THE EFFECT OF DIETARY CYCLOPROPENE FATTY ACIDS ON THE STRUCTURE OF NEW-LAID AND STORED HENS' EGGS: AN OPTICAL AND ELECTRON MICROSCOPE STUDY

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Summary

Two storage disorders, "pink white" and "pasty yolk" are known to develop in eggs from hens with cyclopropene fatty acids (e.g. malvalic and sterculic acid) in their diet. The pink white condition is related to increased diffusion processes in the egg during storage. The pasty yolk condition is related to an increase in the proportion of saturated to unsaturated fatty acids in the yolk. The change in texture becomes evident during storage at normal temperature, but can be induced quickly in any affected egg, even when new-laid, by low temperature. The present investigations were carried out to see if the development of these defects could be related to any structural differences detectable in eggs from hens fed a cyclopropene compound.

Eggs from White Leghorn hens, fed 14 mg methyl sterculate/kg body weight/day, were examined, when new-laid and after storage at 0 and 20°C for periods up to 70 and 50 days respectively, and compared with those from hens fed a normal diet. The optical and electron microscope observations concentrated on the vitelline membrane and the yolk from these eggs.

No marked structural changes were observed in the vitelline membrane to account for the increased diffusion from the albumen and the yolk. This observation supports existing opinion that normal diffusion in the egg is controlled by the physico-chemical organization of the yolk rather than by resolvable structures in the egg. It is likely that these barriers to diffusion are altered when cyclopropene fatty acids are included in the diet. No consistent structural changes which could be related to the occurrence of the pasty condition were observed in the yolk of affected eggs.

I. INTRODUCTION

Two defects of hens' eggs, "pink white" and "pasty yolk", first observed by Sherwood (1928, 1931) in eggs of hens fed cottonseed meal, are now attributed to the presence of dietary cyclopropene fatty acids (e.g. malvalic and sterculic acid) which are found in plants from the order Malvales (Almquist and Lorenz 1933; Shenstone and Vickery, 1956, 1959; Masson *et al.* 1957). A comprehensive review of biological effects of some derivatives of cyclopropene compounds has been made by Phelps *et al.* (1965).

The pink white defect develops during storage and is associated with increased diffusion from the albumen and the yolk. The yolk enlarges at the expense of the albumen and becomes a pink-orange colour, its pH alters, proteins are absorbed into the yolk from the albumen, and iron migrates from the yolk and reacts with the conalbumin of the albumen to form a pink chelate. The pasty yolk condition in the affected eggs gradually appears during storage at normal temperature, but it can be

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induced quickly in any affected egg, even when new-laid, by exposure to low temperature (Shenstone and Vickery 1959). The yolk then remains spherical when separated from the egg and is hard to the touch. Johnson and Shenstone (1964), Phelps *et al.* (1965), Allen *et al.* (1967), and Johnson *et al.* (1967) attributed this change in form and texture to an increase in the ratio of saturated fatty acids (e.g. stearic acid) to unsaturated fatty acids (e.g. oleic acid).

The purpose of this investigation was to compare the structures of the albumen, the yolk, and the vitelline membrane in new-laid and stored eggs from hens fed a cyclopropenoid compound, e.g. methyl sterculate, with those from eggs of hens fed a normal diet, and to attempt to relate any differences in them to the cause of the pink white or pasty yolk condition. The fine structure of the inner layer of the vitelline membrane developing around the ova in the ovaries of hens fed methyl sterculate was also compared with that in normal hens, to see if any structural differences were present in the vitelline membranes when the yolk was being formed. The fine structure of the liver cells from normal and affected hens was also compared, this organ being concerned with the formation of the yolk.

A background to the structure of the yolk was obtained from such investigators as Riddle (1911), Conrad and Warren (1939), Romanoff and Romanoff (1949), and Shenstone (1968), and to that of the vitelline membrane from Needham (1931), McNally (1943), Doran and Mueller (1961), and Fromm (1964, 1967). These structures have been elucidated further by electron microscopy (Bellairs 1961; Bellairs, Harkness, and Harkness 1963; Bain and Hall 1969; Jensen 1969). The investigations of Press (1964), Bellairs (1965, 1967), Wyburn, Aitken, and Johnston (1965), and Wyburn, Johnston, and Aitken (1965) provided a background to the development of the vitelline membrane in the ovary.

II. Materials and Methods

(*a*) *Source of Material*

(i) *Storage at 20°C*

Eggs from White Leghorn hens kept in individual cages at the Division of Animal Genetics, CSIRO, were used. The hens were fed either a commercial laying ration or this ration plus 14 mg methyl sterculate/kg body weight/day. This dose, administered in gelatine capsules as the urea adduct (Allen *et al.* 1967), was sufficient to cause the pink white and pasty yolk defects. Eggs used were those that were laid 14 days after feeding with methyl sterculate began. Eggs designated as new-laid (0 days) were still warm when preparation for microscopy began. The rest were stored at 20°C and 65–80% R.H., their structure being observed at intervals for up to 50 days.

(ii) *Storage at 0°C*

White Leghorn hens, obtained commercially and housed in individual cages in the Division of Food Preservation, CSIRO, were either fed normal rations or methyl sterculate as described above. The eggs from these hens were then stored at 0°C, the structure of the yolk being examined at intervals for up to 70 days.

(*b*) *Microscopy*

(i) *Structure of the Albumen, the Yolk, and the Vitelline Membrane*

(1) *Optical Microscopy.*—The shells of normal and affected eggs were broken and the albumen was separated from the yolk and the vitelline membrane as described previously

(Bain and Hall 1969). The yolk was then placed with the blastodisc uppermost (Fig. 1). The small amount of yolk in the latebra, distinguished from the rest of the yolk by its white colour and more fluid nature, was removed with a Pasteur pipette. The vitelline membrane was then cut to let the rest of the yolk flow out gently. Samples of this yolk were then removed with a microspatula. This method caused less damage to the structure of the yellow yolk than when a pipette was used to pierce the vitelline membrane and remove the samples. Areas of yolk showing least disturbance after very gentle application of a coverslip were photographed at a magnification of $\times 130$. The vitelline membrane was separated from the yolk and examined as described previously (Bain and Hall 1969).

