review

The utilization of sterols by insects

R. B. CLAYTON*

Department of Psychiatry, Stanford University School of Medicine, Palo Alto, California

SUMMARY The experimental data discussed in this review are fragmentary and frequently fail, in a most tantalizing fashion, to allow of precise interpretation. For reasons that are indicated, a shadow of uncertainty hangs over several of the nutritional data discussed. The problems inherent in working with the small amounts of material available from insects' tissues have also contributed to the difficulties of interpretation, and even when an unambiguous experimental result is obtained, it may well be hazardous to generalize from one or two species to all members of the class, which, after all, is the largest in the animal kingdom.

Nevertheless, it is becoming clear that the sterols play a role in insect physiology that seems remarkably similar to that of cholesterol in the mammal. It is evident that both "structural" and metabolic functions are fulfilled, and there is evidence for the existence of mechanisms for the excretion of sterols and their metabolites. If the latter are excretory forms of physiologically active substances, it seems clear that some metabolites other than ecdysone must be involved, since polar derivatives of cholesterol have been detected in the tissues of adult insects in which, presumably, the prothoracic glands have degenerated and ecdysone is no longer secreted.

Studies of the distribution and dynamic state of the tissue sterols of insects show that the sterols per se have diverse roles within the tissues, which for convenience may be described as "structural." Many species of insects can modify the sterols of their diet and presumably such modifications serve to provide a structure that is more appropriate to one or all of the insect's requirements. It is not entirely clear whether phytosterols must always be converted to some extent to cholesterol, but one must conclude from the data available that species differ widely in the extent to which they carry out such conversions. This suggests that while there is probably a basic unity among all species with respect to the types of multimolecular structures into which the sterols are incorporated, there are species differences in the detailed structure of the immediate environment of the sterol molecule. A rewarding aspect of future research in this field may well be the analysis of the relationship between sterols of different structures and the other lipid molecules with which they are associated in the cells of different species. Such studies may shed new light on the manner in which sterols contribute to the stability of subcellular structures.

It is almost certain that with the structure of ecdysone finally established as that of a steroid and its biogenesis from cholesterol demonstrated, there will be increasing interest in the functions of sterols and steroids in insects. It is hoped that this review shows that there is an emergent body of coherent biochemical and physiological information to serve as a basis for future work.

* Established Investigator of the American Heart Association.

INTRODUCTION

THE FIRST demonstration that a dietary supply of sterol was indispensable for the growth of an insect was reported nearly 30 years ago by Hobson (1, 2), who showed that an ether-extracted peptic digest of lean beef was an inadequate medium for the growth of larvae of the flesh fly, *Lucilia sericata*. First the nonsaponifiable portion of the fat and later pure cholesterol were shown to restore the full nutritional value of the medium. Sitosterol or ergosterol could be substituted for cholesterol, though less effectively. On the basis of these findings and of various earlier reports of a lipid growth factor requirement for insects, Hobson suggested that a dietary requirement for sterol was probably general throughout this animal class and that the capacity for sterol synthesis in insects was, accordingly, either entirely absent or grossly deficient. Almost simultaneously with Hobson, Van't Hoog (3, 4) reported similar findings for *Drosophila melanogaster* and shortly thereafter Fröbrich (5) and Offhaus (6) demonstrated a sterol requirement in *Tribolium confusum* and *Silvanus* (*Oryzaephilus*) *surinamensis*. Subsequent work has amply justified Hobson's tentative conclusions. In certain species that do not evince a clear cut dietary requirement for a sterol,

the anomaly has been shown to be due to the activity of intestinal symbionts.

Until recently, most of the work in this field has been nutritional, having as its object the demonstration of the dietary requirement for sterol in a given species and, usually, the identification of utilizable, as distinct from non-utilizable sterols. Radioactive labeling techniques and the newer, refined analytical methods now open the way to studies of the metabolism and functional role of sterols and steroids in insects. Such studies may prove to be particularly rewarding since, as will be seen, there are good indications that the sterols fulfill functions in insects that are closely analogous to those of cholesterol in the mammal. In working with insects, it is possible to exercise close control over the availability of sterols to the tissues since the organism is dependent on supplies of exogenous sterols. Thus studies in insects may permit critical evaluations that would be difficult or impossible in mammals, because the latter have such a large and variable capacity for endogenous sterol synthesis. Moreover, the recently published evidence of Karlson (7, 8) that ecdysone, the growth and moulting hormone of insects, is a metabolite of cholesterol, suggests that the expansion of knowledge in this area may be of value in relation to insect control.

In this review, the results of nutritional studies will be surveyed and related when possible to the information derived from more recent studies at a metabolic level. Newer information concerning the probable functions of sterols in insects will also be discussed. There have been several reviews in recent years (9–14); these have been concerned primarily with the nutritional rather than the metabolic aspects of sterol utilization by insects.

NUTRITIONAL STUDIES

The salient results of studies in which the primary aim has been to compare the effectiveness of different sterols as growth factors for a given species of insect are summarized in Table 1. These experiments have generally been carried out by comparing the growth performance of larval insects with and without the addition of the sterol to a "sterol-free" diet. Most workers have used semisynthetic diets containing such complex natural materials as casein, cornstarch, agar, cellulose powder, filter paper, etc., with or without the addition of small amounts of growth-stimulating materials such as soluble yeast extracts, leaf extracts, or leaf powders. Others have tested sterols for their ability to restore the growth-supporting capacity of the insects' natural foodstuff following its exhaustive extraction with lipid solvents. The uncertainty in these experiments increases with the degree of reliance on undefined dietary components. There is now evidence[1] (15–18) that in the insects the sterols have several functional roles of different structural specificities and a roach[1] (17, 18), a beetle,[2] and a housefly (19) have all been shown to be capable of concentrating into their tissues certain minor components of a mixture of dietary sterols in preference to others that are available in much larger amounts. It is therefore imperative that sterols used in nutritional studies should be of the highest purity.

All species cited in Table 1 utilize cholesterol, but differ in their abilities to utilize other sterols. Other species, not shown in Table 1, for which a requirement for cholesterol has been demonstrated but for which the value of other sterols does not appear to have been studied, are the bean weevil, *Acanthoscelides obtectus* (20); the rice stem borer, *Chilo simplex* (21); the dipteran entomophogous parasite, *Pseudosarcophaga affinis* (22); the clothes moth, *Tineola bisselliella* (23); the rice moth, *Corcyra cephalonica* (24); and the dipteran, *Calliphora erythrocephala* (25).

Several workers have attempted, unsuccessfully, to replace cholesterol in the diet of some species by known biogenetic precursors of cholesterol. These experiments are discussed in a later section.

Quantitative Requirements. The dietary concentration of sterol required for normal growth has been determined for several species and is not the same for all. Low sterol concentrations, of the order of 0.01%, suffice for the growth of the housefly, *Musca vicina* (26); the hide beetle, *Dermestes vulpinus* (15); the carpet beetle, *Attagenus piceus* (27); and the corn borer, *Pyrausta nubilalis* (28). For other species, however, optimal concentrations seem to be closer to 0.1%. The roaches, *Blattella germanica*[3] (29, 37) and *Eurycotis floridana*,[3] the locusts, *Locusta migratoria* and *Schistocerca gregaria* (30); and the dipteran flies, *Lucilia sericata* (2); *Phormia regina* (31); and *Pseudosarcophaga affinis* (22, 32) fall into this category. Frequently a level considerably higher than 0.1% cholesterol has been arbitrarily chosen for species whose true requirements were unknown or were known to be less than the amount used. As a rule, no harm seems to result from this and diets containing cholesterol in concentrations of 1% (*Pyrausta*) (33), 3% (*Acanthoscelides*) (20), 5% (*Eurycotis*)[1] (18), and even 10% (*Tenebrio*) (34), have been found effective for growth. On the other hand, the cowpea weevil, *Callosobruchus chinensis*, could not survive on a diet containing 6.3% cholesterol (35), and 1.4% cholesterol inhibited the growth of *L. migratoria*, though not of *S. gregaria* (30). No evidence of arteriosclerosis has been found in the primitive vascular tree of

[1] N. L. Lasser, R. B. Clayton, and A. M. Edwards, data in preparation.
[2] N. L. Lasser and R. B. Clayton, unpublished observations.
[3] R. B. Clayton, unpublished observations.

