read as negative (0) or positive (+ to + + + +) as shown in the table.

Results. The Elwax 40 film itself was innocuous and did not incite any tissue reaction, nor induce neovascularization, while implants of films containing ovarian extracts induced neovascularization, characterized by capillary growth in the tissue surrounding the implants (fig.). Angiogenic activities of extracts prepared from the ovaries of untreated, hCG-treated and PMSG-treated mice are shown in the table. The control (untreated) extract elicited neovascularizing reaction in 24–29% of mice while those from hCG-treated and PMSG-treated mice induced a significantly higher percentage (32–60% and 77–85%, respectively). At equivalent dose, PMSG-treated extract had a greater angiogenic activity than extracts of hCG-treated mice.

Since neovascularization may occur in association with inflammation, the tissues were examined for signs of inflammatory reactions. Inflammatory cells were seen in some of the specimens. When the number of macrophages and other inflammatory cells around the film exceeded that observed with plain Elwax film (control), these specimens were recorded and considered as inflammation and not scored as neovascularization. Inflammatory reaction was observed in 2 out of 46 mice, 4 out of 50 mice and 5 out of 53 mice in the experiments with untreated, PMSG-treated, and hCG-treated ovarian extracts, respectively. Hence, inflammatory reaction was observed in 5–10% of the mice implanted with films containing ovarian extract.

Discussion. Capillary proliferation has been shown to be a general feature of actively growing tissue, such as the corpus luteum[4], salivary gland[5], granulation tissue[6] and tumor[7]. Neovascularization can be induced by extracts from tumors[8], by secretions from antigen- and phytohaemagglutin-stimulated lymph node cells[9], and by epidermal and fibroblast growth factors[3]. The present finding that ovarian extract from untreated mice showed angiogenic activity (24–29%, table) suggests that the factor is present in the immature ovary. The greater potency of ovarian extracts prepared from PMSG- and hCG-treated mice, indicate that the angiogenic activity was enhanced by hormone administration.

Although the mechanism of neovascularization is not known, it should be pointed out that this phenomenon is not a consequence of an inflammatory reaction. PMSG used in the present study can induce follicular growth since 5–10 IU of this hormone promotes development of mature follicles in mice[10]. Hence, the follicle stimulating activity and the angiogenic activity may be related. Ovarian extract prepared from hCG-treated mice induced neovascularization, albeit at a higher dose. It is well known that hCG induces luteinization of follicular cells and possesses intrinsic FSH activity[10–12]. These findings taken together suggest that gonadotropins may induce the formation of an angiogenic factor that stimulates proliferation of capillaries from the vascular wreath present in the theca layer and thereby promoting follicular development.

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The identification of ecdysterone (20-hydroxyecdysone) in 3 species of molluscs (Gastropoda: Pulmonata)[1]

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Summary. Ecdysterone has been identified in the schistosomiasis vector *Biomphalaria glabrata* Say and in *Helix aspersa* Müller as well as in *Lymmaea stagnalis* L. by chromatography, bioassay, radioimmunoassay, derivatization and by mass spectroscopy. Analysis of the food, faeces and hepatopancreas suggest that the sterol is derived from the diet. The probable function of ecdysterone in relation to calcification of the shell is discussed in this paper.

The procedure for extracting polar sterols from the bodies of the aquatic basommatophoran pulmonate *B. glabrata* (= *B. australorbis* Say) has been described[3], as has the method for rearing these molluscs. Previously a substance extracted from the same species of snail had been tentatively identified as ecdysone[4] but this finding was later questioned[5]. *Mytilus edulis*, the mussel, had been reported[6] to contain 0.2 pg g^{-1} b.wt of a substance active in the *Calliphora* moulting hormone bioassay. Horn[7], therefore suggested that calcification of the shell might be regulated by ecdysteroids in molluscs since this was demonstrable in the exoskeletons of crustacea[8] and the puparia of *Musca autumalis*[9]. Evidence to support this hypothesis was sought[3,10] before the investigation reported in this communication was completed. The support for the presence of an ecdysteroid in the hepatopancreas of *B. glabrata* and *H. aspersa* fed up to 4 days earlier on lettuce *(Lactuca sativa)* was as follows:

a) *Biomphalaria glabrata* (540 adults, 80.3 g) methanol extract after LC on silica gel[3] yielded a fraction eluted by 10% ethanol in chloroform (fraction S_1) with the following properties: 1. It absorbs in the UV (λ max 242 nm), suggesting a level of ecdysteroid of < 1 µg g^{-1} b.wt; 2. The R_f of the component was identical with ecdysterone; 3. The color reaction was olive with vanillin ethanol-H_2SO_4 spray[7]. 5. It was biologically active in a bioassay using ligatured abdomens of stage III *Sarcophaga peregrina* larvae[11], suggesting a level of 720 ng g^{-1} b.wt of moulting hormone; 5. The

Table 1. Distribution of ecdysteroid-equivalents in *L. stagnalis* as determined at 4 intervals by RIA (for each interval 5 adult animals are used)