(2) *Electron Microscopy.*—Smears of yellow and white yolk, removed as above, and whole yolks from which the albumen had been carefully removed, so that the vitelline membrane was *in situ*, were fixed in 1% OsO_4 or 2% $KMnO_4$ for 2·5 and 3·5 hr respectively. Both solutions were buffered with veronal acetate (pH 7·3). A crust formed over the material in the early stages of fixation; small pieces of crust were cut away from the smears and from the surface of the

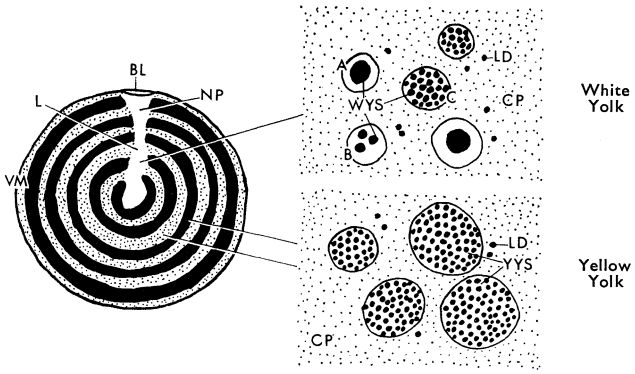


Fig. 1.—Structure of the yolk of the hen's egg. The vitelline membrane (*VM*) separates the yolk from the albumen. The latebra (*L*) contains white yolk and opens into the nucleus of Pander (*NP*) beneath the blastodisc (*BL*); the rest of the yolk is yellow yolk. Yellow yolk spheres (*YYS*), white yolk spheres (WYS), lipid drops (*LD*), and very small granules are found in the continuous phase (*CP*). Three types of white yolk spheres (*A*, *B*, and *C*) are identified.

whole yolk after about 30 min and placed in fresh fixative. Similar samples to those above were placed in 2·5 or 6·0% glutaraldehyde in phosphate buffer (pH 6·8) overnight and post-fixed in the 1% OsO_4 fixative described above. All samples were stained for 1 hr in 2% uranyl acetate after fixation was completed, washed, dehydrated in an alcohol series, embedded in Araldite, sectioned, and examined at 80 kV in a Siemens Elmiskop 1 electron microscope. Better contrast was obtained from material stained with uranyl acetate during preparation than from stained sectioned material. Lead citrate (Reynolds 1963), applied to sections, increased the contrast formed with uranyl acetate.

Isolated vitelline membranes were prepared for examination as described by Bain and Hall (1969).

(ii) *Development of the Vitelline Membrane in the Ovary*

The ovaries of normal hens and those from hens fed methyl sterculate were removed. Each of these was made up of globular structures (follicle plus ovum) ranging from 2 to 36 mm in diameter; the largest ova were ready to be set free from the follicle cells. Series of these globular structures, graded for size, were placed whole into 1% OsO_4 or 2% $KMnO_4$, buffered with veronal acetate (pH 7·3), for 0·5 hr. Small pieces of follicle tissue and underlying ovum were then cut away from the whole structure and placed in fresh fixative for another 2·5 hr. They were then prepared for electron microscopy as above.

(iii) *Fractionated Egg Yolk Sample*

When yolk from an egg of a hen fed methyl sterculate (storage at 20°C) was centrifuged for 0·5 hr at 20,000 r.p.m. (50,000 g) in an angle rotor, a small quantity of material separated out and remained stuck to the sides of the upper end of the tube. This material, approximately 20 mg of it in 5 c.c. of yolk and found only in affected eggs, was fixed in 1% OsO_4 buffered with veronal acetate (pH 7·3) for 4 hr, stained with 1% phosphotungstic acid in 70% ethanol, embedded in Araldite, sectioned, and examined in the electron microscope. Some thicker sections were cut from these Araldite blocks, stained with Sudan III and examined by optical microscopy.

(iv) *Liver Tissue*

Pieces of tissue, approximately 1 mm^3, were cut from livers of hens fed methyl sterculate as above, fixed overnight in 6% glutaraldehyde in phosphate buffer (pH 6·8), post-fixed in 1% OsO_4 for 3·5 hr, stained with 2% uranyl acetate (1 hr), and embedded in Araldite for sectioning. Their fine structure was compared with that of liver tissue taken from a normal hen and prepared in the same way.

III. Results and Discussion

The nature of the yolk material presented problems in examination and photomicrography. Its opacity made observation difficult and the spheres changed shape as the material streamed. Most of the spheres broke when (1) water was added to dilute the material for easier observation, (2) when an aqueous stain, e.g. methylene blue, was added to increase contrast, or (3) when a glass coverslip was placed too heavily on the sample.