TABLE 1 NUTRITIONAL STUDIES OF STEROLS AS GROWTH FACTORS IN VARIOUS SPECIES OF INSECTS*

Species	Lit. Ref.	Cholesterol	7-Dehydrocholesterol	Cholestanol	Ergostanol	Sitosterol	Stigmasterol	Stigmastanol	Epicholestanol	Coprostanol	Zymosterol	Cholest-4-en-3one	Cholest-4-en-3β-ol	Ergosterol
Lucilia sericata	1, 2	+++	—	—	—	++	—	—	—	—	—	—	—	+
Dermestes vulpinus	38	+++	+++	0	—	0	—	—	—	—	0	—	—	0
	15, 16	+++	0	0	0	0	0	0	0	0	0	—	0	0
Tribolium confusum		+++	+++	++	—	+++	—	—	—	—	+	—	—	+++
Lasioderma sericorne		+++	+++	++	—	+++	—	—	—	—	+	—	—	+++
Silvanus surinamensis	43	+++	+++	++	—	+++	—	—	—	—	++	—	—	++
Ptinus tectus		+++	+++	++	—	++	—	—	—	—	+	—	—	+++
Stegobium peniceum		+++	+++	++	—	+++	—	—	—	—	0	—	—	+++
Ephestia kuhniella		+++	+++	+	—	+++	—	—	—	—	0	—	—	++
Attagenus piceus	27	+++	++	0	—	—	—	—	—	—	—	—	—	0
	50	+++	++	—	—	—	—	—	—	—	—	—	—	—
Phormia regina	31	+++	—	—	—	+++	—	—	—	—	0	—	—	++
Musca vicina	48, 26	+++	+	(0)	—	++	+	—	(0)	—	—	++	++	+
Locusta migratoria	30, 49	+++	0	+++	—	+++	0	—	—	—	—	0	—	0
Schistocerca gregaria	30, 49	+++	0	+++	—	+++	0	—	—	—	—	0	—	0
Blattella germanica	29	+++	(0)	—	++	++	++	—	—	—	—	0	—	+ (0)
—	37	+++	0	++	—	+++	+++	—	—	—	—	—	—	0
—	[3]	+++	—	0	—	—	—	—	0	—	—	—	—	++
Gryllulus domesticus	51	+++	—	—	—	+++	++	—	—	—	—	—	—	+++
Bombyx mori	41	+	—	—	—	+++	++	—	—	—	—	—	—	0
Aedes egypti	42	++	0	0	—	+++	—	—	—	++	0	0	—	+
Tenebrio molitor	34	+++	—	—	—	+++	+++	+++	—	—	—	—	—	+++
Callosobruchus chinensis	36, 47	+++	—	+++	—	+++	+++	—	++	—	—	—	—	—
Pyrausta nubilalis	28	++	—	—	—	+++	+	—	—	—	—	—	—	+++
Drosophila melanogaster	3, 4	+++	—	++	—	+++	—	—	0	—	—	++	—	+++
Hylotrupes bajulus	52, 53	+++	—	—	—	—	—	—	—	—	—	—	—	+

* The symbols indicate good (+++), moderate (++), poor (+), or no (0) utilization of a sterol by a given species. Some negative results obtained with the sterol acetates are placed in parentheses. A dash (—) indicates "not studied."

roaches reared on diets containing 5% cholesterol or cholestanol, though abnormal excrescences of the lumen wall of the hind gut are common in such insects. The structure and composition of these nodules are not known, but they do not appear to contain excessive amounts of sterol.[3]

The quantities of sterol required by most insect species are in excess of dietary needs for vitamins of the B group, suggesting, as noted by Ishii (36), different categories of utilization. Gordon (37) pointed out that the insects' generally high dietary requirements for the "lipogenic" factors, choline, inositol, and cholesterol, probably reflected the structural utilization of these compounds. The concept of the structural utilization of sterols in insect tissues has been developed in further detail (15, 16) with the object of rationalizing the relationship between growth-supporting capacity and molecular structure of various sterols.

Utilization of Sterols Other Than Cholesterol. Phytosterols can be utilized by all species listed in Table 1 except for two: *D. vulpinus* and *A. piceus*, both of which are carnivorous predators of stored animal products. Twenty-five unsaturated sterols of the C_{27}, C_{28}, and C_{29} series were tested for their efficacy as substitutes for cholesterol in the diet of *D. vulpinus* and found to be unable to support growth (15, 16). Two sterols, desmosterol and 24-methylene cholesterol, were reported (15) to support the growth of this insect, but later studies with materials of greater purity have failed to confirm the earlier results.[3] Fraenkel et al. (38) pointed out that the contrast between the strict requirement of *Dermestes* for a zoösterol and the ability of omnivorous and phytophagous species to utilize plant or fungal sterols could be related to the feeding habits of the insects concerned. Levinson (14) has emphasized this relationship and more recently (39, 40) has sought to show that this dichotomy of sterol requirements of the carnivorous and phytophagous species reflects different capacities for metabolism of phytosterols in the two types of insects. It is implied that some aspect of utilization of sterols in all

species requires the availability of a C_{27} sterol and that, whereas the phytophagous and omnivorous insects can derive such a sterol metabolically from the C_{28} and/or C_{29} sterols, the carnivores cannot. This view seems also to be favored by Gordon (37). The more direct evidence relating to this point will be discussed below, but it can be seen that nutritional studies (Table 1) suggest that cholesterol is not as effective as some phytosterols for the growth of certain phytophagous species. Sitosterol was utilized more effectively than cholesterol by the silk moth, *Bombyx mori*, the aquatic larva of the mosquito, *Aedes egypti*, and the corn borer, *P. nubilalis*, for which ergosterol and stigmasterol also were more effective than cholesterol. On the other hand, four species, *L. sericata*, *P. tectus*, *M. vicina*, and *B. germanica* grew more poorly on sitosterol than on cholesterol. For the last two species and for *D. melanogaster*, stigmasterol also was less effective than cholesterol. Stigmasterol, differing from sitosterol only in having a Δ^{22}-bond, was utilized less readily than sitosterol by *M. vicina*, *B. mori*, *L. migratoria*, *S. gregaria*, *A. egypti*, and *D. melanogaster*. Eight omnivorous and phytophagous species grew as well with ergosterol as with cholesterol (one, *P. nubilalis*, preferred ergosterol); but for thirteen species ergosterol was less satisfactory than either cholesterol or sitosterol and for seven of these, including the locusts, *L. migratoria* and *S. gregaria*, ergosterol was completely ineffective. It is interesting that, as noted by Dadd (49), the locusts also failed to utilize both 7-dehydrocholesterol and stigmasterol, the $\Delta^{5,7}$-diene of the former, and the Δ^{22}-bond of the latter being structural features that occur together in ergosterol. β-Sitosterol supported the growth of the locusts as well as did cholesterol. The cockroach, *B. germanica*, used ergosterol with difficulty (29, 44, 45) and this sterol was found by one author to be ineffective when used in concentrations that are adequate in the case of cholesterol (37). 7-Dehydrocholesterol was ineffective for the roach just as for the locusts, but unlike the locusts, the roach could utilize stigmasterol at least as effectively as β-sitosterol. The roaches are known to convert ergosterol to $\Delta^{5,22}$-cholestadienol (44, 45), a transformation involving saturation of the Δ^{7}-bond and dealkylation at C_{24}; dealkylation also takes place in this species with β-sitosterol[4] (46) and other C_{28} and C_{29} sterols.[4] The failure of the locusts to utilize the Δ^{22}-C_{24}-substituted sterols may be due to their lack of enzymes for the dealkylation of such compounds.

Since the roaches, the locusts, and the mosquito cannot utilize 7-dehydrocholesterol, it appears that none of these species can reduce the Δ^{7}-bond in this compound. Fraenkel et al. (38) had earlier reported that *D. vulpinus* could utilize 7-dehydrocholesterol even more effectively than cholesterol itself, but when the rigorously purified sterol was employed in more recent experiments by Clayton and Bloch, it was found to be inadequate for replacement of cholesterol in the diet of this insect (16). *A. piceus* and *M. vicina* also grew poorly on 7-dehydrocholesterol as compared with cholesterol. These observations suggest that the growth-supporting action of this sterol in some species, which were the subject of earlier studies, should be re-examined. In reporting the utilization of zymosterol by four species, Fraenkel and Blewett (43) were inclined to attribute the result to the presence of impurities and this seems justifiable, since these insects grew equally well or better on ergosterol, which is frequently a major contaminant of zymosterol.

Saturated Sterols. Stanols appear to be utilized by several species, though cholestanol, the most frequently tested sterol of this group, was as effective as cholesterol in only three species: the locusts, *L. migratoria* and *S. gregaria*, and the weevil, *C. chinensis*. In eight other species, cholestanol was utilized less effectively than cholesterol; and in four: *D. vulpinus*, *A. piceus*, *M. vicina* and *A. egypti*, cholestanol was not utilized. *B. germanica* can grow with cholestanol as the sole dietary sterol under normal conditions but cannot do so when reared aseptically.[3] Ergostanol was utilized by non-aseptic roaches and stigmastanol by *C. chinensis*. Neither compound could replace cholesterol in the diet of *D. vulpinus*, which also failed to grow on epicholestanol and coprostanol. Epicholestanol was also inactive in *M. vicina*, *D. melanogaster*, and *B. germanica* but was reported (47) to support impaired growth and to be recoverable unchanged from the tissues of *C. chinensis*. The evidence for its recovery was tenuous, however. Coprostanol was reported to support the growth of *A. egypti*, though rather poorly. The structures of epicholestanol and coprostanol would be expected to render them unsuitable as replacers of cholesterol in sites of high steric specificity (16) and, in the absence of confirmatory data, the reported utilization of these compounds can only be accepted with reservations.

