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The Influence of Gibberellic Acid on the Permeability of Model Membrane Systems

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ABSTRACT

Gibberellic acid increases the permeability of model membranes composed of various plant-source lipids, a sterol, and dicetyl phosphate. As a result of hormone treatment, the flux of uncharged molecules such as glucose or sucrose, or charged ions such as chromate, through the model membranes (liposomes or micelles) is increased. The revelance of this finding to the *in vivo* effects of the hormone is briefly discussed.

Plant hormones are both fewer in number and less variable in structure and function than their animal counterparts. In only one case, the gibberellins, do plant hormones approach the diversity apparent with animal steroids. These two classes of compounds, steroids and gibberellins, also share the properties of isoprenoid structure (and, thus, a common biosynthetic pathway), similarity in structural diversity (through insertion or deletion of double bonds, radicals, subsidiary ring structures), and control of comparable physiological functions. It would seem possible that gibberellins and steroids also share at least one common trigger or hormonal mechanism.

One of the simplest manifestations of steroid action is the alteration of permeability of synthetic model membrane systems (3, 9, 21). The models are based on the fact that phospholipids, one of the major components of natural membranes, when dispersed in aqueous media, produce self-ordered particles which display many of the properties of natural membranes (6). The physiological pertinence of the models was enhanced by the finding that they demonstrated comparable responses to compounds which affect membranes *in vivo* (3). The permeability of the model structures (micelles or liposomes) is influenced by detergents, some antibiotics, and toxins, which increase permeability, and anti-inflammatory drugs and anaesthetics, which decrease permeability (16). Sterols may either increase or decrease permeability of the liposomes, and in one study, there was a high degree of correlation between the release of acid phosphatase from lysosomes and the increase in permeability of liposomes, with a range of sterols (3). Some sterols which decrease permeability of the model system have also been shown to act in a similar way in higher plant tissue, *i.e.*, they protect red beet tissue from alcohol-induced damage *in vivo* (8). In addition, the *in vivo* effect of the polyene antibiotic, filipin, in increasing permeability of pea stem tissue, red beet, and potato discs (12, 17), closely parallels its effect on liposomes. Furthermore, the effects of filipin, in both the *in vivo* and the *in vitro* systems, can be overcome by cholesterol (12).

An explanation of hormone action in terms of alterations in membrane permeability has been a perennially proposed possibility. In general, the concept has met with only very limited success, for several reasons, and most workers continue to concentrate on more "metabolic explanations." In this and succeeding papers the potential role of gibberellin as a regulator of membrane permeability will be explored.

MATERIALS AND METHODS

To prepare liposomes, lipids, in stock 30 mM chloroform solutions, were deposited on the inner surface of 250-ml round bottom flasks and the solvent was removed with a stream of N_2. Sufficient 0.15 M aqueous solution of a marker compound which served as the leakage indicator was then introduced to bring the lipids to approximately 10 mg/ml. Two or three No. 4 glass beads were added to the flask before it was flushed with N_2, stoppered and shaken on a wrist action shaker. Complete separation of the lipid film from the surface of the glass was usually obtained within 4 hr at room temperature.

When glucose or sucrose was used as the leakage indicator, the liposomes were separated from external glucose or sucrose by gel permeation chromatography. A peristaltic pump regulated the flow of eluent (0.15 M KCl) through a column of Sephadex G-25 coarse grade (Pharmacia K15/30) which was sufficiently large to separate 3.0 ml of the dispersion into liposomes and free glucose. The void volume was approximately 16.0 ml. The columns were run in a cold room in later experiments. One-ml samples of the separated micelles were then combined with the hormone or other compound under test and incubated, under N_2, for 1 hr at 35 C. When gibberellic acid was used, the desired amount of hormone was deposited directly into the test tube from a 5 mM methanolic stock solution. The methanol was removed by a stream of N_2 before the addition of the micelles.

Liposomes and hormones or other test compounds have generally been incubated within dialysis bags (3, 21) but, in this work (with the exception of the experiment illustrated in Fig. 7), individual incubation mixtures were passed down smaller Sephadex G-25 columns (Pharmacia K9/30). The liposomes and leaked glucose were collected as separate fractions which were then assayed by the Somogyi procedure. This technique avoided the uncertainties and potential inaccuracies due to repeated sampling of dialysates, and diminished any mixing time lags.

