The utilization of yolk platelets by tissues of *Xenopus* embryos studied by a safranin staining method

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WITH ONE PLATE

The amphibian yolk platelet is a particular kind of food-reserve granule which may be easily recognized by microscopy and which is abundant in the cytoplasm of amphibian eggs and embryos. Wallace & Karasaki (1963) developed a method by which intact yolk platelets were isolated from eggs of *Rana pipiens* and were shown by electron microscopy to be practically free from other materials. Chemical analysis of such yolk platelets by Wallace (1963a, b) showed that the crystalline main body is made up of two components, a phosphoprotein of similar amino-acid composition to avian phosvitin and a lipoprotein similar to avian \( \alpha \)-lipovitellin, the molecular proportions being 2 to 1 respectively. Surrounding this crystalline main body of the yolk platelet there is a granular peripheral zone which has been reported to contain both protein resembling histone (Horn, 1962) and polysaccharide (Ohno, Karasaki & Takata, 1964). Histochemical work by Ohno, Karasaki & Takata (1964) showed there was no nucleic acid detectable in yolk platelets, and although nucleic acids have often been reported present in isolated yolk fractions subjected to biochemical analysis, it seems probable in view of the experience of Wallace (1963a) that such reports were due to contamination either by follicle cells or cytoplasm. The ultrastructure of the amphibian yolk platelet has been described by Karasaki (1962, 1963a), Ward (1962) and Lanzavecchia (1965). Wallace (1963b) proposed a molecular structure for the main body component of a yolk platelet which appears to fit all known data from previous biochemical, biophysical and electron-optical analyses.

Yolk platelets are formed during the last stages of oogenesis when the oocyte is already quite large, and when reserves of glycogen and lipid have already accumulated (Panijel, 1951; Grant, 1953). The isotopic and serological data of Flickinger & Rounds (1956) support the idea that the yolk proteins are synthesized in the liver of the maternal organism and are then transported to the ovarian ¹

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eggs by way of the blood-stream. Balinsky & Devis (1963) have studied the ultrastructures associated with yolk formation in oocytes in *Xenopus*.

After consideration of biochemical work in which various workers had attempted to draw up a metabolic balance sheet for embryonic development, Barth & Barth (1954) were able to suggest that whereas glycogen and lipid reserves are oxidized to release energy for development, the protein reserves in yolk platelets on the other hand are degraded to amino acids or peptides which are then used by the differentiating cells in the synthesis of structural and other proteins characteristic of the cell type. Studies with amino-acid analogues by Waddington & Perry (1958) and Feldman & Waddington (1955) suggested that the degradation is to amino acids rather than peptides. Glycogen was known to be consumed during and after gastrulation and lipids beginning immediately before hatching; but yolk platelets were not thought to begin their disappearance until after the hatching period was over. These biochemical methods were not suitable for the detection of the early stages in the utilization process where the number of food-storage granules utilized is but a very small proportion of the total number in the embryo.

More recently it has been shown that when amphibian yolk platelets are utilized by the cell, they undergo certain well-marked changes in ultrastructure that have been studied by electron microscopy by Karasaki (1959, 1963b), Sung (1962), Jurand & Selman (1964) and Lanzavecchia (1965). Unfortunately developmental studies made by electron microscopy demand considerably more time than do similar studies made by light microscopy so that at present the appearances of typical structures associated with yolk utilization are known only for a few tissues from any of the species which have been studied. Moreover only for the case of the ventral ectoderm from *Rana pipiens* and *Triturus pyrrhogaster*, studied by Karasaki (1963b), have there been published any quantitative data for the proportion of the yolk platelets being utilized within a tissue at particular stages.

The present paper describes work done by light microscopy using a method which makes it easy to distinguish yolk platelets in the cell which are being utilized from yolk platelets which are not being utilized, by virtue of their different staining properties. Such a method has not been previously described. Indeed it was thought that yolk platelets examined by light microscopy remained in much the same condition until their disappearance at the larval stage (e.g. see the introductory remarks of Karasaki, 1963b). The present paper will describe the yolk utilization pattern in twelve different tissues of *Xenopus laevis* and will give quantitative data based on counted yolk platelets. This paper is not concerned with other food-reserve granules or droplets such as lipochondria or glycogen granules.