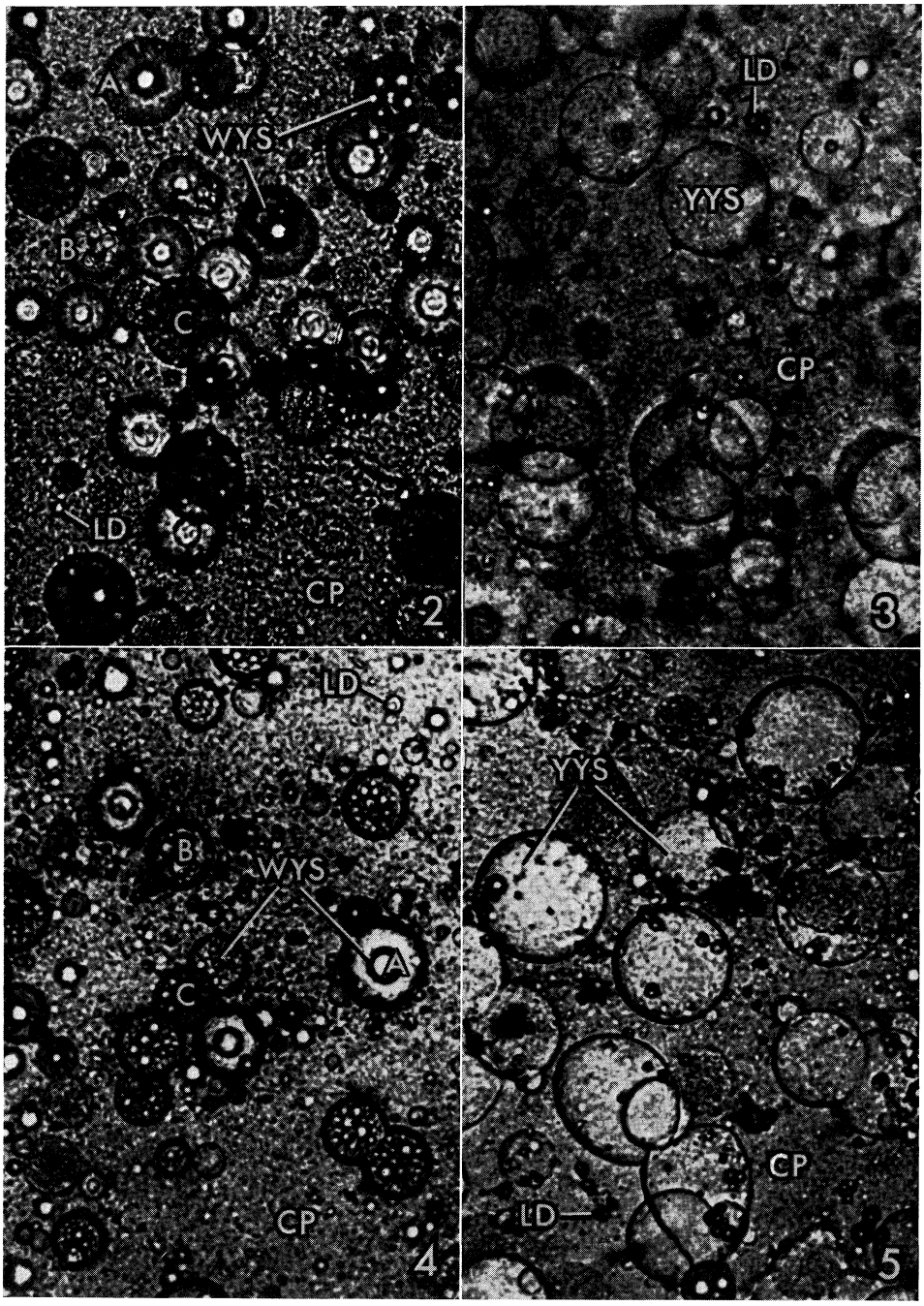
Preparation for electron microscopy also presented problems. Very few yolk spheres were observed in material fixed as smears, most of them apparently breaking in the aqueous fixative; better results were obtained in material taken from yolks which were fixed whole, although only a small proportion of this material was successfully fixed. Osmium tetroxide and potassium permanganate penetrated only the first 1–2 mm; when the yolk was placed in glutaraldehyde overnight only the first 4 mm were fixed. The size of the yolk spheres [4–150 μm diameter according to Romanoff and Romanoff (1949)] made their observation difficult by electron microscopy. It was possible only to examine the smallest of them, a diameter of 4 μm becoming 4 cm at a comparatively low magnification of $\times$10,000. Problems of examination are indicated in Figure 6.

Problems also existed in the interpretation of the structure of the yolk. Two different types of yolk, distinguishable by their colour and by the structure of the yolk spheres in the continuous phase (Figs. 2 and 3), have long been known to occur in the hen's egg. It has generally been accepted that the yolk is made up of concentric and alternate layers of yellow and white yolk laid down around the elongated latebra (see Riddle 1911; Romanoff and Romanoff 1949; and Bellairs 1961); that the latebra, opening into the nucleus of Pander beneath the blastodisc of the unfertilized egg, also contains white yolk; and that the layers of white yolk are deposited during

Fig. 3.—Normal yellow yolk, new-laid. Yolk spheres (*YYS*) with countless subdroplets in them float in the continuous phase (*CP*) of the yolk. Lipid drops (*LD*) and small granules are also present. $\times$260.