Sterol Esters. In general, aliphatic sterol esters have been found to be utilized as readily as the free sterols. No case has been reported in which a sterol ester could be utilized when the parent sterol could not, and it seems clear that the utilization of a sterol ester must depend upon the insect's capacity to hydrolyze it. Cholesteryl acetate has been found effective for several species (26, 27, 29, 38, 42, 43, 49), and the acetates of stigmasterol (29, 42) and stigmastanol (36) were found to serve as well as the parent sterols. Several long-chain aliphatic esters as well as the *p*-aminobenzoate and nicotinate of cholesterol were utilized by *A. egypti* (42). The series: cholesteryl formate, acetate, butyrate, palmi-

[4] F. J. Ritter, R. B. Clayton, and K. Bloch, unpublished observation.

tate, benzoate, diethylacetate, but not cholesteryl trimethyl acetate of *p*-tosylate, were utilized by *B. germanica* (29). Cholesteryl *p*-tosylate was reported (50) to be utilized as readily as the propionate, butyrate, or benzoate in *A. piceus*. These results, and those of a study of some aspects of the intestinal absorption of sterols in the roach, *E. floridana*,[5] suggest that the sterol esterases of insects are generally of rather low specificity (though some evidence to the contrary has been presented [54]). *B. germanica* failed to grow when provided with ergosteryl acetate, though free ergosterol was utilized. This difference may be related to the poor utilization of ergosterol in this species, involving its metabolic conversion to $\Delta^{5,22}$-cholestadienol (44, 45). It is possible that when ergosterol is provided as the ester, its transport to the tissues prior to its conversion to the cholestane derivative is facilitated, with undesirable results (cf intestinal absorption of sterols).

Sparing of the Dietary Requirement for Cholesterol by Other Sterols. In the studies discussed so far, various sterols or their esters were tested for growth-promoting activity when present as the sole dietary sterol. Clark and Bloch (15) showed that a large part of the normal cholesterol requirement of *D. vulpinus* could be spared by any one of a number of sterols, which, by themselves, were inadequate as growth factors for this species. Most striking was the observation that all but 3% of the normal dietary cholesterol requirement of *Dermestes* could be replaced by β-sitosterol, which was shown, in agreement with Fraenkel et al. (38), to be incapable of supporting the growth of the insect when provided as the sole dietary sterol. A similar observation was reported by Bergmann et al. (55) in experiments in which a number of analogues of cholesterol having unnatural side chains were tested as growth factors for the housefly, *M. vicina*, but experimental details were not given.

The initial interpretation of these results (15), that the irreplaceable cholesterol requirement supplied a strictly "metabolic" need for the synthesis of hormonal or other physiologically active substances, and that the major and less specific requirement served a metabolically inert "structural" role, has since been modified. *D. vulpinus*[2] and *E. floridana*[1] (17, 18), reared on diets containing minimal amounts of cholesterol supplemented with a cholesterol-sparing sterol in a ratio of 1:20, do not metabolize most of the cholesterol as predicted from this simple hypothesis, but incorporate it selectively into their tissues. It now appears that the major part of the minimal cholesterol requirement fulfills a highly specific structural role throughout the tissues. The sparing sterol presumably plays a different structural role of lower specificity. These results do not, of course, rule out the possibility that some metabolism of cholesterol occurs, and, in fact, evidence for this is available.

An attempt was made by Clark and Bloch (15) and by Clayton and Bloch (16) to explore the steric and electronic characteristics of the functional spaces into which the sparing sterols must be bonded in the tissues of *D. vulpinus*, by testing the cholesterol-sparing capacity of more than 30 3-hydroxy sterols of the cholestane, ergostane, and stigmastane series. The results indicated that cholesterol was spared only by sterols having a generally planar molecular shape and a 3β-(equatorial)-hydroxyl group; coprostane and 3α-hydroxycholestane derivatives were inactive. Cholestanol was the only fully effective sparing sterol of the saturated series; removal of the C_{27}, C_{26}, and C_{25} carbon atoms, or substitution of 1 or 2 carbon atoms at C_{24}, progressively reduced the cholesterol-sparing efficiency. The sparing activity of some of these less active compounds was restored or improved by the introduction of a double bond into the nucleus at C_5, C_7, or $C_{8(14)}$, but not at C_1, C_4, C_6, $C_{8(9)}$, or C_{14}. The presence of a Δ^{22}-bond in the side chain of sterols of the C_{28} and C_{29} series impaired their cholesterol-sparing activity. The results could not be rationalized entirely on the basis of molecular shape, but suggested that the bonding of the sterol molecule into its functional space might depend upon three types of interaction with surrounding structure: (*a*) hydrophobic bonding involving some parts of the molecule (probably especially the side chain); (*b*) hydrogen bonding involving the 3β-hydroxyl group with considerable steric specificity; and (*c*) some form of interaction (hydrogen bonding or dipole-dipole interaction) between a nuclear double bond at Δ^5 or Δ^7 and some specific structure in the environment of the sterol. Double bonds at C_1, C_4, C_6, $C_{8(9)}$, or C_{14} could enter into interactions of type (*c*) leading to inappropriate orientation of the molecule within the functional space. The acceptability of the $\Delta^{8(14)}$-sterols may merely reflect the low polarity contribution of the double bond in this position (56).

Sterol Analogues. Δ^4-Cholestenone was utilized to some extent by *M. vicina* and *D. melanogaster* but was inactive in the locusts, the roach (as the enol acetate), and the mosquito, in which it was found to be growth-inhibiting, whether supplied as the free ketone or as the enol acetate. The utilization of this ketone by the first two species awaits confirmation. *M. vicina* was also found capable of growth on Δ^4-cholestenol, which could be the physiologically active derivative of Δ^4-cholestenone. (However, Δ^4-cholestenol was inactive in *D. vulpinus* either as a sparer or replacer of cholesterol [16].) Δ^5-Cholestenone was inactive for both *B. germanica* (29) and *M. vicina* (26), the growth of which was inhibited by cholestanone (26, 48). Steroid hydrocarbons have invariably failed to support the growth of insects

[5] R. B. Clayton, D. A. Smith, P. C. Hinkle, and A. M. Edwards, *Comp. Biochem. Physiol.*, in press.

(26, 36, 38, 43), as have steroids of the androstane (26, 36, 42, 49, 50), pregnane (15, 42), and bile acid (4, 15, 34, 36, 37, 49) series, estrone (4, 15, 26, 49), and calciferol (4, 25, 31, 34, 38, 43, 49). Cholestanol derivatives having an extra oxygen function at C_4, C_5 and C_6 (42), C_6 (36, 42), or at C_7 (26, 27, 36, 38, 42, 43, 49), were inactive for all species tested. Diosgenin and a series of saponins were inactive for *M. vicina* (26); and in *Blattella*, negative results were obtained (29) with a series of cholesteryl ethers, thiocholesterol and its acetate, cholesteryl amine and its acetate, cholesteryl iodide, bromide, and chloride, and with a number of other more bizarre derivatives. In *Drosophila* (4), several cholestane derivatives, in which the oxygen function at C_3 was replaced by other groups, were inactive.

In summary, the bulk of the evidence of these nutritional studies indicates that for effective growth-supporting activity in insects, a sterol must have an intact nucleus, which is generally planar and has a 3β-hydroxyl group (free or esterified) and a hydrocarbon side chain at C_{17}. The few reported cases of the utilization of sterols whose structure departs from these conditions (coprostanol, epicholestanol) are unconfirmed. The utilization of 3-keto steroids could depend upon their reduction to a 3β-sterol, but no evidence bearing on this point has been presented. Evidently there are considerable species differences with respect to modifications of both nucleus and side chain that are consistent with growth-promoting activity, but no sterols having supernumerary oxygen functions in the nucleus have been found to be active in any species.

Sterol Analogues as Growth Inhibitors. Several workers have reported that certain analogues of cholesterol inhibit the growth of insects, though never very dramatically. Compounds with which such effects have been noted are: cholesteryl chloride (26, 27, 29, 39, 57); cholestanone, epicholestanol, 7-ketocholesteryl chloride, and digitonin (26); Δ^4-cholestenone (and enol acetate), $\Delta^{4,6}$-3-ketocholestadiene, 3β,5α,6β-cholestanetriol (42); cholesteryl methyl ether, thiocholesterol, and thiocholesteryl acetate (57).