**MATERIAL AND TECHNIQUE**

Aquarium specimens of *Xenopus laevis* were induced to lay by injections of gonadotropin. Fertilized eggs were allowed to develop at room temperature in
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mains tap-water. Most of the slides analysed in the main experiment were from embryos derived from a single ovulation. The gelatinous layer surrounding the eggs or embryos was removed with fine forceps in Holtfreter's saline. The vitelline membrane was also removed from eggs and embryos at later stages than neurula. The vitelline membrane was not removed from gastrula stages because it was desired to avoid distortion of the embryo and any loss of yolk-platelets from the yolk-laden cells.

Ovaries were obtained from mature females 6 or 8 weeks after oviposition, when they were presumed to have recovered from the effects of gonadotropin. Mature toads were anaesthetized with MS-222 and decapitated. Portions of ovary were removed by abdominal incision in air.

Specimens were immediately transferred to Smith's fixative, prepared according to Rugh (1948), and after 24 hr. fixation they were washed in running tap-water for a further 24 hr. The specimens were dehydrated in an ascending series of ethyl alcohols to 95 per cent. alcohol, washed briefly in absolute ethyl alcohol, cleared in terpineol, embedded in paraffin wax and serial sections were cut at 7 μ or 10 μ. The slides were immersed in xylene to remove the wax, washed in absolute alcohol, taken through 94 per cent. and 70 per cent. alcohol and then stained for 2 days in a solution which consisted of 3 per cent. red safranin dissolved in 50 per cent. ethyl alcohol. Slides were then drained of excess stain, rinsed briefly in 70 per cent. alcohol, stained for 30 sec. in a solution of 1 per cent. Fast Green FCF in 95 per cent. ethyl alcohol, dipped in 95 per cent. alcohol and then transferred to two changes of absolute alcohol for 5 min. each, cleared in xylene and mounted in Canada Balsam.

The standard staining procedure described above was used to obtain the histological results to be described. Many modifications of the method were also tested. A longer period in the fast green solution gives a more intense green stain which may subsequently be reduced in 95 per cent. alcohol before transfer to absolute alcohol in which the staining is almost stable. After the slides have been dewaxed, they may be taken down through graded alcohols to water and then given a standard hydrolysis and Feulgen staining (e.g. as in Darlington & La Cour, 1960) before the standard safranin and fast green procedure. This modification gives improved nuclear staining and the subsequent staining for yolk is unaffected. Many modifications of safranin staining were tried, including staining at acid or alkaline pH, at different alcohol concentrations and after a 4 per cent. aqueous solution of ferric ammonium sulphate as mordant for 2 hr. None of these modifications gave more intense safranin staining. The standard procedure was tried using a number of different batches of red safranin from different sources, including some labelled Safranin O and Safranin WS. The safranin staining for yolk was of varying intensity from faint to intense but there was no variation in stain-specificity and no sample gave negative staining for yolk. It is not possible to recommend the best source of safranin since the best results were from a sample labelled only as safranin with the retailer's name.
Conn (1946) comments on the variability of safranin and the difficulties of standardizing safranin. We were unable to distinguish between the good and poor safranin by paper chromatography with 4:1:5::butanol:acetic-acid:water as solvent.

Tests for the staining-affinities of safranin and fast-green were made by allowing certain known substances to dry out from solution so as to form a dry patch adhering to a clean glass microscope slide. The slide was then placed in Smith's fixative and then processed and stained exactly as in the standard staining schedule, except that the slides were not mounted but were examined after dehydration in absolute alcohol. For comparison the same substances were also similarly stained after fixation in acetic alcohol (3 alcohol:1 acetic acid).

The stage numbers used in the present work are those for the normal development of *Xenopus* described by Nieuwkoop & Faber (1956). This work describes the internal morphology and the development of the tissues and organs but does not illustrate it. The present authors have also consulted the illustrated accounts of internal anuran development given by Huettner (1949), Kerr (1919), Kamel & Ramadan (1960) and Mahmoud (1957). Provisional stage numbers were assigned to embryos at the time of fixation and these were carefully checked by consideration of internal criteria when the serial sections were examined.

Yolk platelets were classified according to their staining affinities and counted under the oil-immersion objective of the microscope for all tissues except notochord for which a dry objective of one-sixth inch focal length was mainly used. The counts were made with the aid of a 5-key laboratory counter, operated with one hand, while the other hand adjusted the microscope and the eyes concentrated continuously on the field of view.

**Observations**

Examination of the slides of serially sectioned *Xenopus* embryos showed that in the largest oocytes, in mature eggs and in embryos before gastrulation, the yolk platelets were all of a roughly similar ovoid shape (except for a low proportion of nearly spherical ones) and with similar staining properties. They showed a range of size from a maximum diameter of about 12 μ down to about 1 μ, the larger yolk platelets being concentrated towards the vegetative hemisphere of the egg. The bulk of each yolk platelet was stained a deep rose pink with safranin. At the outer surface of most yolk platelets there was a zone of material stained deeply with fast green.