The crude lecithin (soybean lecithin type II-S, Sigma Chem. Co.) used in these experiments is grossly impure, consisting of about 75% polar and 25% nonpolar lipids. Phospholipids comprise about 50% of the polar fraction and there is slightly

more phosphatidyl ethanolamine than phosphatidyl choline. The mixture also contains sterols, free fatty acids, and glycerides, although there is no detectable protein. Further purification was undertaken with well established techniques of column and thin-layer chromatography (13, 14). Phospholipid was converted to Pi and assayed by the method of Bartlett (4).

Measurement of radioactivity was performed directly on the aqueous solutions from the Sephadex columns by dispersing aliquots in water-miscible Bray's formula scintillator (5) and counting in a Packard Tricarb for statistically suitable periods.

RESULTS

All of the existing information about the effects of hormones on model membranes has been obtained with animal hormones and animal membrane components. However, there are important differences between plant membranes and the membranes of higher animals. The differences include: (*a*) the generally higher degree of unsaturation of the fatty acid moiety of plant phospholipids; (*b*) the presence of lipids not found in animals, *e.g.*, galactolipids and many sterols; and (*c*) the flexibility of membrane lipid composition during the seasonal cycle of the plant (1, 10, 11, 20). For these reasons, our initial membrane systems were composed of a commercial crude lecithin preparation from soybeans, cholesterol and dicetyl phosphate (a negatively charged ion used to ensure the separation of the individual layers of the multimembrane micelle). In later experiments (as indicated), other plant sterols, as well as other phospholipids, were investigated.

Although the membrane systems used in these experiments are relatively simple, GA_3 was found to increase their permeability. When applied to membranes composed of crude lecithin, cholesterol, and dicetyl phosphate (60–20–20%, w/w), the leakage of entrapped glucose increased from 20 to 57% (Fig. 1). When β-sitosterol, which is a more common plant sterol than cholesterol, was used, essentially similar results were obtained (Fig. 2). It is difficult to distinguish the effect of small amounts of GA_3; however, when the number of replicates was doubled, a significantly increased leakage response to 5 μl of 5 mM GA_3 (8.65 μg) was detected (Fig. 3). This represented a GA_3 to lipid proportion (by weight) of about 0.0015–0.002 to 1.0 since 5 to 6 mg of lipid was present in each incubation flask.

The nature of the sterol incorporated into the micelles does not appear critical, although the amount of sterol has a marked effect on the semipermeable qualities of the membranes, and hence on the response. Increasing the proportion of β-sitosterol over the range of 0 to 30% increases the capacity of the micelles to entrap and retain glucose (Fig. 4). This is also reflected in an increased responsiveness of the liposomes to GA_3 (Fig. 5).

The use of the negatively charged ion, dicetyl phosphate, is not essential for the manifestation of the GA_3-induced response (Fig. 6), though it has been included in most of our preparations. It is important to note, as well, that the hormone-induced increase in permeability of the model membrane systems is not restricted to the passage of glucose through the membranes. Increased permeability in response to the hormone is also evident when the leakage indicator is a charged ion like chromate (Fig. 7). In this case the membranes were somewhat different in composition although the qualitative response was essentially identical. In addition, when glucose was replaced by sucrose, the qualitative aspects of the response were unchanged, in spite of a decreae in the control leakage rate due to the increased molecular size of the leakage indicator (Table I).

In several experiments, with a diversity of both treatments and membrane components, radioactive glucose was used as

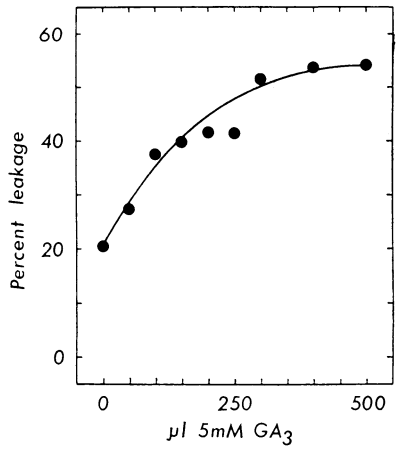

FIG. 1. Influence of GA_3 on the permeability of model membranes to glucose. Liposomes was prepared from crude lecithin, cholesterol, and dicetyl phosphate (60:20:20 mole %, 95 mg total lipid in 10 ml 0.15 M glucose), shaken for 4 hr at 25 C and freed of excess glucose by gel permeation. Liposome suspension was incubated for 1 hr at 35 C with indicated amounts of GA_3.