The most closely studied of these compounds is cholesteryl chloride, which was reported by McKennis (27) to impair the growth of *Attagenus piceus*. The effect was readily reversed, however, by concentrations of cholesterol close to the normal requirements. Noland (57) studied in considerable detail the effects of cholesteryl chloride, cholesteryl methyl ether, and thiocholesteryl acetate in relation to the utilization of cholesterol by *B. germanica*. The minimal optimal dietary level of cholesterol was shown to be about 0.05% (29). With this concentration of cholesterol, each of the above compounds produced growth inhibition and failure of maturation when added at a level of 0.1%. The data were interpreted to indicate competitive inhibition of cholesterol utilization by all four analogues, of which thiocholesteryl acetate and cholesteryl chloride showed the most marked effects. When similar experiments were carried out with cholesteryl acetate in place of free cholesterol, the inhibition by the chloride and thiocholesteryl acetate was halved and some maturation occurred. It was suggested (29, 57) that these effects were due to inhibition of cholesterol esterases whose function was essential for transport of cholesterol through the intestinal wall. However, in the absence of a direct experimental demonstration of such an esterase inhibition, Noland's interpretation remains speculative and has been questioned by Robbins et al. (58). These workers fed *B. germanica* for 2 weeks with C^{14}-cholesterol as 0.05% of the diet and studied the effect on the level of incorporation of C^{14} into the insect's tissues when cholesteryl chloride was fed simultaneously as 0.05% of the diet. This procedure reduced the incorporation of the cholesterol by only 11%. In a study of sterol absorption from the intestine of the roach, *E. floridana*, Clayton et al.[5] failed to find a significant effect of cholesteryl chloride or cholesteryl methyl ether on either the absorption or esterification of cholesterol in this species.

Monroe et al. (59) examined the effects of cholesteryl chloride, cholestan-3-one, cholesteryl methyl ether, thiocholesterol, and thiocholesteryl acetate on reproduction in the housefly. No deleterious effects of any of these compounds, fed at a level 10 times the dietary cholesterol concentration, could be detected, whether adult survival, number of eggs produced, or viable egg hatch were used as criteria. It was also stated, though without experimental data, that these compounds had no inhibitory effect on the growth of the larvae of this insect and that, to the contrary, some of them (not specified) could supply the insect's sterol requirement either completely or in part.

The diversity of results that have been reported can only be said to leave undecided the question of whether these analogues have any inhibitory effects at all. Since two of them (cholesteryl chloride and cholesteryl methyl ether) have been shown to be absorbed from the gut approximately as efficiently as cholesterol itself,[5] it seems likely that any inhibitory effects they have are not attributable to simple interference with intestinal absorption of sterols. It is, of course, possible that the effects of these "inhibitors" are different in different species.

METABOLIC STUDIES

The recent developments in the field of gas-liquid chromatography of sterols and steroids (56, 60, 61), and improved isotopic assay techniques, have opened

up for study several facets of the physiology and biochemistry of sterol utilization in insects.

Studies of Sterol Biosynthesis in Insects. Sterol biosynthesis in an insect was first examined by isotopic tracer techniques by Bloch et al. (62), who showed that *D. vulpinus* failed to incorporate C^{14} from ingested acetate-l-C^{14} into cholesterol. In these early experiments, the incorporation of C^{14} into a long-chain hydrocarbon fraction was interpreted to indicate that squalene was synthesized by the insect. However, in later experiments with the same organism (63), no incorporation of C^{14} from either acetate or generally labeled fructose into either squalene or cholesterol could be detected. These experiments pointed to an extensive deletion of the enzymic mechanisms of sterol biosynthesis and confirmed the results of nutritional tests in which various known biological precursors of cholesterol, including mevalonic acid, squalene, and lanosterol, failed either to replace or to spare the normal cholesterol requirement of the insect.

Isotopic tracer techniques have also been used to demonstrate the absence of cholesterol synthesis in two other species. Kodicek and Levinson (64) and Sedee (25) showed that *Calliphora erythrocephala*, reared aseptically on a semidefined diet, did not incorporate C^{14} from acetate into cholesterol. Squalene could not replace cholesterol in the insect's diet and it did not become labeled when acetate-C^{14} was administered (25). The housefly, *M. domestica*, failed to incorporate either acetate-1-C^{14} (65) or mevalonate-2-C^{14} (66) into squalene or sterols, though both substrates were metabolized to yield labeled materials in both the saponifiable and nonsaponifiable fraction. As in *Dermestes* (63), higher hydrocarbons and higher alcohols became labeled with C^{14}.

Symbiotic Organisms and Sterol Biosynthesis in Insects. Symbiotic organisms make a significant contribution to the nutrition of many insect species, either as providers of accessory food factors or in promoting the breakdown of ingested food materials (67–69). In some species, special modifications of the intestinal tract (mycetomes) accommodate symbionts, which are of nutritional significance (67, 70). Sterols may be supplied to an important extent by the intestinal symbionts of some species. Pant and Fraenkel (71, 72) showed that the beetles, *Stegobium paniceum* and *Lasioderma sericorne*, reared in the absence of yeasts, which are normal intestinal symbionts, became markedly more dependent upon the dietary supply of sterol and B vitamins. This dependence could be relieved by reinfection of the insects with cultures of the symbiotic yeast.

In the cockroaches, *P. americana* (54), *B. germanica* (45, 73), and *E. floridana*,[3] sterols become labeled with C^{14} following administration of acetate-1-C^{14}. (See, however, Louloudes et al. [74], who found extremely low incorporation of the acetate-C^{14} into sterols in *P. americana*.) Since the roaches are known to harbor an extraordinarily wide range of intestinal microorganisms (75), the intestinal flora were suspected as the source of newly biosynthesized sterol. However, these insects' relatively high requirement for dietary sterol (see Quantitative Requirements), clearly implied that this synthesis did not come close to satisfying their entire needs. Clark and Bloch had shown (44) that in utilizing ergosterol, *B. germanica* converted this sterol to $\Delta^{5,22}$-cholestadienol. The major labeled sterol of *Blattella*, isolated after feeding acetate-1-C^{14}, was also identified as $\Delta^{5,22}$-cholestadienol (45). Moreover, when ergosterol and acetate-1-C^{14} were fed simultaneously, the label was found to be present in ergosterol subsequently reisolated from the insect's tissues. Under conditions of aseptic culture (76), the incorporation of acetate-1-C^{14} into sterols in this insect was reduced to only 0.2% of that in normal roaches, but the transformation of ergosterol to $\Delta^{5,22}$-cholestadienol took place with undiminished efficiency. It was concluded (45) that almost the whole of the sterol synthesis in *Blattella* was due to a primary synthesis of ergosterol by intestinal organisms, followed by the conversion of this sterol to $\Delta^{5,22}$-cholestadienol in the tissues of the insect.

Under aseptic conditions, 0.2% of the normal incorporation of C^{14} from acetate into roach sterols took place, giving cholesterol as the only detectable labeled sterol, and cholesterol was also synthesized in similar small amounts in the non-sterile insects. This minute synthesis of cholesterol might occur in parasitic organisms not eliminated by the technique of external sterilization of the oöthecae. On the other hand, it may indicate a residual biosynthetic capacity, which, as Clark and Bloch have suggested (63), might still be present in the more primitive insects, of which the roach is one. In an insect even more primitive than the roach, the silverfish, *Ctenolepisma*, it was found that incorporation of acetate-1-C^{14} into cholesterol under non-aseptic conditions, occurred without detectable synthesis of $\Delta^{5,22}$-cholestadienol (77). The site of this synthesis remains to be determined, but this result would seem to indicate that if, as in the roach, sterol synthesis depends upon the ability of the intestinal flora to produce ergosterol, the silverfish, unlike the roach, can reduce the Δ^{22}-bond.

From this account, it will be evident that the total absence of sterol biosynthesis in insects in general is far from rigidly established. Reasonably adequate proof has in fact been presented for only three species. In considering whether cholesterol synthesis might take place in insects, their extraordinarily high degree of functional specialization should perhaps not be overlooked. Certain highly localized tissues of some species may retain this biosynthetic capacity, conceivably as a

part of a mechanism for synthesizing some other end product. Under these circumstances, it may well be a matter of considerable difficulty to detect cholesterol biosynthesis by examination of the whole insect, as in the experiments described.

It seems unlikely that the absence of sterol synthesis should be taken to imply a general inability of insects to synthesize terpenoid compounds. Methylheptenone, according to Cavill et al. (78), may constitute as much as 4% of the dry weight of the ant *Iridomyrmex detectus*, and iridomyrmecin, also a monoterpenoid, is found as about 1% of the body weight of *I. humilis* (79). These and related volatile terpenoid substances are important in the corporate life of the ants since their release by threatened or injured individuals probably serves both defensive and warning functions. While the insects' food materials cannot be ruled out as a source of terpenoid precursors of these alarm substances, the high concentration of terpenoids suggests an active de novo synthesis. The visual system of the honeybee has been shown (80) to involve the photosensitive pigment retinene, and coenzyme Q has been isolated from larvae of *Calliphora* (81).