The larger platelets could be examined in optical section under an oil-immersion lens, so that the three dimensional disposition of the stained areas could be observed. The green material often took the form of two cap-shaped regions on opposite surfaces of the platelet at opposite ends of a minor axis (Text-fig. 1). Thus there was a very close correspondence between the homogeneous and crystalline main-body component of the yolk platelet described by
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electron microscopy and the safranin-positive zone. Similarly the fast-green staining zone corresponded to the irregularly-packed granular zone of electron microscopy.

In less than 2 per cent. of the yolk platelets a thin layer of the green material also extended between the cap-like regions so as to divide the safranin-positive central zone in two, and such yolk platelets therefore had a 'twinned' appearance (see Text-fig. 1). These twinned yolk platelets have been found in the largest oocytes, and in eggs as well as in various developmental stages and the frequency of their occurrence has not been correlated with any particular developmental stage of any tissue.

**TEXT-FIG. 1.** The forms which normal yolk platelets may assume when observed by microscopy at stages before their utilization. The commonest form is that shown in the top left-hand corner. The double or twinned forms are relatively rare. Those parts of the drawings shown in black represent material which stained with fast-green. The interior (unshaded) zones were safranin-positive. The dotted line in one case represents a line of fast green material at a different focal plane.

The first change in the staining affinities of the yolk platelets was seen within certain endodermal cells lining the archenteron during neural closure Stages 14 to 20. Ten embryos were serially-sectioned and examined at these stages and all showed these changes. All or very nearly all the yolk platelets of the affected cells showed an increased affinity for fast-green, so that the change could be noticed easily at low magnification (Plate-fig. D). A considerable proportion of the cells lining the archenteron was affected but never those along the dorsal midline. At the sides of the archenteron, the affected cells formed a layer not more than a single cell in depth. On the ventral surface of the archenteron the affected cells often formed a layer two cells in depth. In those cells least-affected the yolk-platelets were still predominantly safranin-positive, but each safranin-positive zone was crossed by several green stripes which ran in the same direction as the longest axis of the platelet (Plate-fig. C). On closer examination the stripes were found to consist mostly of sheets but occasionally of rods of material.
stained with fast-green. Sometimes the sheets or rods did not extend across the whole length of the yolk platelet but only part way from the two pointed tips of the yolk platelet into its interior. In those cells affected to a greater extent by the changes, the yolk platelets were entirely green and no safranin-positive regions could be seen. In many such cases the stripes could still be seen as darker green lines crossing the lighter green of each yolk platelet. The degree of change in staining-affinity of a yolk platelet was characteristic of the cell to which it belonged and all yolk platelets within the cell were affected to the same extent. One frequently observed a cell whose yolk was mainly safranin-positive but with green stripes, bordered on one side by a cell all of whose yolk was stained normally and on the opposite side by a cell all of whose yolk was green. In this way cell boundaries between endoderm cells were unusually easy to follow.

In a subsidiary study embryos between Stages 14 to 20 were cut into two hemispherical halves through the archenteron and the halves were fixed immediately. The staining pattern in this case was similar to that observed with whole embryos; so the possibility is excluded that the staining observed for cells lining the

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**EXPLANATION OF PLATE**

All photographs are from material fixed in Smith’s fixative; and stained with safranin (red) followed by fast-green. In figs. A and B nuclei were stained with a standard Feulgen procedure before staining with safranin and fast green. Figures A to G are from sectioned *Xenopus* embryos.

**FIG. A.** A normal medium-sized oocyte at the stage when small yolk platelets begin to appear in the cytoplasm bordering the follicle cells. Within the germinal vesicle note the nucleoli, some stained with safranin and others with fast-green. Magnification ×190.

**FIG. B.** Follicular atresia within a large oocyte. Phagocytes have invaded the cytoplasm and yolk platelets are being absorbed. The phagocyte nuclei are Feulgen-positive. Yolk platelets being absorbed are stained with fast-green; others are safranin-positive. Magnification ×320.

**FIG. C.** Endoderm cells just ventrally to the archenteron of a stage 21 neurula. Cells with yolk platelets safranin-stained but with green stripes are in the centre of the field. Cells with all-green yolk, to the left, are at a more advanced stage of yolk absorption. Cells to the right of the field have normal safranin-positive yolk. Magnification ×380.