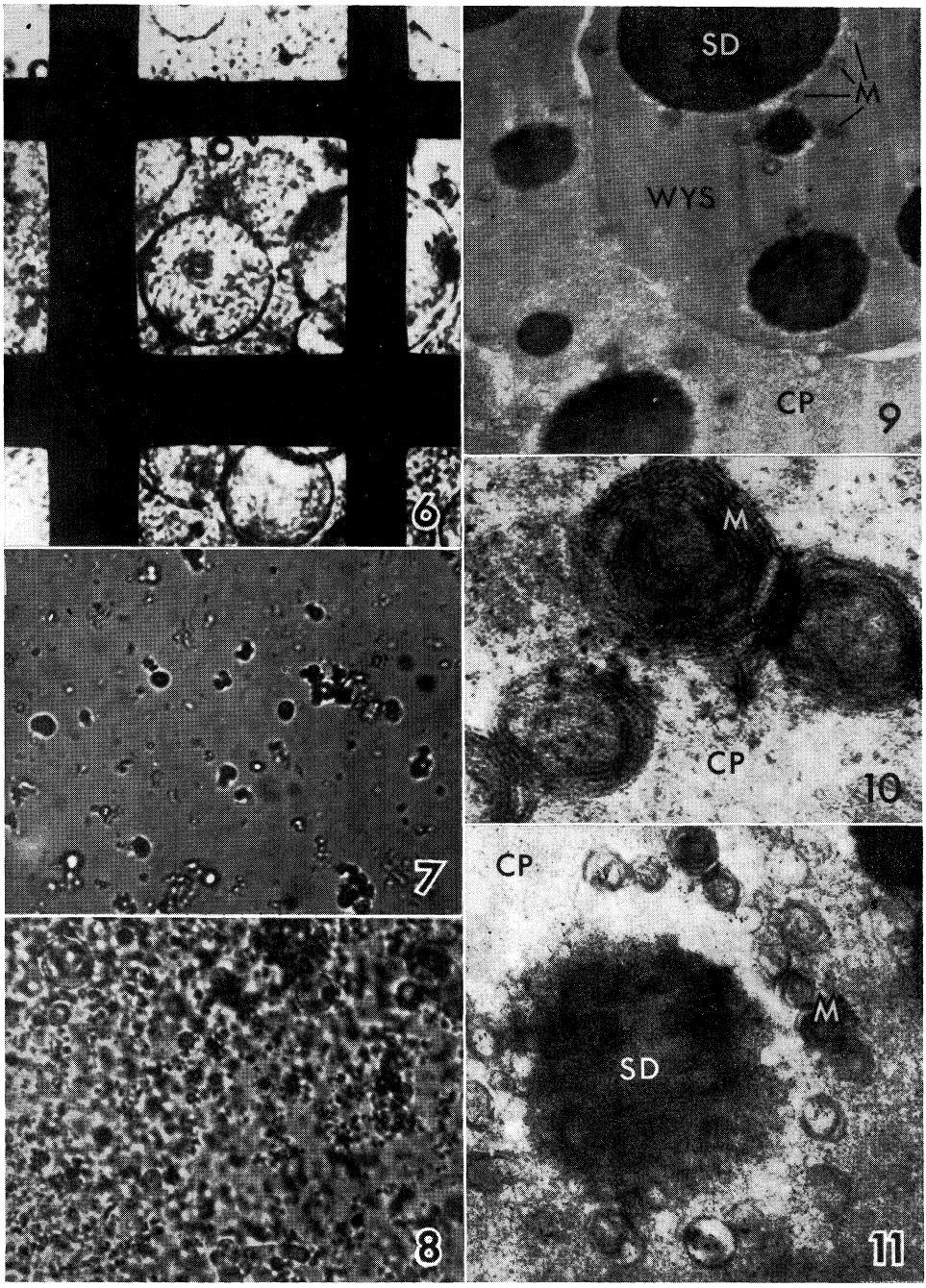
Fig. 4.—Affected white yolk, new-laid. Structure comparable with that in Figure 2. $\times$260.

Fig. 5.—Affected yellow yolk, new-laid. Structure comparable with that in Figure 3. $\times$260.



Figs. 2–5.—Optical micrographs of unstained material.

Fig. 2.—Normal white yolk, new-laid. Three types (*A*, *B*, and *C*) of yolk spheres (*WYS*) are conspicuous in the continuous phase (*CP*). Lipid drops (*LD*) and small granules are also present. ×260.



Figs. 6–8.—Optical micrographs of unstained material.

Fig. 6.—Normal yellow yolk. An optical micrograph of part of a specimen grid has been superimposed on an optical micrograph of yellow yolk (same magnification) to indicate the difficulties associated with the electron microscopy of this material. The small amount of material which can be photographed on a plate ($6 \cdot 5 \times 9 \cdot 0$ cm) in the microscope at a magnification of $\times 5{,}000$ is judged if the present magnification is increased $12 \cdot 5$ times. $\times 390$.

the early hours of the morning after the hen has metabolized the food eaten the previous day. Such a structure was believed to account for the stratification observed after a yolk has been hard boiled. Other investigators [e.g. Balbiani 1879 (cited by Marza and Marza 1935); Conrad and Warren 1939; and Shenstone 1968] have doubted that the yolk is made up of alternating layers of structurally different yolk. They maintain that the visible banding is probably caused not by structurally different layers but by layers with a different degree of pigmentation, and that the bulk of the yolk is yellow yolk with the white yolk confined to the latebra. Present observations support the latter interpretation of yolk structure (Fig. 1). Although the internal organization of the yolk was disturbed as soon as the vitelline membrane was ruptured, a small amount of white material could always be distinguished in it. This material (Fig. 2), identified as coming from the latebra, always had the typical structure of white yolk as reported by Riddle (1911), Romanoff and Romanoff (1949), and Bellairs (1961). Samples from the rest of the yolk were always typical of yellow yolk (Fig. 3). The yolk spheres in the bulk of the yolk of a hard-boiled egg, though somewhat deformed by processing, were recognizable as coming from yellow yolk; the white yolk in the latebra was still liquid in this egg and the spheres were recognizable as coming from white yolk. The distribution of yolk spheres could not be examined in frozen material because they broke at low temperature.

Observations with the electron microscope confirmed the above interpretation. Diagrams of the morphology of the yolk have always represented the outer layer as white yolk continuous with that of the latebra; electron micrographs of yolk fixed with the vitelline membrane *in situ* indicated that yellow yolk was outermost, except in the vicinity of the nucleus of Pander, where white yolk was indicated.

(*a*) *Storage at 20°C*

(i) *Changes in the Albumen and Yolk*

The time taken for the pink white condition to appear varied even in eggs from the same hen, although the pasty yolk condition could be induced any time on exposure to low temperature. In some instances the pink white condition was discernible a few days after laying, these eggs also showing a tendency to pasty yolk development; an occasional egg was pasty when new-laid. This early appearance of the defects is believed to have been related to the genetic characteristics of some of the hens, which had a tendency to be internal layers; this perhaps could account for the

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Figs. 7 and 8.—Affected albumen, stored 30 and 40 days respectively. No trace of pink had developed and the yolks appeared normal. ×260.

Figs. 9–11.—Electron micrographs of material stained with uranyl acetate before embedding, and with lead citrate after sectioning.