Intestinal Absorption of Sterols. The metabolic events that accompany absorption of sterols from the gut have been studied[5] in *E. floridana*. The principal site of absorption of sterols in this species is the crop, with some uptake probably occurring also in the gastric caeca. These findings are in accord with the early observations of Petrunkiewitsch (82), Sanford (83), Abbott (84), and others concerning the sites of lipid absorption from the roach intestine. Using the technique of simultaneously feeding C^{14}-sterol and H^3-sterol ester, it was shown that in the tissues of the crop, ingested cholesterol was progressively esterified and ingested cholesterol ester was hydrolyzed, until approximately 50% of each was in the free and esterified form. After feeding amounts of cholesterol from 0.05 μg to 2 mg, the uptake into the carcass was consistently proportional to the total concentration of cholesterol taken up in the crop but (at dose levels of 0.1–2 mg) was unrelated to the concentration of esterified sterol in the crop. No data are yet available concerning the rate of turnover of the esterified cholesterol pool of the crop tissues but these results suggest that the esterification which takes place in the crop is not an *obligatory* step in cholesterol transport, though at lower and more normal dosage levels, it could play a facilitating role. That esterification in the crop is not essential for transport is further suggested by the finding that epicholestanol was absorbed into the body tissues without detectable esterification in the crop (though some occurred in the body tissue) and that the non-esterifiable analogues, cholesteryl chloride and cholesteryl methyl ether, were absorbed as effectively as cholesterol itself and were recovered from the tissues unchanged. Ingested cholesteryl esters were transported intact to the tissues during the period (2–3 hr) immediately following feeding and were recovered largely unchanged. In general, cholesterol fed in the esterified form was more readily absorbed into both crop and body tissues than the free sterol. However, cholesterol fed as the free sterol was almost entirely unesterified in the hemolymph during the period of absorption even when appreciable esterification had taken place in the crop.

It seems likely that in the gastric caeca, the sterols and sterol esters are handled in a manner somewhat similar to that found in vertebrates (85–87) where, apparently, hydrolysis of sterol esters occurs in the lumen prior to absorption.

An examination of the intestinal absorption of several sterols other than cholesterol showed some differences between them, both as to the efficiency with which they were absorbed and the degree to which they were esterified, both in the crop and in the body tissues, but indicated no clear relationship between absorption and esterification. Transport of the sterols by the hemolymph to the body tissues is probably mediated by specific lipoprotein acceptors (88).

Distribution of Sterols in Insect Tissues. The distribution of sterols in the tissues of both *Periplaneta americana* and *Eurycotis floridana* has been studied in considerable detail, though by different techniques and with somewhat different results. Casida et al. (54) used digitonin precipitation and Liebermann-Burchard colorimetric estimation to analyze the tissue sterols of *P. americana* that had been reared on a diet of Purina chow. The sterol was found to be distributed throughout the tissues of the insect, with highest concentrations in the mid-gut and Malpighian tubules (~1 μg/mg fresh tissue) and the lowest in the muscle and the cuticle (~ 0.15 μg/mg). The nerve was reported to contain 0.7 μg/mg and most other tissues to contain less than 0.55 μg/mg. Esterification ranged from about 50% in the hemolymph and salivary glands to 12% in the hind intestine. The nerve tissue and muscle were both found to have about 40% of the sterol in the esterified form. Sterols that were "fast acting" in the Liebermann-Burchard reaction were present in all tissues, generally accounting for 10–20% of the total sterol, but amounting to more than 50% in mid-gut and gastric caeca. This last observation is consistent with the presence in these tissues of relatively large amounts of ergosterol formed by the gut flora. No analysis of the sterols of the diet were reported.

An analysis was made[1] (17, 18) of the sterol content of the tissues of *E. floridana* reared aseptically on an artificial diet in which the sole sterol was cholesterol-4-C^{14}. The specific activity of the dietary sterol was used in

TABLE 2 DISTRIBUTION OF CHOLESTEROL AND "POLAR STEROIDS" IN TISSUES OF E. FLORIDANA REARED TO ADULTHOOD ON A DIET CONTAINING 0.1% CHOLESTEROL-4-C^{14}

Tissue	Total C^{14} Steroid	Relative Amounts of C^{14} Present as: Esterified Cholesterol	Free Cholesterol	Polar Steroid
	μg/mg	%	%	%
Crop	1.38	18.9	77.9	3.0
Proventriculus	0.68	5.8	87.9	6.2
Mid-intestine	1.07	1.4	87.0	11.4
Hind-intestine	1.01	3.1	93.5	3.3
Rectum	1.85	30.9	66.7	2.2
Gastric caeca	1.04	8.5	79.4	15.0
Malpighian tubules	1.63	2.0	86.3	3.9
Fat body	1.22	47.4	57.0	1.3
Salivary glands	2.98	1.2	92.9	2.7
Muscle	0.33	7.7	85.0	3.1
Nerve	3.25	32.9	62.7	4.1
Cuticle	0.57	22.0	73.0	4.9
Reproductive organs (male)	0.58	1.3	98.2	0.6
Reproductive organs (female)	1.78	3.8	91.5	4.6

conjunction with assays for C^{14} to estimate the total concentrations and percentages of the different tissue sterol fractions (ester, free, and "polar steroid"), which could be separated by alumina chromatography. The results (Table 2) were in general higher than those of Casida et al. for *Periplaneta*. The highest concentrations of cholesterol in *Eurycotis* were found in the nerve (3.25 μg/mg) and salivary gland (2.98 μg/mg). All other parts of the insect contain less than 2 μg/mg with the lowest concentration in the muscle with 0.33 μg/mg. It is interesting to compare some of these values with those found in vertebrates. The concentration of cholesterol in the adult insect's nerve is only about one-tenth of that found in the cerebral tissue of adult mammals (89) but is little different from that of fetal whole brain, a fact which is doubtless related in the primitive myelination of insect nerve (90). The concentration in the muscle, on the other hand, though somewhat lower than that of most mammals, is close to that recorded for the light meat of the adult hen (91). Levels of esterification of cholesterol varied markedly from tissue in tissue, the highest value, approaching 50%, being found in the fat. More than 30% of the cholesterol in the nerve was esterified (in contrast to almost complete absence of sterol ester from mammalian myelinated nerve tissue [92]).

A small percentage of recovered radioactivity of all tissues was present as "polar steroids" of unknown identity. A significantly higher concentration of this material was found in the gastric caeca and mid-intestine than in any other tissues.

It has been pointed out above that, like *Dermestes vulpinus*, *Eurycotis floridana* can be reared under conditions in which 95% of the total sterol requirement is supplied as a sterol, which, by itself, is incapable of supporting the growth of the insect, provided that a minimal cholesterol requirement is supplied. Table 3 shows the analyses of C^{14}- and H^3-sterol found in the tissues of *E. floridana* reared on a diet containing 0.1% cholestanol-7α-H^3 (the "sparing" sterol) and 0.005% cholesterol-4-C^{14} (17, 18).[1] In almost all tissues, an enrichment of C^{14}-sterol vs. H^3-sterol has taken place as compared with the dietary sterol mixture. A comparison of the relative efficiencies of uptake of cholesterol and cholestanol from the insect's intestine showed that this enrichment was not due to discrimination between the two sterols at the site of absorption.[5] The enrichment is most striking in the nerve, where the ratio C^{14}-sterol/H^3-sterol approaches 1:1. Further, the tritium sterol in these insects is esterified to an appreciable extent but the cholesterol-C^{14} is almost entirely unesterified. The results point to a specific structural role of unesterified cholesterol that cannot be fulfilled by the sparing sterol. In order to determine some further characteristics of these different sterol pools, *Eurycotis* was fed throughout its early growth phase (about 3 months) on a diet containing cholesterol-C^{14} (0.005%) and cholestanol-H^3 (0.1%) and thereafter on a diet containing either the same proportions of unlabeled sterols or 5% unlabeled cholesterol. Individuals from the colony were analyzed both before the change of diet and several months later so that the fate of the different sterols that had been taken up into the tissues early in life could be observed. When due allowance was made for gain in weight, the results[1] (18) showed that,

TABLE 3 CONCENTRATION OF C^{14}-STEROID AND H^3-STEROID IN TISSUES OF E. FLORIDANA REARED ON A DIET CONTAINING 0.1% CHOLESTANOL-7α-H^3 AND 0.005% CHOLESTEROL-4-C^{14}

Tissue	C^{14}-Steroid*	H^3-Steroid*
Crop	0.119	0.720
Proventriculus	0.101	0.347
Mid-intestine	0.080	1.116
Hind-intestine	0.180	2.729
Rectum	0.320	2.141
Gastric caeca	0.063	0.957
Malpighian tubules	0.104	1.159
Fat	0.080	1.173
Salivary gland	0.513	2.718
Muscle	0.047	0.353
Nerve	0.934	0.996
Cuticle	0.095	0.384
Reproductive organs (male)	0.177	0.277
Reproductive organs (female)	0.348	1.065

* All data are given as μg/mg of fresh tissue.

whether the diet during the second phase of the experiment contained the minimal (0.005%) level of cholesterol or a thousand times this level (5%), the cholesterol that had been incorporated early in the life of the insect largely failed to exchange with the newly ingested sterol. The labeled unesterified sparing sterol, on the other hand, exchanged to a very significant degree with the sterol taken up in the second phase of the experiment. Even when the diet contained 5% unlabeled cholesterol, the crop, with the largest flux of cholesterol of all the tissues, retained more than 30% of the labeled cholesterol that had been incorporated during the earlier growth period, though almost all the labeled sparing sterol was displaced. The fat, unlike all the other tissues of the body, showed a net gain in the concentration of H^3-labeled (sparing) sterol during the second half of the experiment. This gain took place entirely in the esterified sterol fraction and accounted for more than half of the sterol that had been displaced from the other tissues. It would appear, therefore, that in the growing insect the fat body traps and stores (as the ester) sterol that has been displaced from other tissues by subsequently ingested sterol. This observation may reflect a need to conserve sterols for incorporation into the tissues of the reproductive system during maturation or for subsequent egg production.