**FIG. D.** A low-power view of an oblique section through a Stage 21 neurula to show the band of green-stained endoderm cells lining the archenteron. In these cells all yolk platelets have stained with fast-green. Magnification ×100.

**FIG. E.** Tissue from the most anterior loop of the gut of a stage 40 embryo to show yolk platelets stained with safranin, and others, in the course of utilization, stained with fast-green or stained brown or in intermediate stages. Magnification ×380.

**FIG. F.** As for Figure E. Magnification ×320.

**FIG. G.** Part of a transverse section through the trunk region of a stage 40 embryo to show differentiated muscle cells from myotome with yolk platelets not utilized or in various stages of yolk utilization. Magnification ×450.

**FIG. H.** A single cell, tissue cultured for 2 days from neural ectoderm tissue of *Rana pipiens*. Individual yolk platelets are stained with fast-green. Others are safranin-positive. The dots are pigment granules. The nucleus stains with fast-green. Magnification ×800.
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archenteron might be partly due to poor penetration of fixative. In unfixed halved embryos at these stages it may be significant that certain cells lining the archenteron appear darker than others.

At Stages 20 to 29 degenerating yolky cells with green-stained yolk platelets were found inside the lumen of the archenteron or gut. At later stages, green-staining or brownish amorphous material was also observed in the lumen of the gut. These observations made on endoderm cells between Stages 14 and 29 are all consistent with the idea that certain of such cells degenerate into the archenteron during this period.

In all other tissues the earliest observable change in the yolk-platelets is the loss of the green cap-shaped regions from edges of the safranin-positive body of the platelet. In myotome and notochord, the platelets all retain their green borders up to Stage 20, but they nearly all are absent at Stage 23 and they were never observed at Stage 25 or later. In ectodermal tissues the yolk platelets are smaller so that only the largest platelets may be observed to have the normal green borders which they certainly retain up to Stage 19. In no case were green borders observed in platelets within ectoderm after Stage 25, and most of the green borders had been lost from the larger platelets before Stage 23. In the endodermal cells which will form the tissues of the gut, the green borders seem all to be retained to Stage 34. By Stage 40, in endodermal tissues, most platelets are without green borders and green borders were never observed at Stage 45 or later.

In the case of all tissues, the disappearance of the normal green-staining border to the yolk platelets immediately preceded the first signs of cell differentiation. At earlier stages the tissues were recognizable by virtue of the positions they occupied within the embryo. For the case of myotome tissue, Stage 23 when the green borders have just been lost is also the stage when cell differentiation is first noticeable by virtue of changes in cell shape. At Stage 25 the first cross-striations of muscle fibres were seen in a few muscle cells. Stage 25 was also the earliest stage at which further signs of yolk utilization were observed in one instance involving only a few cells. Further signs of yolk utilization involved less than 1 per cent. of yolk platelets in the muscle tissue even as late as Stage 35, after which yolk utilization increased rapidly as more and more stripy muscle developed in more muscle cells. However the yolk utilization within differentiating cells involved a different pattern of change in the staining affinity of yolk-platelets, and this pattern will now be described.

At the earliest stage of cell differentiation the yolk platelets stained entirely red with safranin. At a later stage the outer edge of some platelets stained with fast green, so that there was an even green band round the pink interior. Other yolk platelets, at a presumed later stage of yolk utilization, had broader green borders and correspondingly less of a pink interior. Between the green and red staining zones there was a ring of unstained yolk which appeared slightly yellowish so that the change from green to pink appeared gradual. In other yolk
platelets the safranin staining had been entirely replaced by fast-green staining and the yolk platelet was stained a strong clear and uniform green. At later stages of differentiation some of the green-stained yolk platelets were bounded by a brown envelope or shell. There was a sharp demarcation between the green and brown parts of a platelet. (It is not certain how the brown colouration arises but it may be produced by the fixative.) Some yolk platelets have been observed in optical section to have a red centre surrounded by a green anulus which in turn is bounded by a brown envelope. At late stages of differentiation when only a few yolk platelets remain, a high proportion of these are brown with a green interior or are entirely brown.

During cell differentiation only one, two or three yolk platelets within a particular cell may show staining changes associated with yolk utilization while the other yolk platelets are all safranin-positive. There appears to be no coordination between the progress of yolk utilization for one yolk platelet and for any other platelet. This is in sharp contrast to the yolk utilization process described previously for the endoderm cells round the archenteron. In the differentiating tissues studied there was no evidence of cell degeneration.