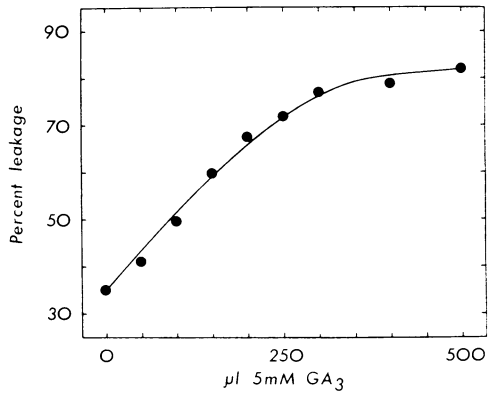

FIG. 2. Effect of replacement of cholesterol by β-sitosterol on GA_3-enhanced leakage of glucose from liposomes. Liposomes were prepared from crude lecithin, β-sitosterol and dicetyl phosphate (60:20:20 mole %, 95.8 mg total lipid in 10 ml 0.15 M glucose), shaken for 4 hr at 25 C and freed of excess glucose by gel permeation. Liposome suspension was incubated for 1 hr at 35 C with indicated amounts of GA_3.

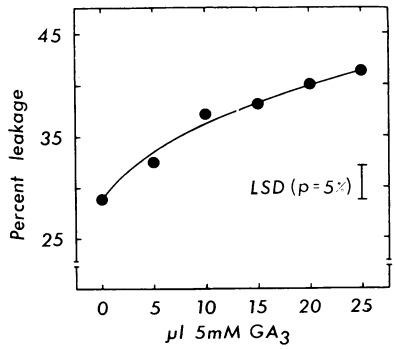

FIG. 3. Glucose leakage induced by small amounts of GA_3. Liposomes were prepared from crude lecithin, β-sitosterol and dicetyl phosphate (60:20:20 mole %, 95.8 mg total lipid in 10 ml 0.15 M glucose), shaken for 4 hr at 25 C and freed of excess glucose by gel permeation. Liposome suspension was incubated for 1 hr at 35 C with indicated amounts of GA_3; each point is the average of eight determinations.

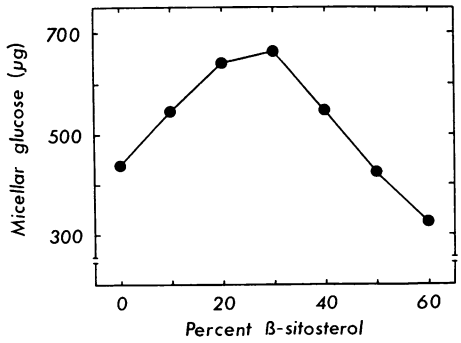

FIG. 4. Effect of β-sitosterol on glucose-trapping capacity of liposomes composed of crude lecithin. Liposomes prepared from 22 mg/ml crude lecithin and 12.4 mg/ml β-sitosterol chloroform solutions mixed in appropriate amounts, and dried with N_2, before suspending in 2.0 ml of 0.15 M glucose for 4 hr at 25 C. Micellar glucose determined as sum of amounts retained and leaked following incubation in GA$_3$.