Casida et al. (54) studied the distribution of cholesterol-4-C^{14} in the tissues of the *P. americana* 16 hr after injection into the body cavity and found that it entered all of the tissues, including the nerve, with highest concentration appearing in the gut tissues. More recently, Ishii et al. (93) followed the distribution of labeled cholesterol in the different tissues at intervals of 1, 10, and 20 days after its injection into *P. americana*. After 1 day, the heaviest labeling was found in the fat body with the mid- and fore-gut also relatively highly labeled. With time, the concentration of labeled sterol in these tissues declined while that in the hind gut showed some increase. The labeled material recovered from the tissues was separated chromatographically into esterified, unesterified, and "polar" fractions. In most tissues the percentage of esterification of labeled material increased during the 20-day experimental period but in no case was this increase so pronounced as in the fat, where a rise from 8% after 1 day to 43 and 44% after 10 and 20 days, respectively, was observed. The authors noted that these observations seemed to conflict with those of Casida et al. (54), who found the esterase activity of the fat to be lower than that of any other tissue. The "polar" steroid fraction remained at a concentration of only 1–3% of the total labeled material for most tissues throughout the 20-day period, but increased to 13% in the mid-intestine and gastric caeca by the 20th day and comprised 8–11% of the labeled sterol in the hind gut at all times. These results, like those obtained with *Eurycotis floridana*[1] (18), suggest that the mid-gut tissues of the roaches may be involved in the elaboration of some excretory metabolites of cholesterol. The formation of small amounts of polar metabolites of cholesterol has also been shown to take place in *Blatella germanica* (58) and in the housefly *Musca domestica* (94). The structures of these metabolites remain to be elucidated.

Sterol Ester Formation. Very little is known about the enzymes responsible for the formation and breakdown of sterol esters in insects with respect either to their characteristics or to their distribution, and the chemical constitution of the sterol esters of only one species of insect has so far been described. Clément and Frisch gave evidence for the presence of a cholesterol esterase in the intestine of the wax moth, *Galleria mellonella* (95), and a study of the specificity of the sterol esterase of homogenates of various tissues of *P. americana* was described by Casida et al. (54). The esterases of several tissues of this species were reported to be inactive with phytosterols and coprostanol, but catalyzed the esterification of cholesterol, Δ^7-cholestenol, and 7-dehydrocholesterol. Bade and Clayton (96) found the most abundant cholesterol ester of *E. floridana* to be oleate (~80%) with smaller amounts of esters of saturated (1–9%) and diunsaturated (10–17%) fatty acids. The major component of the diunsaturated fatty acid ester fraction was apparently linoleate. Palmitate was the principal saturated ester. The proportion of cholesteryl oleate was remarkably constant, whether the diet contained oleate, mixed saturated acids, mixed saturated and unsaturated acids, or pure stearic acid.[6] The chief influence of these dietary modifications seems to be to increase the saturated ester fraction at the expense of the diunsaturated fraction when a high proportion of saturated fatty acid is consumed. The formation of a high percentage of cholesteryl oleate, even when the dietary fatty acid is all saturated, is readily accounted for by the efficient and direct desaturation of stearate to oleate that has been demonstrated to occur in these insects.[7] The origin of the linoleic acid moiety of cholesteryl linoleate is less obvious, however, since this acid is synthesized extremely poorly in the aseptic roach. Gordon (37) has shown that although dietary linoleate is not required for growth and maturation of the first generation of *B. germanica*, it is needed for the production of viable eggs. It is therefore possible that during oögenesis, the egg receives a supply of linoleate that suffices for the formation of cholesteryl linoleate required throughout life. Louloudes et al. (74) have presented evidence that acetate-1-C^{14} incorporated into fatty acids of non-

[6] M. L. Bade and R. B. Clayton, unpublished results.
[7] M. L. Bade, data in preparation.

aseptic *P. americana*, is found predominantly in oleate and mixed palmitate and palmitoleate. Gas-liquid chromatographic peaks corresponding to linoleate and linolenate also contained appreciable radioactivity.

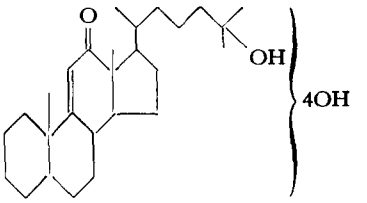
TRANSFORMATIONS OF THE STEROL MOLECULE

Dietary cholesterol is retained in the tissues of cockroaches almost entirely unchanged. The conversion of minor amounts of cholesterol to polar materials in *E. floridana*, reared aseptically, has been noted (Table 2), but otherwise esterification seems to be the only major chemical change which cholesterol undergoes in this insect[1] (17, 18, 96). Similarly, cholesterol was recovered unchanged to the extent of 95% from *B. germanica* (58) and 97% from *P. americana* (93). Very minor amounts of cholesterol were reported to be converted to 7-dehydrocholesterol in both the latter species, but since these insects were not aseptically reared, the significance of this observation is uncertain. No conversion of cholesterol to 7-dehydrocholesterol has been observed in *E. floridana* reared aseptically. The desaturation of cholesterol to 7-dehydrocholesterol took place in the housefly, *M. domestica* (94), in which the $\Delta^{5,7}$-sterol comprised 30–40% of the total labeled sterol found in the eggs. Cholesterol was also converted to a sterol that was fast acting in the Liebermann-Burchard reaction and was tentatively characterized as 7-dehydrocholesterol, in *Tribolium confusum* (97). However, the accumulation of unchanged cholesterol in the tissues of *Dermestes*,[2] when this sterol is supplied in the diet, and of *Bombyx mori* (98), *Platysamia cecropia*,[8] and probably many other species (39, 99) as a metabolite of phytosterols, indicates that this sterol can function effectively without modification in the tissues of many species other than the roaches.

Excretion of Sterols and Sterol Metabolites. When the excreta of house flies injected with cholesterol-4-C^{14} were examined for labeled cholesterol metabolites, only a minor amount (~10%) of the material showed acidic properties. The major portion of the label was recovered as neutral sterol or more polar steroid (94). A somewhat similar pattern of excretion appears to apply in the cockroach, *B. germanica*, where a neutral sterol (probably mainly cholesterol) accounted for about 70% of the excreted radioactivity and the remainder consisted of neutral polar steroids. No coprostanol was detected in this material (58). By the use of double labeling techniques, it has been found[1] (18) that 90% of the polar steroid that can be isolated from the mid-intestine and gastric caeca of *E. floridana* retains the label of cholesterol 26-C^{14} as well as of cholesterol-3α-H^3. If, as seems likely, these polar materials are excretory metabolites, these results suggest that pathways of sterol degradation that occur in mammals are absent or are of only minor significance in this insect.

Horning (100) found that 30% of the label of cholesterol-26-C^{14} was lost by enzymic oxidation when the sterol was incubated with an acetone powder of larvae of the sawfly, *Neodiprion pratti pratti*, but both the physiological significance and the nature of the products of this reaction are unknown.

Cholesterol as a Precursor of an Insect Hormone. Although the bulk of the dietary cholesterol apparently undergoes little modification in the tissues of most insects, evidence has recently been presented (7, 8) for the formation of the moulting hormone, ecdysone, as a metabolite of cholesterol. This substance, the existence of which was indicated by the pioneering work of Kopeć (101), Wigglesworth (102), and Fraenkel (103), stimulates the processes of growth and ecdysis in insects. It is secreted by the prothoracic glands during larval or nymphal life (104) under the control of an "activation hormone" secreted by neurosecretory cells of the brain (105). The isolation of crystalline ecdysone from silkworm pupae was described in 1954 by Butenandt and Karlson (106). In this first isolation, only 25 mg crystalline material was obtained from 500 kg of pupae. A molecular formula of $C_{18}H_{30}O_4$ derived on the basis of earlier work (107) has been revised and the following structure is now proposed (7) for ecdysone, which is a highly polar oxidation product of cholesterol having 27 carbon atoms:



Its biogenesis from cholesterol has been demonstrated (8) in experiments in which cholesterol-H^3 was injected into pupae of *Calliphora erythrocephala* and labeled ecdysone was isolated by using the pure crystalline hormone as a carrier.