Within any tissue during differentiation, yolk platelets are to be seen at all stages of yolk utilization as revealed by the changes in staining affinity. Nearly all the staining patterns observed may be fitted into a single series (illustrated in Text-fig. 2), and it is significant that this series is very similar to that used by Jurand & Selman (1964) to interpret the yolk utilization in notochord of newt as studied by electron microscopy, provided the assumptions are made that the safranin-positive zone corresponds to the crystalline core whose ultrastructure was interpreted by Wallace (1963), that the green-staining zone corresponds to the irregularly-packed granular zone observed by electron microscopists and that the brown zone corresponds to the yolk envelope consisting of multiple layers of lamellae (Jurand & Selman, 1964). The remainder of the staining patterns observed in yolk platelets from differentiating tissues all showed green bands precisely similar to those observed in the endodermal cells lining the archenteron at Stages 14 to 20. In differentiating cells the banded pattern was normally observed only in a few randomly scattered yolk platelets however and such platelets were usually surrounded in the same cell by other safranin-positive platelets not being utilized or perhaps by a few others showing different staining patterns associated with yolk utilization. In nearly all differentiating tissues at all stages, the striped yolk patterns were but a small proportion of all the yolk-utilization staining patterns, but they do represent an alternative pathway in yolk utilization (see Text-fig. 2). The only exception was found at the last observed stage (Stage 46) round the alimentary canal where a majority of the utilization patterns were striped.

The staining patterns associated with yolk platelet utilization are distinctive and contrast so well with the normal staining of yolk that when sections of tissue are rapidly scanned at a microscope magnification between 100 and 400 diameters,
those tissues in which yolk utilization is taking place may be immediately noted when less than 1 per cent. yolk platelets are involved in utilization. The staining method is therefore of particular value for the identification of the stage at which the yolk utilization process begins.

In order to be able to describe the progress and rate of yolk utilization at particular stages in a more quantitative manner, counts were made of the numbers of yolk platelets showing particular staining patterns. Graphs were

\[Yolk \text{ platelets in successive stages of utilization}\]

(i) As observed by electron microscopy:

(ii) As observed by light microscopy:

\[\text{TEXT-FIG. 2. In the case of yolk utilization observed by light microscopy, the extent of each zone within a yolk platelet which stained red, green or brown is shown with a distinctive style of shading.}\]

plotted for each tissue to show, at each stage, the percentage of normal safranin-positive yolk platelets, the percentage that were green (i.e. fast-green positive to show utilization) and the percentage that were predominantly brown. For the case of notochord the graph (Text-fig. 3) shows that the percentage of the predominantly brown plus the green platelets rises as the percentage of red platelets falls, but that whereas the percentage of brown platelets is greatest at the latest stage plotted (when the platelets per cell are few), the percentage of green platelets is greatest at an earlier stage. These observations were characteristic of all tissues, and they support the interpretation that has been put upon the changes in staining affinity of the platelets.

Detailed observations and counts of yolk platelets during the stages of
utilization were made for epidermis, neural ectoderm, myotome, myocardium, parachordal cartilages, pronephros, notochord, pharynx, oesophagus, liver and intestine. The percentage of yolk platelets showing staining patterns associated with utilization was plotted against stage number for each tissue (Text-fig. 4). For greater realism, the scale of the abcissa is hours of development at 23° C. (from Nieuwkoop & Faber, 1956). Data for all these tissues appears on the same graph to facilitate comparisons between tissues. The yolk utilization for the whole embryo was also expressed diagrammatically (Text-fig. 5).

There are several advantages inherent in expressing the numerical results in the present manner so that the platelets showing staining patterns associated with utilization are expressed as a percentage of the total number of platelets within the volume of tissue that was examined. Firstly such results do not depend critically upon the differences that may exist between the same tissue sectioned and stained on different slides, and so reproducible figures were obtained for similar tissues on different slides. Secondly the results of the yolk utilization observed by electron microscopy by Karasaki (1963b) in ventral ectoderm were expressed in a similar manner and direct comparison may be made. On the other hand it would be interesting to obtain reliable figures proportional to the average amount of yolk utilization taking place per cell. With this in mind counts of platelets were made using an oil-immersion objective for two tissues, notochord and myotome, while observing the numbers of cell nuclei included
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in the same volume of tissue under examination. For each stage ratios were evaluated for the average number of platelets observed to be in utilization per cell and for the total number of yolk platelets observed per cell. These ratios were considered inaccurate particularly in the case of myotome. They appeared to depend upon a number of factors such as the plane of the section-cutting, the shape of the cells at the different developmental stages and small differences in staining intensity between slides. All these factors affect the size of the smallest