Source	$\bar{x}$ ± SEM ng g^{-1} b.wt	06.00 h	12.00 h	19.00 h	24.00 h
Hepatopancreas-gonad mass	560.4 ± 114.8	631.1	251.8	557.2	801.2
Albumen gland	123.7 ± 29.6	56.1	101.7	141.7	195.1
Hemolymph	5.2 ± 0.4	5.3	4.0	5.6	5.8
Rest of body	82.3 ± 20.0	111.9	52.2	43.7	121.5
Total	771.6	804.4	409.7	748.2	1123.7

Table 2. Distribution on TLC plates of ecdysteroid - equivalents determined by RIA which were present in the hepatopancreas - gonad mass (HPG), food and faeces

TLC region*	Rf**	HPG*** ng g^{-1}	Lettuce	Faeces
Less polar	0.67-1.00	158.7	2.5	90.0
Ecdysone	0.58-0.67	34.2	2.8	5.3
Ecdysterone	0.49-0.58	63.2	1.3	1.9
More polar	0-0.49	9.0	2.0	0.6
Total		265.1	8.6	97.8

* Kieselgel $60F_{254}$; ** solvent system - 20% methanol in chloroform; *** 5 masses were extracted.

Table 3. Methanol-extractable labeled compounds derived from 24 *Helix aspersa* injected 52 h earlier with a total of 20.4 µCi 4-^{14}C-cholesterol (in 10% ethanol-saline)

Fraction of methanol extract (see Whitehead[3])	dis min^{-1} Hepatopancreas	Mantle
Petroleum ether 1st	52,967	676,994
2nd	32,589	600,065
Aqueous phase	874	270
n-Butanol phase	20,423	7,091
Rf 0.40-0.60 region of TLC*	3,143	500

* On silica gel using 50% methanol in chloroform.

low resolution electron impact mass spectrum exhibited characteristic peaks[7] at 81, 99, 327, 345, 363, 480 and 426 mass units.

b) *Helix aspersa.* A sterol identical with ecdysterone was extracted from 7.586 g of hepatopancreas obtained from the terrestrial pulmonate stylommatophoran *H. aspersa* fed on lettuce[10] by the procedure described for *B. glabrata*[3]. The *O*-carboxymethyloxime derivative of ecdysterone had been prepared earlier by the method of Borst and O'Connor[12]. 270 µg of the steroid isolated from *H. aspersa* was mixed with 2 µCi of purified ecdysterone ^{3}H(G) (1 Ci $mmole^{-1}$; New England Nuclear, Boston) before reaction with carboxymethoxylamine in pyridine. The main product obtained was identical (λ max 253 nm, yellow with vanillin-H_2SO_4, R_f 0.25) with ecdysterone-6-(-*O*-carboxymethyl) oxime and moreover, the sp. act. (6.5-6.7 Ci $moles^{-1}$) did not vary after repeated purification by column chromatography and TLC in 2 separate solvent systems as described[12]. The extinction coefficient (18,900) was also identical with that of the ecdysterone-6-(-*O*-carboxymethyl) oxime[12].

New evidence was then sought using *Lymmaea stagnalis,* the basommatophoran pond snail. The animal is of special interest because a neurosecretory growth hormone has been isolated from it[13]. The methods used for holding and extracting the various parts of the snail together with the food (lettuce) and faeces, were essentially the same as those described[3,14].

However, a more detailed analysis of the ecdysteroids in the extracts of lettuce, faeces and the hepatopancreas with gonads was achieved using radioimmunoassay (RIA) after separation by TLC on Kieselgel $60F_{254}$. The RIA[12] was performed as described[15] using the antisera H21B[15,16] and DLW[15] with added bovine serum albumen (6 g l^{-1} borate buffer, pH 8.4) which had been checked for unspecific binding of the (23, 24-$^3H_4$) ecdysone (56.1 pg, 0.121 pmoles, 8.24 µCi) used in each assay.

The results are shown in table 1. 73% of the ecdysteroids found in the snail were present in the hepatopancreas - gonad mass (HPG). 24 and 13% of this appeared to be ecdysterone and ecdysone respectively (table 2). The largest proportion (60%) of the ecdysteroid - equivalents was accounted for by unidentified substances less polar than ecdysone itself. These substances were present in smaller proportion (29%) in the lettuce fed to the snails. However, ecdysterone (15%) and ecdysone (33%) were also present, as suggested[17] for other compositae such as *Artemisia dracunnculus* and *Chrysanthemum* species. Therefore, it must be assumed for the present that ecdysone and ecdysterone accumulate in the HPG when *L. stagnalis* are fed on lettuce. As assumed earlier[3,10], excretion of these sterols (table 2) would presumably reduce the pool in the HPG if the diet were withheld or changed to cellulose alone. Injection of 20.4 µCi of 4-^{14}C-cholesterol (53.7 mCi $mmole^{-1}$ sp. act.; Radiochemical Centre, Amersham) through an optic tentacle[10] of 24 *H. aspersa,* followed 52 h later by extraction of the hepatopancreas with methanol and chromatography of the n-butanol-soluble substances in the manner described[3], resulted in only 0.007% of the label (table 3) incorporated into ecdysterone. Therefore, the possibility of ecdysteroids in pulmonates deriving from a source (like cholesterol) in the diet other than from a phytoecdysone is unlikely. Nevertheless, if the pool of free cholesterol in *H. aspersa* is as large as in, say, hematophagous arthropods, then incorporation of the label would in any case have been extremely slow. Indeed tissues of other stylommatophoran pulmonates do contain variable but sometimes high cholesterol levels[18].