Although the moulting hormone of only one species has been characterized, its interspecies activity has been demonstrated by several workers (108–111) so that the activity of a single hormone throughout the class seems to be indicated. Moreover, Karlson (112) has demonstrated the presence of substances in crustaceans, which show ecdysone activity in *Calliphora*, though apparently of a lower potency than that of similar extracts from insects.

Recent publications by Kobayashi and co-workers (113–115) have implicated cholesterol itself as the brain hormone responsible for stimulating the production of

[8] R. B. Clayton and C. M. Williams, unpublished observations.

ecdysone by the prothoracic glands. These workers described experiments in which the effects of brain hormone were produced in diapausing *Bombyx mori* pupae by the injection of solutions of crystalline material, having the properties of cholesterol, which had been isolated from the brains of other insects of the same species. Similar results were claimed for cholestanol and 7-dehydrocholesterol. These observations involve the apparent paradox that a compound, which is already present in relatively large amounts throughout the insects' tissues, has a central hormonal role such that the injection of as little as 0.02 μg gives a physiological response. Ichikawa and Ishizaki (115*a*, *b*) have presented evidence that the brain hormone is a peptide or heat-stable protein, while Gersch and co-workers (115*c*) have isolated four neuro-secretory substances from the nervous tissues of *Periplaneta*, three of them in crystalline form. One of these is claimed to behave as the activation hormone and is said (115*d*) to be thermostable and soluble in both Ringer's solution and ethanol. Ichikawa and Ishizaki (115*a*) consider it unlikely that their material is identical with that of Gersch et al. For reviews of the biological aspects of insect endocrinology the reader is referred to Wigglesworth (116) and Novak (117).

Desaturation of the Sterol Nucleus. Cholestanol is converted in both *Eurycotis* (118, 119) and *Blattella* (118–120) to Δ^7-cholestenol. The transformation takes place slowly and apparently irreversibly in *Eurycotis;* only 30% of the cholestanol incorporated into the tissues was desaturated during 3 weeks after the ingestion of 100 μg labeled sterol. The reaction is an interesting one from both the mechanistic and physiological points of view. It is the first desaturation of the steroid nucleus to be described that does not involve a possible activation of hydrogen atoms by an adjacent unsaturated center. It was shown by the use of 7α-H^3 and 7β-H^3-labeled substrates to involve the loss of the 7β- and 8β-hydrogen atoms without the participation of either the 7α- or 7β-hydroxy sterol as intermediate. Thus, if an 8β-hydroxy steroid were an intermediate, its dehydration directly to the Δ^7 derivative would require elimination of the axial 8-OH group with the 7β-equatorial hydrogen atom rather than with the sterically more favored 7α-hydrogen atom. There is evidence for a localization of the enzymes responsible for the transformation in the gastric caeca of the insect. Differences between the distributions of cholestanol and Δ^7-cholestenol in the tissues seems to indicate different functional roles[1] (18). It has been pointed out that in *Dermestes*, a Δ^7-stenol is more effective than the corresponding stanol in sparing the cholesterol requirement and some primitive molluscs and starfish contain Δ^7-sterols almost exclusively.

Metabolism of Phytosterols. Among the first sterols of insect tissues to be studied were those of the pupa of the silkworm *Bombyx mori* and the constitution of this crystalline material, "bombicesterol," has been the subject of several conflicting reports. The late W. Bergmann (98) resolved it into cholesterol (85%) and β-sitosterol (15%), though without putting an immediate end to the controversy (99). In light of later knowledge, this early work constitutes evidence for a metabolic capacity that is probably widespread among phytophagous and omnivorous insects: the ability to convert the C_{28} and C_{29} sterols of plants to sterols with 27 carbon atoms. Bergmann (99) has cited the identification of cholesterol as the sole sterol of the cabbage butterfly, *Pieris brassicae*, (121) as further evidence for this type of conversion. The replacement of the C_{24} alkyl group by a hydrogen atom is the essential feature of this transformation, but other changes may also occur in the nucleus. Thus, Beck and Kapadia (97) reported that *Tribolium confusum*, whether reared on a diet containing cholesterol, 7-dehydrocholesterol, cholestanol, sitosterol, or ergosterol, always contained a similar mixture of two sterols in its tissues. The major component appeared to be 7-dehydrocholesterol and the minor component, cholesterol, but these sterols were not rigorously identified.

An interesting biological approach to this problem was used by Bergmann and Levinson (122) and Levinson (39, 40) who exploited the strict dietary requirement of *Dermestes* for cholesterol. Larvae of *Dermestes* were shown to grow normally on a diet of ground, desiccated pupae of houseflies, *M. vicina*, which had been fed a diet containing only β-sitosterol. The pupal residue after exhaustive lipid extraction lost its capacity to support the growth of *Dermestes*, but full nutritional value was restored by addition of the extracted sterol or cholesterol. It was concluded that some conversion of sitosterol to a zoösterol adequate for the growth of *Dermestes* had taken place in the housefly. Similar experiments with two other phytophagous species, *Orgyia antiqua* and *Colias hyale*, led to similar conclusions. Subsequently 14 other phytophagous insects (including *T. confusum*) were tested by the same technique for their capacity to modify the structure of phytosterols (39). The tissues of all these species, as well as of the mullusc, *Helix aspersa*, and the mold-eating decapod, *Armadillium vulgare*, were shown to contain sterols, which, unlike those of their vegetarian diets, were adequate growth factors for *Dermestes*. Paper chromatography of the tissue sterols of several of these species was described, and showed the presence of spots corresponding in R_F value to cholesterol, but a complete chemical characterization was not carried out. These results were interpreted as indicating that the ability of phytophagous insects to utilize the plant sterols of their natural diet was contigent upon their capacity to metabolize them to cholesterol or some closely related zoösterol.

The conversion of ergosterol to $\Delta^{5,22}$-cholestadienol in *B. germanica* was demonstrated unequivocally by Clark and Bloch (44) and by Clayton (45). In this species, no enzymic mechanism for reduction of the Δ^{22}-bond was found. The insect can reduce the Δ^{7}-bond of the $\Delta^{5,7}$-diene either in ergosterol itself or in an intermediate in the conversion to $\Delta^{5,22}$-cholestadienol, but not in $\Delta^{5,7}$-cholestadienol (29, 37). *E. floridana* is also unable to reduce the Δ^{7}-bond in Δ^{7}-cholestenol (118, 119). It is not clear whether the poor utilization of ergosterol by the roach is due to the slowness of the conversion to the cholestane derivative or to an inherent physiological unsuitability of the product.

More recently, the removal of the ethyl group of β-sitosterol in *B. germanica* to yield cholesterol has been demonstrated by Robbins et al. by gas-liquid chromatographic and radiochemical methods (46). Preliminary evidence from GLC analyses of the tissue sterols of *B. germanica* reared on diets containing a variety of sterols of the ergostane and stigmastane series suggests that C_{24} dealkylation can take place in this insect with sterols in which the nucleus is either saturated or unsaturated, with double bonds in the Δ^{5}-, Δ^{7}- or $\Delta^{5,7}$-positions.[4]

The concept that utilization of phytosterols by insects invariably involves a dealkylation at C_{24} has not received universal experimental support. On the basis of the evidence of a wide range of physical analytical methods, Agarwal et al. (123, 124) concluded that the major sterol of the adults and eggs of the housefly, *M. domestica*, reared on a fermenting vegetarian diet, differed from cholesterol in the structure of its side chain. The properties of this compound (designated "muscasterol") did not correspond with those of any known sterol, however, and it was later shown by Thompson et al. (19) with the aid of GLC analysis, that "muscasterol" was in fact a mixture of campesterol (24α-methyl cholesterol) and β-sitosterol in a ratio somewhat greater than 3:1. Similar analysis of the sterols of the medium showed β-sitosterol to be the major component, with campesterol a minor constituent. When the flies were fed synthetic diets containing mixtures of sterols (campesterol + β sitosterol, cholesterol + campesterol, or cholesterol + β sitosterol), they selectively retained cholesterol or the sterol (campesterol) most similar to it. This last observation was reminiscent of findings with *E. floridana*[1] (17, 18).