Text-fig. 4. For a number of tissues, the graphs show the total percentage of yolk platelets observed to be undergoing utilization at known stages. A yolk platelet showing any fast-green or brown staining was judged as being utilized. Abbreviations: Cart., parachordal cartilage. Proneph., pronephric tubules. N. Ect., neural ectoderm. Ant. intest., anterior loop of the intestine. Oes., oesophagus.

yolk platelets whose staining affinities may be clearly observed, and upon this, all estimates of numbers of platelets per cell are dependent. On the other hand the percentages are scarcely dependent at all upon such considerations, since the visibility of all classes of yolk platelet of whatever staining affinity is almost equally affected by the conditions of observation. In order to assist the interpretation of such results it is of interest to note that for the case of notochord the average number of yolk platelets of observable size per cell was about 26 at Stages 23 and 25, before utilization had affected their numbers; it was about 22 at Stage 28, about 19 at Stage 29, about 8 at Stages 33 and 34 and about 2 at Stage 40. The corresponding estimate for the average number of platelets in utilization per cell was 0 at Stages 23 and 25; it varied between 0·2 and 2·0 between Stages 28 and 34, while individual cells were commonly found to contain any number between 0 and 6 platelets in utilization; at Stage 40 the
ratio was 1.5. In myotome between Stages 28 and 45 the average number of yolk platelets observed per cell appeared to fall from 12 to 2, while the corresponding average number of yolk platelets in utilization per cell appeared to increase steadily from 0 to 2.0. The boundaries between adjacent cells could be clearly seen in the sections so long as the boundary did not lie parallel or nearly parallel to the plate of sectioning.

In the case of a number of tissues including the neural ectoderm, myotome and notochord, the observable signs of development proceed in an anterior to posterior direction, and in these cases the pattern of yolk utilization was also observed to take place with a time-lag for the more posterior regions. The

TEXT-FIG. 5. A diagrammatic chart to show the utilization pattern of yolk platelets for tissues of *Xenopus*. The duration and roughly the rate of utilization is indicated by the areas of cross-hatching to either side of each line of development. This line terminates at the latest stage for which safranin-positive yolk platelets may still be found. The arrows indicate the stage of disappearance of the fast-green positive cap-shaped border of platelets. Abbreviations: G., gastrula stages. N., neurula stages. T.B., tail-bud stages. H., period of hatching.
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Numerical data which has been presented for these tissues refers to the mid-trunk region of the young larva at a level roughly midway between the anterior tip of the notochord and the anus. For the notochord, utilization is first complete in this region when the cells have become greatly vacuolated and the sheath has thickened. Meanwhile unutilized yolk may still be found in the notochord of the tail of the larva where the cells are still at a less advanced stage. To a lesser extent, yolk platelets also persist at the extreme anterior tip of the notochord. At stage 29, for notochord of the mid-trunk region there appeared to be many more smaller yolk platelets than were noticeable at later stages, which may indicate that smaller platelets are utilized more quickly.

Observations on yolk platelets are rather difficult for the epidermis. Not only are the platelets themselves small but the tissue is pigmented and becomes increasingly flattened. Counts made on the platelets apparently show that the yolk of the epithelium in the tail region is utilized slightly ahead of the yolk in the mid-region. The yolk utilization pattern for the caudal epidermis was very similar to that plotted for notochord in Text-fig. 3. In the case of the cephalic epidermis and the cement gland (sometimes known as the sucker) which is derived from it, rather a low proportion of yolk-platelet utilization figures were observed, and these were the only tissues for which the present staining method might therefore be regarded as less than satisfactory. For the cephalic epidermis the maximum proportion of yolk utilization figures was observed at Stage 33 and amounted to 7 per cent. of the yolk present. For the cement gland a maximum of 15 per cent. of yolk platelets in utilization was observed for Stage 34. For both tissues there was practically no yolk to be seen at Stage 40.

Most cartilage in the head region is laid down after all yolk platelets have disappeared. The parachordal cartilages on the other hand are well developed by Stage 45, which is before all the yolk platelets have been utilized.