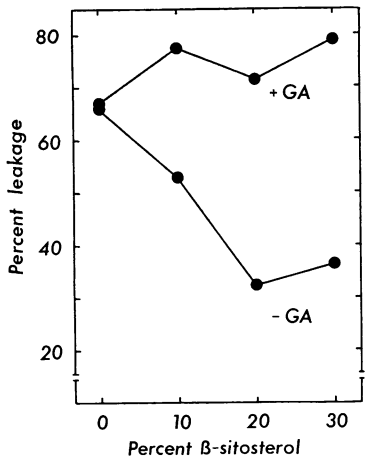

FIG. 5. Influence of β-sitosterol on the glucose permeability of liposomes in the presence and absence of 200 μl of 5 mM GA$_3$ (final concentration of 1 mM). Liposomes were prepared from crude lecithin and various amounts of β-sitosterol (22 mg crude lecithin dispersed in 2 ml 0.15 M glucose for 100% lecithin), shaken for 1 hr at 25 C and freed of excess glucose by gel permeation. Liposomal suspension was incubated for 1 hr at 35 C.

the leakage indicator. The accuracy of the colorimetric assay was determined in a regression analysis against results obtained with scintillation counting (Fig. 8). A correlation coefficient of 0.946 was obtained, indicating that, although the colorimetric technique was more cumbersome and perhaps less elegant, it could be recommended for equal sensitivity and considerably lower cost.

Control, or non-GA$_3$ treated micelles or liposomes, demonstrate a relatively slow rate of glucose leakage during the first 30 min of incubation (Fig. 9). Subsequently, the rate falls off even more, though glucose continues to leak out. In the presence of GA$_3$, the initial rate of glucose leakage is considerably enhanced, although after about 20 to 30 min it also decreases. This suggests that initially the GA$_3$ is probably not evenly distributed throughout the various concentric layers of the liposomes. The results also demonstrate that the micelles are not in equilibrium with the external medium, with respect to the leakage indicator, even after 60 min, and this implies that the physical barriers imposed by the presence of the concentric liposomal layers may be limiting the response.

Finally, attempts were made to assess the importance of

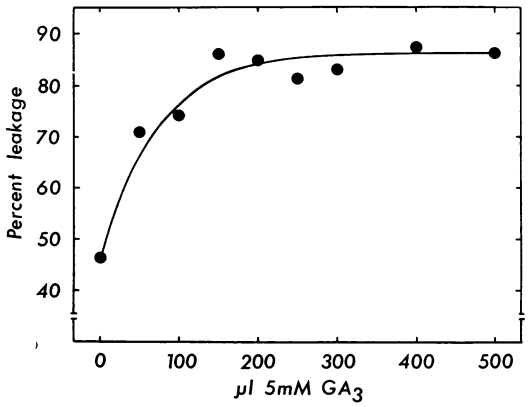

FIG. 6. Influence of GA$_3$ on glucose permeability of liposomes constructed without dicetyl phosphate. Liposomes were prepared from crude lecithin and β-sitosterol (75:25 mole %, 78.4 mg total lipid in 10 ml 0.15 M glucose), shaken 2.5 hr at 25 C and freed of excess glucose by gel permeation. Liposomal suspension was incubated for 1 hr at 35 C with indicated amounts of GA$_3$.

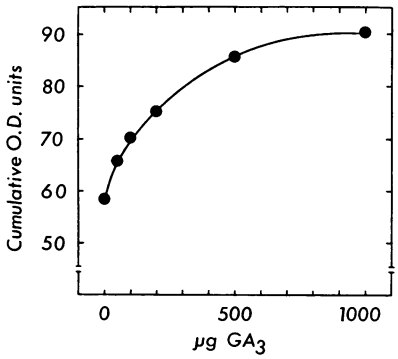

FIG. 7. Influence of GA$_3$ on leakage of chromate ions from liposomes composed of crude lecithin, β-sitosterol, cetyl pyridinium bromide and linoleic acid (70:15:10:5 mole %, 97 mg total lipid in 10 ml 0.15 M potassium chromate). Liposomes were prepared by shaking for 4 hr at 25 C, and freed of excess chromate by gel permeation prior to dialysis at 30 C against 0.15 M KCl. Methanol, 0.1 ml, containing indicated amount of GA$_3$ was added to dialysis sacs, each containing 2 ml liposome suspension, before closure. Controls received 0.1 methanol without GA$_3$. Liposomes were incubated at 37 C for 1 hr. Dialyzing medium (5 ml) was changed four times (at 15 min intervals), and optical density at 370 nm was determined.