According to Thompson et al. campesterol and β-sitosterol together comprised about 96% of the total sterols of *M. domestica*, the remainder consisting of two unidentified materials of lower retention time on GLC. This result was in general agreement with the earlier findings of Agarwal et al. (123, 124) who, by silicic acid chromatography, were able to isolate only very small amounts of three materials besides "muscasterol." These minor components were considered to be Δ^{7}-, $\Delta^{5,22}$- and 4α-methyl-Δ^{7}-compounds, and no cholesterol was found. More recently, Kaplanis et al. (125) studied the metabolism of dietary H^3-labeled sitosterol in adult houseflies and were unable to detect H^3-cholesterol in either the adults or their eggs. It is clear, therefore, that in this species the conversion of phytosterols to cholesterol is not quantitatively important and may only occur as a stage in the formation of ecdysone or other physiologically active metabolites.

The apparent lack of concurrence between these findings and those of Bergmann and Levinson (122) may be due to species differences. On the other hand, since as much as 97% of the normal cholesterol requirement of *D. vulpinus* can be replaced by β-sitosterol (15), the use of this organism for the detection of cholesterol as a product of dealkylation of phytosterols may be more sensitive than some of the chemical techniques that have been used. It was noted by Levinson (39) that the dealkylation of phytosterols was incomplete in several species, including *Calliphora* and *Musca vicina*, though quantitative data were not presented. In any case, there is no lack of evidence for the accumulation of major amounts of dietary C_{24}-substituted sterols in the tissues of species other than houseflies. The bee, *Apis mellifica*, accumulates 24-methylene cholesterol (126, 127), which is a constituent of various pollens (128), and β-sitosterol is apparently the principal sterol of a cantharides beetle (129) and of the Colorado potato beetle, *Leptinotarsa decemlineata* (130). In connection with this last observation, the recent demonstration (131) that cholesterol is present in the stems and leaves of *Solanum tuberosum*, the host plant of *Leptinotarsa*, as well as in *Dioscorea spiculiflora*, raises the question of whether the quantity of cholesterol in such plants may make a significant contribution to the sterol requirement of insects that use them as food.

FUNCTIONS OF STEROLS IN INSECTS

The insects' relatively high quantitative requirements for sterols and the relatively constant concentrations of sterols found in their tissues throughout development[1] (18, 132, 133), strongly suggest that the bulk of their dietary sterol is utilized per se as an element of cellular structure. There is abundant evidence that unesterified cholesterol plays an indispensable role in the maintenance of membranous structures in the mammal, where it occurs in the red cell membrane (134), the endoplasmic reticulum (135), the mitochondria and their particulate derivatives (136), and, most abundantly, in the myelin sheath of nerves (92). The probable manner of incorporation of the cholesterol molecule into the alternating lipid and protein layers of cell

membranes (137) is suggested by Finean's (138, 139) schematic representation of the structure of myelin (itself the product of accretion of multiple layers of the Schwann cell membrane (140) or elements of its endoplasmic reticulum [141]).

It thus seems likely that sterols are an essential component of subcellular membrane structures in all the Metazoa and that this is the primary basis of their utilization in insect tissues. In confirmation of this view, the analysis of the tissues of *E. floridana* by differential centrifugation showed the free sterol to be almost entirely associated with the particulate fractions.[2] The existence in all the tissues of this insect of at least one unesterified cholesterol pool having a slow rate of turnover[1] (18) is also consistent with the involvement of cholesterol in highly stable membrane structures. The identification of other stable components in the tissues of *Eurycotis* may give new insight into the type of intermolecular associations in which cholesterol is involved. In this connection a knowledge of the nature of the other lipids in the tissues would be of great interest but these compounds remain to be characterized. At the present time, there is only fragmentary information concerning the structures of complex lipids present in lower Metazoa (142–144) and still less is known of their dynamic state.

There is evidence for other functions of sterols in insects besides this structural role. Two of these, conversion to the growth and moulting hormone, and the possibility that cholesterol or some related steroid may have brain hormone activity, have already been discussed. It is also possible that cholesterol, or a metabolite of cholesterol, is involved in control of reproduction. Chauvin (51) recorded that while *B. germanica* could be reared to adulthood on a diet of flour and 10% yeast, it could not reproduce unless this diet was supplemented with fresh lettuce, in which the physiologically active factor was considered to be a sterol. This interpretation has been confirmed in this laboratory,[3] where *E. floridana* and *B. germanica* have been found to survive many months (the former species almost two years) on aseptic sterol-free diets. Egg production under these conditions quickly ceases, however.

The role of sterols in reproduction in the housefly, *M. domestica*, has been studied in some detail by Monroe and Robbins and coworkers. Cholesterol deprivation did not shorten the life of the adult of this species, but drastically reduced the viability of the eggs, without, however, diminishing the numbers of eggs laid (145, 146). The fermenting vegetarian diet (19), that was used for routine rearing of housefly larvae initially contained only phytosterols (cf Thompson et al. [19]). When this diet was supplemented with only 0.01% cholesterol, the viable egg production of the emergent adults was doubled, but β-sitosterol, even at a concentration of 1.0%, had no such effect (146*a*). It was emphasized that the effect was apparently not due to storage of cholesterol, since there was little variation in the total tissue concentration of 3β-hydroxy sterols, whether the larval insects received the unsupplemented diet or a diet containing 0.1–1.0% additional cholesterol. The significance of this observation is limited, however, in view of the later report from the same laboratory (19) that cholesterol when present in trace quantities in the diet is concentrated into the tissues of the housefly in preference to more abundant phytosterols. In a further development of this study, Robbins and Shortino (147) have shown that provision of dietary cholesterol (but not β-sitosterol) during larval life, allows full ovarian development and viable egg production by adult houseflies maintained after emergence on a diet of sucrose and water. Previous work had led to the belief that adult houseflies required some dietary protein for ovarian development. A specific metabolic role for cholesterol as a hormone precursor, such as was postulated by Clark and Bloch (15), might be involved in these effects. On the other hand, Kaplanis et al. (94) have shown that a major route of elimination of injected cholesterol-C^{14} from adult houseflies is via the eggs, suggesting that at least one function of cholesterol may involve its incorporation into the egg and the fulfillment of a specific role in the developing embryo.

Another function attributed to cholesterol by Dennell and coworkers, largely from histological and histochemical evidence, is that of assisting in the hardening and tanning processes that occur in the insect cuticle immediately following moulting. It is suggested (148, 149) that the sterol is secreted via the pore canals to the new exocuticle where some form of interaction between protein, sterol, and phenolic substances takes place (cf review by Dennell, 150). While the chemical evidence upon which these views are based is somewhat tenuous, this aspect of sterol utilization seems to merit more detailed study. It is evident from radiochemical analyses of sterols in the tissues of *E. floridana* nymphs[1] (18) that the sterol content of the cuticle is extremely variable, as might be expected if some such secretory activity, fluctuating with the moulting cycle, were taking place. Sterols may well be common constituents of the surface wax of insect cuticles. In the mormon cricket, *Anabrus simplex*, cholesterol was found to comprise 2–3% of the total cuticular wax (151).

A relationship between cholesterol deprivation and an increased susceptibility of insects to bacterial infection has been noted (2, 48, 14). This relationship has never been subjected to rigorous study and it seems likely that it merely reflects a deterioration and loss of integrity of the tissues of sterol-deprived insects rather than some specific bactericidal role for cholesterol.

SOME COMPARATIVE ASPECTS

The *Insecta* are not the only metazoan class in which sterol biosynthetic mechanisms have been shown to be absent or defective. The edible snail, *Helix aspersa*, probably requires a dietary supply of sterol (152) and a crustacean, the crayfish *Astacus astacus*, has been shown to be incapable of incorporating acetate-1-C^{14} into either squalene or cholesterol (153). Several marine Annelida are apparently able to carry out the complete synthesis of cholesterol, while *Lumbricus*, a terrestrial form, cannot carry the synthesis beyond the squalene stage (154). These observations suggest that many sterols of invertebrates (99), which frequently show structural features characteristic of phytosterols, may be of dietary origin.

Several species of microorganism have been found to require sterols for growth and a considerable amount of work has been done to characterize the types of sterols that are adequate growth factors for different species. A general similarity between the structural specificity of sterols as growth factors for insects and microorganisms has been noted (42, 16). This field has recently been reviewed by Hutner and Holz (155) and will not be considered further except to point out that in one species, the ciliate *Tetrahymena corlissi*, a phenomenon resembling the cholesterol sparing effect in insects was observed. In these experiments (156) the sterol requirement was spared by DL-α-glycerophosphate, oleic acid, and some synthetic phosphatides. The authors drew attention to the similarity between their findings and those of Golberg and DeMeillon (42), who were able to spare the cholesterol requirement of the mosquito, *A. egypti*, with lecithin and cephalin. The results of Golberg and DeMeillon do not appear to have been confirmed, but it is possible that they indicate that in certain subcellular structures in the mosquito, as in *Tetrahymena*, sterols and phospholipids may function interchangeably. Further information on this point would be highly interesting.

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