At Stage 40 an impressive display of clear yolk utilization patterns was observed in 27 per cent. of the large platelets within the large anterior loop of the intestine in transverse sections cut at the level of the liver and slightly posterior to it (Plate-figs. E and F). At this stage the intestine is in process of formation. Whereas formerly the gut had consisted merely of a canal through a mass of irregularly shaped yolk-laden cells, the tissue now condenses to form a cylindrical tube of large columnar cells and the tube coils as it lengthens. This process takes place in an anterior to posterior direction down the alimentary canal and there is a wave of yolk utilization within the intestine while the changes in cell shape take place. At Stage 40, in transverse sections cut at a level posterior to the region of transition less than 8 per cent. of the yolk platelets show utilization patterns. At Stage 45 these cell changes are complete and there is a minimum in yolk utilization; only 4 per cent. of the platelets in the intestine are being utilized. At this stage however a few degenerating cells with green yolk were observed in the lumen, together with some brown amorphous material. At Stage 46 however all of several sectioned larvae showed increased yolk utilization in the intestinal
cells, and it is likely that this was the final phase of yolk utilization. By this stage the intestine was the only tissue within which yolk could be found and the larvae were of course feeding. At Stage 46 the staining patterns of the platelets in utilization were mostly striped and this was in sharp contrast to their low proportion at all earlier stages, if the utilization in the cells lining the archenteron between Stages 14 and 22 is excepted.

When the *Xenopus* ovaries were examined in stained serial sections it was found that a proportion of the oocytes were in the process of degeneration or follicular atresia (Brambell, 1956). The degeneration may occur for any size of oocyte and when the oocyte is large the yolk is reabsorbed. It is characteristic that the thin layer of follicular epithelium which normally bounds the oocyte is replaced by a broad zone of loosely packed cells of similar shape to fibroblasts which appear to spread inwards and aid the reabsorption by phagacytosis. In follicular atresia the yolk platelets, before they are absorbed, pass through similar staining changes to those observed during embryonic development (Plate-fig. B). The safranin-positive main body component is always replaced by fast green positive material before it is absorbed.

When amphibian embryonic cells are cultured in vitro while they spread in thin layers on a supporting cover-glass, as in the work of Jones & Elsdale (1963), their differentiation is known to be accompanied by utilization of the yolk platelets. This can be observed by noting an obvious diminution in the number of platelets over a time-interval. Some apparent splitting of the larger yolk platelets may also be seen by phase-contrast microscopy. When such cultures of differentiating cells, kindly provided by Drs Jones & Elsdale, were fixed and stained on their coverslips in an identical manner to the sectioned embryonic material, the results were similar. Both the normal safranin-positive platelets and the fast-green positive yolk utilization stages were observed (Plate-fig. H). It was clear that the number of yolk platelets shown to be in utilization by the staining method was several times greater than might have been inferred by observations of yolk fragmentation made with phase-contrast.

Safranin is a basic dye and fast green is an acid dye. Tests were made in an attempt to understand the mechanism of the safranin staining of yolk platelets. It seems essential to use a fixative containing anionic chromium. When fixation in Smith’s fixative was replaced by fixation in acetic alcohol, no safranin staining of yolk platelets was obtained. However when slides, after fixation in acetic alcohol, were soaked in potassium dichromate solution and the usual staining procedure was followed, then safranin positive yolk was obtained once more. It would seem that the Smith’s fixative chromes as well as fixes the yolk material and the acetic alcohol does not extract the Safranin-positive material in yolk platelets.

When staining tests were made with known substances dried on slides and then treated with Smith’s fixative, safranin staining was obtained for several phospholipids and very faintly for starch and ribose nucleic acid, while the
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proteins albumin, globulin, collagen and fibrinogen only took up fast-green. When the tests were repeated using acetic alcohol in place of Smith’s fixative, only cephalin was safranin-positive. However none of these test substances stained with safranin after acetic alcohol fixation followed by treatment with potassium dichromate. No phosphoproteins have been successfully tested. A sample of phosvitin from chicken yolk was found to be too soluble for this kind of test.

A number of extraction tests with proteolytic enzymes were made by our colleague Dr Kato on the embryo sections themselves before staining—all with negative results. However after Smith’s fixative, which contains anionic chromium as well as formalin, the tests themselves may be of dubious value. In another test after acetic-alcohol fixation, extraction with hot trichloracetic acid (Taft, 1951) was followed by treatment with potassium dichromate and safranin-positive yolk was still observed, so that nucleic acids can at least be excluded from involvement in the staining of the normal platelet. Hot ether extraction, after Smith’s fixation, did not affect the safranin staining of yolk.

From a consideration of all the observations, and the known composition of the main body component of amphibian yolk platelets (Wallace, 1963b), it seems possible that the phosphate groups of the phosphoprotein bind the safranin in the present staining method. It has been shown that other substances with phosphate groups bind safranin but there is no direct evidence for the involvement of the groups in the case of yolk. The phosphate groups are also considered by Flickinger (1960) to be involved in the process of yolk solubilization.