Table I. *Effect of GA$_3$ on the Per Cent Leakage of Glucose and Sucrose*

Membranes were composed of 78% lecithin (purified by chromatography on alumina column), 11% cholesterol and 11% dicetyl phosphate. Liposomes were incubated at 35 C for 1 hr.

Treatment	Glucose		Sucrose	
	Retained	Leaked	Retained	Leaked
	%			
None	31.7	68.3	91.8	8.2
GA$_3$ (200 μg)	17.7	82.3	79.4	20.6
Enhancing effect of GA$_3$		14.0		12.4

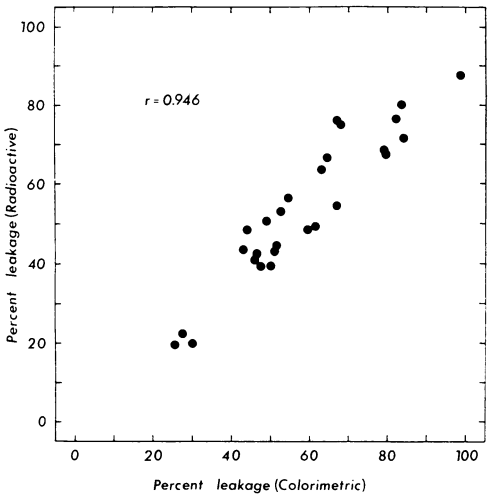

FIG. 8. Correlation between simultaneous colorimetric and radioactive measurements of glucose leaked from liposomes. The experiments include a variety of liposome compositions and hormone (GA_3 and diethylstilbestrol) concentrations. The slope (b: 0.9505 ± 0.065) is not significantly different from 1.0 at the 10% level.

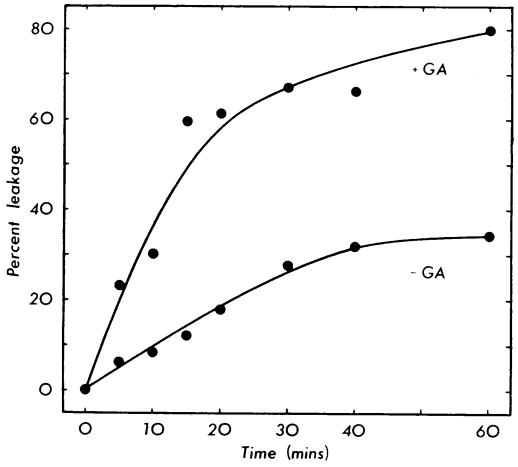

FIG. 9. Time dependent leakage of glucose from liposomes incubated varying lengths of time in the presence or absence of 200 µl of 5 mM GA_3 (final concentration 1 mM). Liposomes were prepared from crude lecithin, β-sitosterol and dicetyl phosphate (60:20:20 mole %, 95.8 mg total lipid in 10 ml 0.15 M glucose), shaken 4 hr at 25 C and freed of excess glucose by gel permeation. Liposomal suspension and hormone were incubated at 35 C.

variations in the nature of the major constituent of the liposomes, the phospholipid component. The commercial crude lecithin preparation used in these experiments contained a relatively low amount (about 15%) of phosphatidyl choline, and varying amounts of other phospholipids. It was of interest, therefore, to determine whether the responsiveness of the liposomes to GA_3 could be ascribed to any of the major component phospholipids, and what, if any, was the effect of the different phospholipids on the nature of the response. The purified phospholipids (at least 99% pure and demonstrating only one spot after thin-layer chromatography) were obtained by chromatography of the original crude lecithin and micelles, prepared from the various phospholipids, were assayed for their responsiveness to GA_3. In addition, the liposomes were also assessed for their efficiency as semipermeable membranes by measuring their glucose-entrapping attributes.

The results of these experiments (Table II), arranged in order of control rates of leakage of the glucose indicator, demonstrate the inability of the various purified phospholipids to form hormonally-responsive liposomes, at least in the proportions tested. In general, also, purification of the phospholipids resulted in an increased control rate of leakage of the glucose indicator, thus making it more difficult, if not impossible, to measure a response to GA_3. It is apparent that, with phosphatidyl choline as the major phospholipid, small amounts of other lipids produced considerable effects. In general, the presence of phosphatidyl serine, for instance, substantially reduced the control rate of glucose leakage, while phosphatidyl inositol increased it. The more complex the composition of the micelles, the greater the control of leakage, but only the original crude lecithin demonstrated a substantial GA_3-induced enhancement of the glucose leakage rate.

