrogen bond both with the carbonyl of the adjacent 1-substituted ester as well as with the phosphate hydroxyl of the lecithin molecule on the other side. Any water trapped in this space could also be involved. Space-filling molecular models substantiate this observation. The apparent overlap of the steroid hydrogens in the figure as drawn is caused by the methyl groups which are actually above the plane of the linear hydrocarbons.

This hydrogen-bonded bridge could be the link that holds lecithin molecules together in a membrane monolayer. It would be stronger in the semipolar surface of the lipoidal phase than in a wholly aqueous environment. Steroids are known to strengthen membranes, and hydrogen bond disrupting agents like urea and guanidine are known to weaken and fragment them. Moreover, nearly all biologically active steroids have a β-hydroxyl which is exactly that steric configuration necessary to participate in such an H-bonded bridge.

Equally pertinent is the fact that unsaturated fatty acids in lecithins are usually 2-substituted to glycerol. In our model, if they were in the 1-position, then the carbonyl would not be as available for H-bonding to an adjacent steroid as long as this plane of the hydrocarbon chains was maintained. Tinoco and McIntosh (22) have recently shown that the same phosphatidyl choline we have used for our illustration here, namely the 1-palmitoyl-2-linoleoyl compound, complexes with cholesterol. So did 1-stearoyl-2-linoleoyl, but its inverse 1-linoleoyl-2-stearoyl (the only one tested) did not.

The dimensions and properties of our postulated membrane cavity are being further explored by using lipid derivatives substituted along the chain and with other sterols. This also permits examination of the effect of conformational stereoisomers, and the nature of the lipid layer above the lecithin molecules. The results will be reported in future papers of this series.

Relevance to Hormone Action. Mitchell *et al.* (10) have recently reported an effect of lipids, apparently similar to ours, but on a bean hypocotyl assay. They suggest this is due to a new class of hormones, which they name "brassins." After 12 years work with our active lipids, we would still hesitate to accord them hormonal status. To be sure, at micromolar concentrations they do modulate the growth and respiration of an isolated organ. But to be a hormone, transport from a regulating source must be demonstrated. In several years of unpublished work in our laboratory, Miss Jean B. Obreiter found in pea extracts a wealth of bioassay-active spots on thin layer chromatograms. In retrospect, we interpret these as due to the effects of any sufficiently long neutral lipid molecule on our bioassay. Unless some of these have exceptionally high specific activity or can be shown to be translocated in a potentially regulatory manner, it is too soon to create a new class of hormones. In any event phenomena of this type are not limited to brassicaceous extracts, and origin need not be commemorated in the name.

A more appropriate class name should reflect the chemistry and action of the compounds. Our substances appear to match a membrane matrix and to stimulate respiration and growth. Among established hormones, the insect juvenile hormones appear to be the closest analogs (13, 18), and they too maintain respiration and growth (19). All such substances fitting a steric site in a membrane might be termed "lipometrins." In the specific case of the pea-active lipids, the term "oleanimin" would not only imply the animating action of our oily substances on respiration (the Latin anima means breath), but would resurrect the old pharmacological usage of anima to mean the active principle of a vegetable drug. For the present, their hormonal status can remain moot.

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