Fig. 9.—Normal white yolk, new-laid. Myelinic figures (*M*) associated with the subdroplets (*SD*) of the yolk spheres (*WYS*) and the subdroplets of the continuous phase (*CP*) of the yolk. Glutaraldehyde + OsO_4. ×18,000.

Fig. 10.—Normal white yolk, stored 2 days. Myelinic figures (*M*) in the continuous phase (*CP*) of the yolk. Glutaraldehyde + OsO_4. ×180,000.

Fig. 11.—Normal white yolk, new-laid. Myelinic forms appeared to develop from the subdroplets (*SD*) in the continuous phase (*CP*). Glutaraldehyde + OsO_4. ×45,000.

apparent increased effect of the cyclopropenoid compound in some eggs. The contents of the affected egg appeared normal in some instances for at least 40 days, while others stored for 50 days were severely affected. It was difficult to keep the pink albumen and the orange-pink yolk separate in these eggs. The fragile vitelline membrane broke as the shell was broken and the contents mixed and formed a pink solution. Most of the albumen disappeared with further storage and the yolk became gelatinous.

Little structure was observed in the albumen of new-laid normal or affected eggs. Marked changes occurred in that of affected eggs during storage. Small inclusions appeared in the albumen before the pink colour became discernible (Fig. 7). They increased in size and number and became massed together during further storage (Fig. 8). No such change occurred in the normal albumen.

The structures of the white and yellow yolk were similar in new-laid normal or affected eggs (Figs. 2–5). Yolk spheres were conspicuous in the continuous phase; small refractive droplets and smaller granules also floated in this phase. Yellow yolk spheres had a uniform structure, each sphere containing a large number of subdroplets with a refractive index similar to that of the continuous phase. These spheres were very susceptible to breaking, especially when from affected eggs, and varied in size [25–150 μm according to Romanoff and Romanoff (1949)]. Pairs of spheres were observed in close contact, indicating that it was possible for them to merge. White yolk spheres were smaller than those in the yellow yolk [4–75 μm according to Romanoff and Romanoff (1949)] and varied in structure. Three types of sphere (*A*, *B*, and *C*) were recognized in the white yolk (Fig. 1), each type occurring in approximately equal proportions. Type *A* contained one large refractive subdroplet; type *B* contained several refractive subdroplets, distinguishable as individual structures; type *C* contained an indistinguishable number of subdroplets. These three types were also found in the white yolk from eggs of the bantam, the guinea fowl, the turkey, and the emu, the size of the spheres tending to increase with the size of the bird; white yolk from the duck egg contained only type *C* spheres.

There was no marked alteration in the form of the normal yellow or white spheres during storage, but they showed increasing susceptibility to breakage on handling, especially the yellow spheres; the degree of disorganization varied within a sample of yolk. The yellow yolk spheres often appeared larger than in new-laid yolk, probably due to the merging of two spheres. In the white yolk, type *A* spheres were least susceptible to breaking, the proportion of type *B* and *C* spheres becoming increasingly

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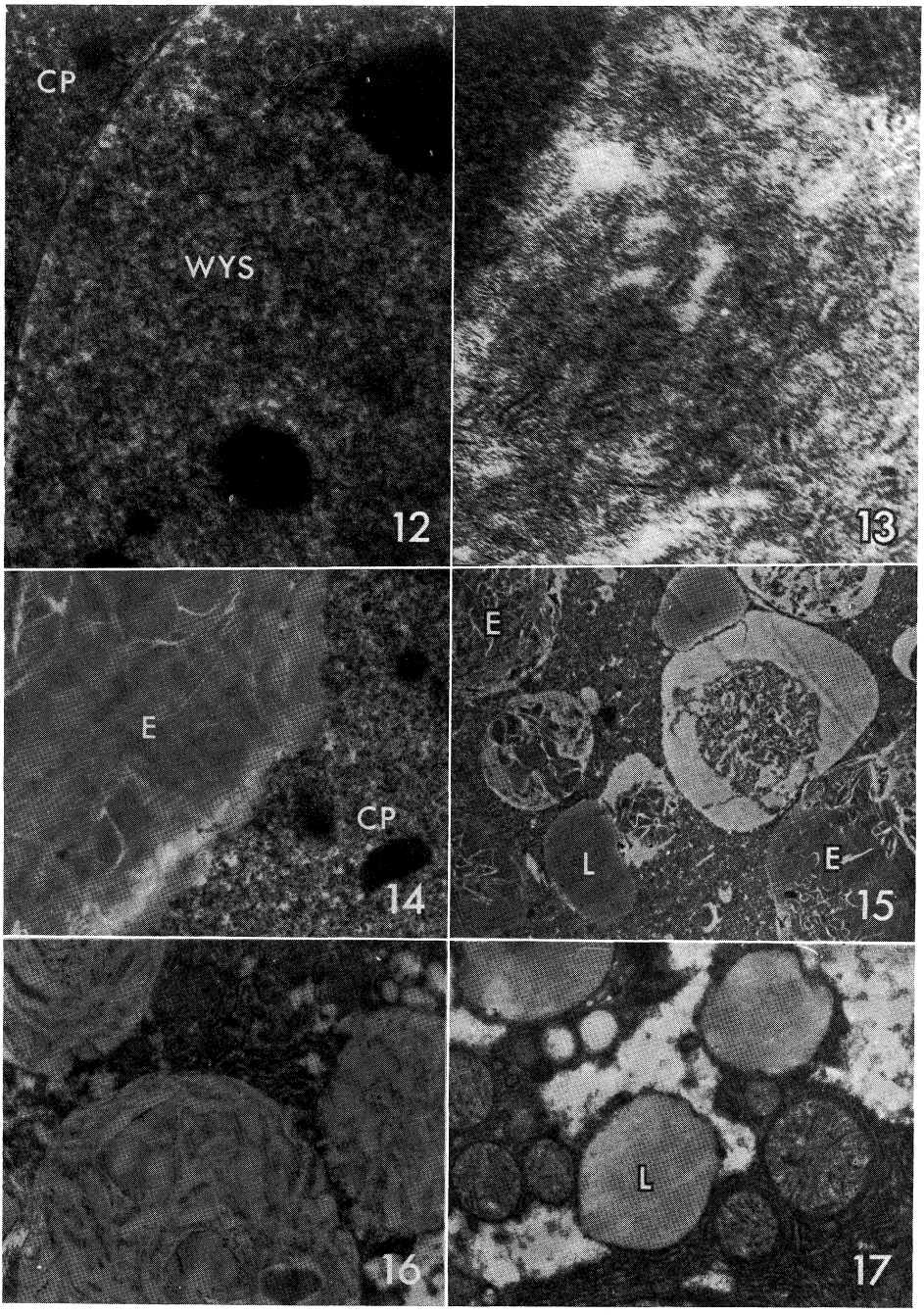
Fig. 13.—Affected white yolk, stored 5 days. Greater detail of the elongated particles in the continuous phase of the yolk. OsO_4. ×120,000.