When the micellar compositions were arranged on the basis of their glucose-entrapping properties (Table III) it became apparent that liposomes containing phosphatidyl serine had the

Table II. *Influence of Phospholipid Composition on GA_3-induced Leakage of Glucose from Liposomes*

Liposomes composed of 20% cholesterol, 20% dicetyl phosphate, 10% phospholipid other than phosphatidyl choline, remainder as phosphatidyl choline. All phospholipids (but crude PC sample) were purified by column and thin-layer chromatography. PS was from a bovine source and PI was from yeast. Liposomes were incubated at 35 C for 1 hr.

Phospholipid[1]	Glucose Leakage		Effect of GA_3
	Control	200 µl 5 mM GA_3	
	%		%
PC + PI	74.8	82.0	+9.1
PC + PE	63.0	64.5	+2.4
PC + PI + PS	54.6	52.3	−4.2
PC + PS	50.8	49.1	−3.3
PC + PS + PE	47.3	46.6	−1.5
PC + PI + PE	43.8	45.9	+4.8
Crude PC	43.8	72.6	+65.7

[1] PC: phosphatidyl choline; PI: phosphatidyl inositol; PE: phosphatidyl ethanolamine; PS: phosphatidyl serine.

Table III. *Glucose-trapping Capacity of Various Liposomal Compositions*

Liposomes composed of 20% cholesterol, 20% dicetyl phosphate, 10% phospholipid other than phosphatidyl choline, remainder as phosphatidyl choline. All phospholipids but crude PC sample were purified by column and thin-layer chromatography. PS was from a bovine source and PI was from yeast. Liposomes were incubated at 35 C for 1 hr. Abbreviations are as in Table II.

Phospholipid	Glucose Trapped	
	Per µg Pi	Per µg Lipid
	µg	
PC + PS + PE	11.7	0.49
PC + PS	10.7	0.45
PC + PI + PS	9.4	0.39
Crude PC	8.3	0.28
PC + PE	7.6	0.32
PC + PI + PE	6.1	0.29
PC + PI	5.6	0.24

highest values in spite of their nonresponsiveness to the hormone. The very marked effect of phosphatidyl serine on glucose entrapment by crude lecithin, and the interaction with sterol level, are demonstrated in Figure 10. Those micelles containing only crude lecithin trap about one-third of the amount of glucose trapped when 20% phosphatidyl serine is included. However, at none of the levels of sitosterol were the phosphatidyl serine-containing liposomes responsive to GA_3, in spite of their high glucose-entrapping ability.

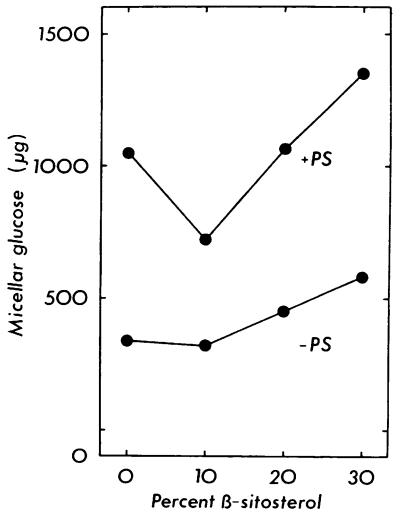

FIG. 10. Influence of phosphatidyl serine on glucose-entrapping capacity of crude lecithin liposomes containing various proportions of β-sitosterol. Crude lecithin, 22 mg, was dispersed in 2 ml 0.15 M glucose for 100% lecithin; bovine phosphatidyl serine replaced 20 mole % of the lecithin. Lipids were shaken in glucose solution for 1 hr at 25 C, and freed of excess glucose by gel permeation. Micellar glucose was determined as sum of amounts retained and leaked following an incubation in GA_3.