The origin and nature of the comparatively large amount of material, less polar than ecdysone, which reacted with DLW and H21B antiserum (table 2) is still a mystery. Further analysis is required. The role of ecdysteroids in pulmonates is not clear[3,10] especially if they are derived solely, as they may be, from phytoecdysones in the diet. The wide fluctuation in levels detected by RIA at noon and at night (or early morning) need to be confirmed. They are not easily explained (table 1) unless it can be shown, say, that light catalyses rearrangement of ecdysterone in the enol form to pentahydroxycholecalciferol or hexahydroxydihydrotachysterol. Chemically the reaction is highly feasible; so too would be the shift of the equilibrium back to the enol in the dark. (A. Hassanali, personal comunication). Nevertheless, the precursors of vitamin D in vertebrates, like ergosterol, 7-dehydrocholesterol, and $\Delta^{5,7,22}$-cholestatrienol, have been reported[18] in molluscs but there is no reason *per se* to suppose that the calcification hormone in

invertebrates should be identical to the vertebrate hormone. In fact use of 25-hydroxy and 1,25-dihydroxycholecalciferol did not promote incorporation of $^{45}Ca^{++}$ into the shell of B. glabrata whereas under the same conditions ecdysterone did[3]. Hemolymph Ca^{++} levels were not affected in *Helix pomatia* by infusion of ecdysterone, c-AMP, dibutyryl c-AMP or an ionophore (A23187. Eli Lilly)[19]. However, ecdysteroids or their metabolites positively promote calcification in crustaceans[8] and even in an insect[9], although the mechanism might possibly be a passive one accompanying promotion of glycoprotein synthesis and growth.

The existence of a growth hormone from the light green cells of *L. stagnalis*[13] does not, we think, preclude that penta- or hexahydroxy forms of vitamin D could be involved (with calcium binding protein) in calcification of the matrix of the exoskeleton of molluscs or crustacea. A glycoprotein isolated by gel filtration from the mucus of *B. glabrata* does indeed bind $^{45}Ca^{++}$ effectively (Whitehead, unpublished report to Ministry of Overseas Development, U.K. (1972)). In view of the economic importance of invertebrates possessing a calcified exoskeleton, greater effort should be made to understand calcification and the role hormones play.

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Inhibition of steroidogenic activity in the adrenal cortex of rats fed benzene hexachloride (hexachlorocyclohexane)

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Summary. Feeding various dosages of benzene hexachloride (100, 250, 750 and 1500 ppm) in the diet to weanling male albino rats for 90 days resulted in marked hypertrophy of the adrenals with large, vacuolated cells in the cortex at 750 and 1500 ppm. Accumulation of cholesterol-positive lipids and marked reduction in the activities of steroidogenic enzymes such as Δ^5 3β HSDH, 11β HSDH, G-6-PDH and SDH were seen using histochemical methods in the adrenal cortex of rats fed 750 and 1500 ppm. The results are suggestive of steroidogenic inhibition at 750 and 1500 ppm of dietary BHC while 100 and 250 ppm did not produce any discernible changes in the adrenal cortex.

Nelson and Woodward[2] were the first to report that chronic administration of o,p'-DDD [1,1-dichloro-2,2-bis(chlorophenyl)ethane], a derivative of the insecticide DDT, causes atrophy of the adrenal cortex of dogs and it was later confirmed by others[3,4]. It was shown that the atrophied gland secretes less than normal levels of corticosteroids in response to ACTH[5,6]. Later studies revealed that o,p'-isomer of DDD inhibited ACTH-induced corticosteroid production by interfering with the ACTH-dependent conversion of cholesterol to pregnenolone in the mitochondria[7-9]. These observations were confirmed by Hart et al.[10], who found degenerative ultrastructural changes in the mitochondria in the inner cortical zones, which were correlated with the inhibition of steroidogenesis in dogs given DDD isomers. This pharmacological property of DDD was exploited for therapeutic use in man to check excessive production of corticosteroids in adrenocortical carcinoma and Cushing's syndrome[11,12].

All these reports deal mainly with the effect of DDD on the dog adrenal, and surprisingly little is known about the effects of other organochlorine pesticides on the adrenal cortex of experimental animals[13]. This is important in view of the fact that the adrenal is one of the principal sites for accumulation of organochlorine pesticide residues[14,15].

Benzene hexachloride (BHC or hexachlorocyclohexane), a widely used organochlorine insecticide, has low acute toxicity and high chronic toxicity[16,17]. Short-term feeding of BHC at various dietary levels is known to produce histopa-