Fig. 14.—Affected yellow yolk, new-laid. Deposit of "etched" material (*E*) found in the continuous phase (*CP*) of the yolk. Glutaraldehyde + OsO_4. ×14,000.

Fig. 15.—Affected yolk, new-laid. Two types of material were observed in the fraction on top of a yolk after centrifugation. The first was identified as lipid (*L*). The second had an "etched" appearance (*E*) and was similar to the material shown in Figure 14. OsO_4. ×4,500.

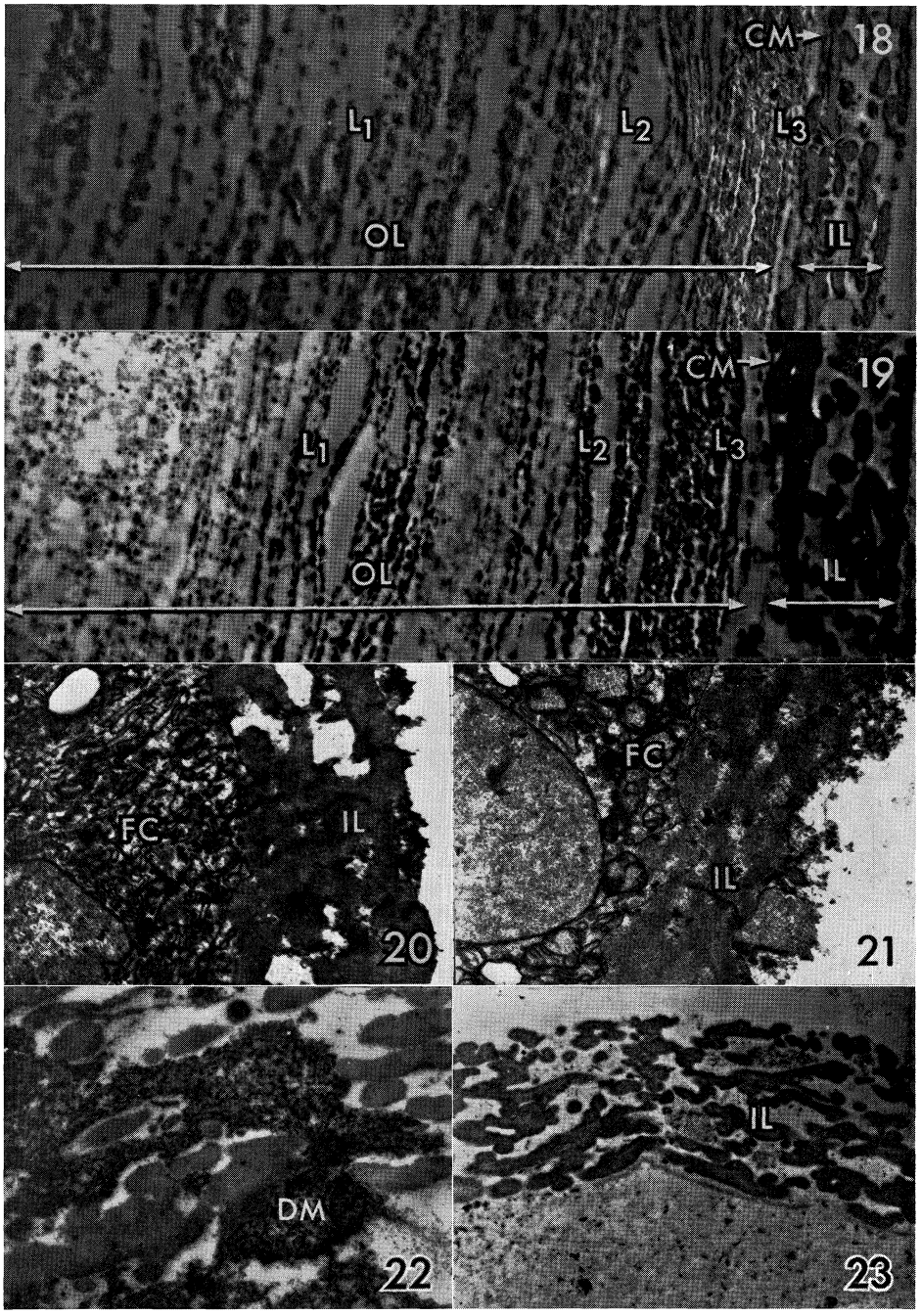
Fig. 16.—Affected liver tissue. The "etched" form of storage material (see Fig. 14) was frequent in this tissue. Glutaraldehyde + OsO_4. ×14,000.

Fig. 17.—Normal liver tissue. Only deposits of lipid material (*L*) were found in this tissue. Glutaraldehyde + OsO_4. ×14,000.



Figs. 12–17.—Electron micrographs of material treated as in Figures 9–11.