DISCUSSION

This work demonstrates clearly, for the first time, that GA_3 can influence the permeability of model membranes, composed of plant constituents, which have many characteristics in common with their more complex and sophisticated natural counterparts (2, 3). The alterations in permeability demonstrated are not mediated by enzymes, do not involve carrier systems, and do not depend on the presence of charges on the permeating molecules. The response of the model membranes, on the contrary, seems to be to either a biophysical alteration of one or more of the components of the liposomes by the hormone through some sort of bond formation, or to an alteration of the "pore" sizes of the micelles through insertion of the hormone into the membrane.

The latter possibility was examined with radioactive GA_3 and the direct association of the hormone with the membrane was found to be rather low. We were unable reliably to detect partitioning of ^{14}C-labeled GA_3 into the lipid phase by the technique of Seeman *et al.* (15), over a range of aqueous concentrations of 2.3 μM to 1 mM. The loss of counts from the aqueous phase into 19 mg/ml lipid did not exceed 1.5%, nor could we find any evidence of an increase in radioactivity in the sedimentable pellet. At present the hormonal effect seems more likely to involve an alteration in the characteristics of one or more of the membrane components, and this possibility will be considered in the next paper in this series.

At least two aspects of the work require comment. Although several methods of purifying the crude lecithin have been attempted, we have not yet isolated or identified a component demonstrating comparable activity. However, lipids in general are notoriously liable to oxidation, and the active agent(s), if there is any, may have been altered during our experiments. The other aspect which is related, and which is perhaps even more unsatisfactory, is the fact that liposomes composed of purified phospholipids have not yet been shown to respond to the hormone. This can be due to at least two possibilities. First, there is a real likelihood that the purified phospholipids we tested were not the "right" ones, in terms of the fact that each of the four main classes of phospholipids, phosphatidylcholine, -ethanolamine, -serine, and -inositol, may be subdivided into an almost infinite series depending on the nature, position, and type of linkage of fatty acid substituents. Secondly, and perhaps more likely, the absence of a response of purified phospholipids from a plant source may be due to the high control leakage rates of the liposomes prepared from them. Thus, when it becomes possible to prepare "nonleaky" liposomes from purified components, it may also be possible to demonstrate a more general hormonal response. A third possibility, that some other component than phospholipids is responsible for the hormonal response, is considerably less likely since liposomes prepared with purified phospholipids respond to steroids (3), and since, also, we have been able to demonstrate an effect of GA_3 on the NMR spectrum of at least one purified phospholipid.

The specificity of the membranes constructed in these experiments is low. GA_8 produced as strong a response as GA_3, and even IAA increased leakage of the indicator molecule. Kinetin, on the other hand, was completely inactive up to 216 μg per incubation reaction. In spite of the low specificity, however, it is obvious that changes in the components of the model membranes alter the responsiveness of the micelles to the hormone.

The question of the physiological importance of hormone-induced responses of model systems has frequently been resolved in favor of nonrelevance because of the usually high amounts of hormone required to produce the response. In a consideration of these results, however, it seems more pertinent to assess the amount of hormone required to produce a response *in relation to the amount of lipid present in the system.* As indicated in the text above, and in Figure 3, a GA_3/lipid ratio of about 0.001 is sufficient to produce a reliable response. What does this mean in terms of an *in vivo* system which is responsive to GA_3? An aleurone layer of about 3 mg contains 1.7% polar lipids of which about two-thirds may be phosphorylated. Assuming, then, that 1% of the aleurone layer (30 μg) is phospholipid, and that 10% (3 μg) of the phospholipid is actually located in active membranes, at a GA_3/lipid ratio of 0.001, 3 ng GA_3 would be required to produce a detectable response of the nature observed with the model membranes. These calculations illustrate that the hormonally-induced alterations in membrane permeability demonstrated above fall well within the concentration range required for biological feasibility and importance.

The physiological significance of steroid-induced changes in membrane permeability is presently thought to be most closely associated with hormonal effects on lysosomes (19). Close similarities in *in vivo* and *in vitro* effects strongly support the contention (3). Furthermore, the recent demonstration of the lysosomal nature of two of the GA_3-induced enzymes of the cereal aleurone layer (7) affords further circumstantial evidence for at least one common hormonal mechanism for steroids and gibberellins.

The nature of the hormonal effect on membrane components will be explored further in a later paper.

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