Fig. 12.—Affected white yolk, stored 1 day. Elongated particles in fluid phase of a yolk sphere (*WYS*) and in the continuous phase (*CP*). OsO_4. ×30,000.



Figs. 18–23.—Electron micrographs of material treated as in Figures 9–11.

Fig. 18.—Normal vitelline membrane, new-laid. Transverse section. The inner layer (*IL*), adjacent to the yolk, was separated from the outer layer (*OL*), adjacent to the albumen, by the "continuous membrane" (*CM*). The outer layer was divided into three layers. (L_1) marks the

less with time; large refractive drops accumulated in the continuous phase of the white yolk as the number of whole spheres decreased. The structure of the yolk of the affected egg appeared similar to that of a normal egg, even when the albumen was slightly pink. Later the yolk spheres disappeared with increased severity of the disorder.

The fine structures of the yellow and white yolk in new-laid normal and affected eggs were comparable and similar to that described by Bellairs (1961). The structural composition of the yolk is difficult to relate to its chemical composition [48·7% water, 32·6% lipid, 16·6% protein, and 1·0% carbohydrate according to Romanoff and Romanoff (1949)]. Using Sudan III, Bellairs (1961) identified the subdroplets of the spheres and droplets in the continuous phase as lipid material, and assumed that the non-staining continuous phase and the fluid phase of the yolk spheres consisted largely of proteins (or possibly lipoproteins) in aqueous solution. Sudan black gave a similar staining reaction in the present investigation but, in addition, very small non-staining granules were observed in the continuous phase. Although histological evidence indicated that much of the structure of the yolk was made up of fat material, electron micrographs did not indicate that the subdroplets were made up of lipid (see Fig. 9), only an occasional deposit resembling lipid in cells of other animal tissue being present in the continuous phase. Chemical evidence indicates that no free fat exists in the yolk (Burley and Cook 1961; Burley 1969) and that the subdroplets and droplets previously regarded as lipid are likely to be lipoprotein, with the granules of the continuous phase possibly protein plus some lipid.

The more extensive use of osmium tetroxide and glutaraldehyde fixation has yielded additional information concerning yolk structure. In normal eggs, myelinic figures were prominent features in both the yolk sphere fluid and the continuous phase in smears of new-laid yellow and white yolk after fixation in glutaraldehyde and post-staining in osmium tetroxide (Fig. 9); they were also observed after fixation with osmium tetroxide alone, but to a lesser degree. The components of the figures were concentric, each consisting of a central light area (*c.* 7·0 nm across) bounded by two dark lines (Fig. 10); the figures were closely associated with the subdroplets of the continuous phase and with those of the yolk spheres (Fig. 11). Grodziński (1946) noted myelin forms surrounding yolk spheres when the osmotic pressure was increased rapidly in the yolk suspension, and he attributed these to the hydration of phospholipids

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limit of the chalaziferous layer and the underlying second layer. (L_2) marks the boundary between the second and third layers and (L_3) indicates the boundary between the inner layer and the entire outer layer. OsO_4. ×5,400.

Fig. 19.—Affected vitelline membrane, new-laid. Transverse section. Structure comparable with that shown in Figure 18. ×5,400.

Fig. 20.—Normal vitelline membrane in ovary of hen. Transverse section of an ovum ready to be liberated from the ovary. The inner layer of the vitelline membrane (*IL*) is surrounded by the follicle cells (*FC*) on its outer side. $KMnO_4$. ×6,000.

Fig. 21.—Affected vitelline membrane in ovary of hen. Structure similar to Figure 20. ×7,000.

Fig. 22.—Affected vitelline membrane, stored 1 day. Electron-dense material (*DM*) enmeshed in spaces of the inner layer of the vitelline membrane. Transverse section. OsO_4. ×15,000.

Fig. 23.—Affected vitelline membrane, stored 50 days. The outer layer of the vitelline membrane has been lost, only the inner layer (*IL*) remaining. Transverse section. OsO_4. ×5,000.

present in the supposed semipermeable membranes surrounding the yolk spheres. Revel, Ito, and Fawcett (1958) produced myelinic figures (repeating unit 5·0–6·0 nm) *in vitro*, by hydrating phospholipid extract of hens' eggs; structures produced were strikingly similar to cellular membranes, although no protein was present. Stoeckenius (1959) hydrated brain phospholipid, then added globin, and obtained myelinic figures with a repeating unit of 5·0–6·0 nm. Such results suggest that the myelinic figures found in the present work could be caused by hydration of phospholipids in the yolk and that they are not an integral part of its structure. Myelinic figures were not apparent in yolk of affected eggs.

Only two structural differences from normal yolk were observed in the affected yolk. In some instances there was a distinct change in the shape of the particles in the fluid phase of the spheres and in the continuous phase of the yolk. Instead of the particles being rounded (Bellairs 1961), they were elongated, their length being approximately three times their width (Figs. 12 and 13). This condition, observed in new-laid and stored eggs, was not evident in all eggs from hens fed methyl sterculate nor was it a uniform feature of the yolk. Large deposits of material were occasionally located in sections of the yolk from affected eggs. These deposits had an "etched" appearance (Fig. 14) and were distinct from other occasional deposits of dense material, presumably lipid, which were found in normal and affected eggs. It is not known whether this etched pattern was normal or whether it was produced in the material by the solvents used during the embedding process. These two types of globular deposits were observed also in the sample separated by centrifugation (Fig. 15). Under the optical microscope, the lipid-type droplet appeared red and the etched droplet mauve, when stained with Sudan III.

Examination of the liver tissue taken from hens fed methyl sterculate showed marked increase in the amount of material deposited in the cells (Fig. 16). Most of the material was similar to the etched droplets found in the yolk of the affected eggs, only a small porportion of it resembling the lipid form found in normal liver cells (Fig. 17). Further investigation would be needed to determine whether the occurrence of such reserves could be related to the pasty yolk defect.

(ii) *Structure of the Vitelline Membrane*

The vitelline membrane is relatively strong when removed from the new-laid egg and can be separated into four, sometimes five, layers. It has been long regarded as the permeability barrier between the yolk and the albumen, but an increasing number of investigations concerned with the physical properties of the yolk and the vitelline membrane tend to dispute this view (see Shenstone 1968). Electron micrographs show that the vitelline membrane is an open meshwork of fibres and fibrils, often with large particles enmeshed in it (Bellairs, Harkness, and Harkness 1963; Bain and Hall 1969; Jensen 1969), and it is difficult to see how it could be a real permeability barrier unless the "continuous membrane" (50–100 nm) separating the inner and outer layers of the vitelline membrane was a semipermeable membrane (see Fig. 18). Transverse sections of the vitelline membrane show that the width of the continuous membrane varies considerably; several instances were observed where this layer appeared broken. Iordonov, Georgiev, and Boyadzhieva-Mikhailova (1966) investigated the permeability of the vitelline membrane to proteins and lipoproteins

from the egg and estimated that it was permeable, in both directions, to particles having diameters $\leqslant$ 30–50 nm.

Initially it was thought that some of the increased permeability found in the affected eggs might be related to obvious structural differences in the vitelline membrane of the new-laid normal and affected eggs. All four layers were present to an equal extent in the normal and affected eggs and their total width was comparable (Figs. 18 and 19). Increased permeability in the affected eggs did not appear to be related to the structure of the continuous membrane; this membrane appeared thicker, in several instances, rather than weaker as might be supposed. No differences were observed in the structures of the inner layer of the vitelline membranes of ova about to be liberated from the ovaries of normal and affected hens (Figs. 20 and 21). Increased amounts of extraneous matter were found in the framework of the vitelline membranes from affected eggs, especially the inner layer (Fig. 22), possibly indicating greater movement of material in these eggs. Deposits of lipid-like material were found on one occasion between the inner and outer layers. An etched deposit similar to those deposits found in the yolk, the yolk fraction, and the liver cells was observed also in this same position.

During storage the vitelline membrane became progressively difficult to handle, being easily broken when separating the albumen from the yolk, especially in the affected eggs. Such morphological changes can be related to structural alteration observed in the vitelline membrane. All electron micrographs showed decreasing thickness of the outer layer of the vitelline membrane with time, the loss being greatest in the affected eggs, only the inner layer remaining in these eggs (Fig. 23) at the end of storage. No obvious changes occurred in this tougher, narrower, inner layer adjacent to the yolk. The delicate nature of the outer layer of the vitelline membrane makes comparative measurements of thickness difficult, the fibrils of the lattice work of the outer layer breaking away easily during the process of separation or embedding, but it was concluded that material was lost during storage rather than during preparation of the samples, as a considerable number of samples were involved in this investigation.

(*b*) *Storage at 0°C*

Low temperature delayed the appearance of the pink white defect in affected eggs. The albumen was tinged with pink after 46 days' storage; the yolks were then a normal colour. The pasty yolk defect was apparent in all eggs after several hours at 0°C. Optical or electron microscopy gave little information on the structure of normal or affected yolk at this temperature. Yellow yolk structure was disorganized in all these eggs, the majority of the yolk spheres being broken; the white yolk spheres in the latebra were unaffected by low temperature. An occasional deposit of dense material, similar to the etched deposits observed in the yolk of affected eggs at 20°C, occurred in electron micrographs of the yellow yolk.

(*c*) *Cause of Pink White and Pasty Yolk Defects*

Present results have shown no constant structural changes in the vitelline membrane which could be related to accelerated diffusion and the resulting pink white condition in eggs of hens fed methyl sterculate; the quicker dissipation of the outer

layer of the vitelline membrane in these eggs was considered to be related to the accelerated changes in the pH of the albumen which are a feature of the physiology of eggs of hens fed cyclopropenoid compounds.

It is not certain whether the alteration of the shape of the particles in the matrix of the continuous phase and that of the yolk spheres (elongated rather than rounded in section) could be associated with the development of the pasty yolk, when this change was observed only intermittently. The occasional etched deposits observed in the sections of the yolk and similar deposits in the centrifuged fraction from affected yolk were associated, however, with the presence of methyl sterculate in the diet. The great abundance of this additional material in the livers of hens fed the cyclopropene compound supported this conclusion, but it was not possible to relate it to the cause of the pasty yolk defect.

Stearic acid accumulates in tissues of hens fed cyclopropene compounds (Johnson and Shenstone 1964; Phelps *et al.* 1965; Allen *et al.* 1967; Johnson *et al.* 1967), the enzyme system which desaturates stearic acid to oleic acid in normal hens being inhibited (Allen *et al.* 1967; Johnson *et al.* 1967). It is possible that this accumulation of stearic acid may have been associated with the occurrence of the etched droplets observed in electron micrographs of tissue and eggs from affected hens.

Previous investigators (e.g. Maurice 1952; Maurice and Fidanza 1952, 1954; Shenstone 1968, 1969) have suggested that the barrier to diffusion between the albumen and the yolk exists in the physicochemical organization of the yolk rather than in a resolvable structure such as the vitelline membrane. Maurice (1952) showed that the vitelline membrane had a high electrical conductivity, that the conductivity of the disturbed yolk was high, and that the conductivity of undisturbed yolk was low. Shenstone (1968, 1969) suggested that water diffusion into the yolk could depend on a lower level of hydration in the yolk proteins or on a molecular transfer either within protein structures or through lipid bodies or through layers of yolk. It has been suggested that the cyclopropene compounds affect these unresolvable barriers to diffusion within the yolk, causing the increased diffusion in the affected egg (Shenstone 1968, 1969). It has also been suggested that the diffusion process in such eggs may be influenced by the increase in stearic acid mentioned above, the normal control to diffusion being disturbed at the yolk surface or in the yolk itself (Allen *et al.* 1967).

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