Safranin was not found bound to many other parts of cells than yolk platelets. The exceptions may be worth noting. The cytoplasm of the outermost ends of the long cells of the cement gland stained pink with safranin. Normally cytoplasm was either colourless or stained weakly with fast green. Chromosomes sometimes stained with safranin. Often chromosomes stained more strongly with fast green.

Some nucleoli stained strongly with safranin. At a stage before yolk synthesis, some of the many nucleoli in the germinal vesicle stained strongly with safranin and others within the same section stained strongly with fast green (Plate-fig. A). In the germinal vesicle of large oocytes containing yolk platelets, there were large safranin-positive nucleoli within which there were zones containing material which stained strongly with fast green. These nucleoli closely resembled those from invertebrate oocytes photographed and published in colour by Bolognari (1961). When the staining was made after extraction with hot trichloracetic acid, a larger number of nucleoli stained with fast green rather than with safranin, especially for the case of the smaller oocytes. This seems to confirm the conclusion of Brown & Ris (1959) that the nucleoli contain widely varying proportions of ribose nucleic acid, but the fact that some nucleoli remain safranin-positive after extraction may mean that there is also a variable proportion of some other safranin-positive constituent. In differentiated cells, nucleoli stained either with safranin or fast green. In muscle cells from myotome, nuclei have been seen with two large nucleoli, one stained green and the other stained red.
DISCUSSION

Other light microscopists have observed structure within amphibian yolk platelets. For instance the difference in staining-affinity between the material in the border and in the main-body of the platelet has been studied by Di Berardino (1954) and Ohno, Karasaki & Takata (1963). Holtfreter (1946) showed that when yolk platelets undergo intracellular digestion they may be split into parallel discs or rods and that this splitting could also be obtained in vitro when yolk was exposed to weak acids. The present study of yolk utilization with the light microscope is believed to be the first to give results that may be compared to those obtained by electron microscopy. The two methods are complementary. Greater detail is readily obtained by electron microscopy but light microscopy is quicker and often more convenient.

Save for two exceptional cases, the intracellular utilization of yolk platelets has been observed to take place entirely during a period of progressive cell differentiation at stages before the larva can feed. This is not surprising since cells in differentiation synthesize proteins, characteristic for the tissue, from amino acids supplied by the breakdown of yolk platelets. The utilization of the main-body component of some yolk platelets was observed to begin at a stage just after the first signs of differentiation, but the disappearance of the borders of the yolk platelets took place just before the first signs of differentiation. Cell differentiation in these tissues then could be dependant upon the ability of the cells to break down their yolk platelets. The work of Jones & Perry (1964) seems to support this idea.

The exceptional cases both concern endoderm cells. There is no obvious reason why endoderm cells should need so much more yolk than the other tissues. The yolk-laden cells round the archenteron which are broken down and absorbed between Stages 14 and 29 may play a rôle in development which is now obscure. Glucksmann (1951), in a review of cell death in normal vertebrate ontogeny, refers to the reported degeneration of some yolk endoderm cells during gastrulation (Vogt, 1913) and hints at a metabolic rôle for the broken-down yolk, but Deuchar (1963) emphasizes that at these early stages the break-down products could only serve the needs of adjacent endoderm cells. The other exceptional case concerns the considerable amount of intracellular yolk utilization which takes place in the cells of the intestine at about Stage 40. This period of yolk utilization accompanies the rearrangement of cells and the changes in cell shape that occur as the endodermal epithelium is formed. These changes resemble the cell movements which occur in other tissues during gastrulation or immediately afterwards but it is usual to refer to these as morphogenesis rather than as differentiation and they were not found to be accompanied by yolk utilization in the present work. It may be noted that Dorris (1935), who studied the developing gut in Amblystoma showed that the enzyme amylase first appeared at Harrison Stage 40 and the proteolytic enzymes pepsin and trypsin at Harrison Stage 43.
Wallace (1963) measured the changes in nucleolar volume in five differentiating tissues in *Xenopus* embryos. Nucleolar growth occurred during early differentiation up to the functional stage. For somite tissue in which the increase was greatest, nucleolar growth commenced at Stage 20; it was greatest between Stages 25 and 32 and maximum nucleolar size was reached at about Stage 40, after which the nucleolus became smaller. It appears that for myotome between Stages 25 and 45 the number of yolk platelets being utilized within the tissue is roughly proportional to the average nucleolar size. The situation in the pronephros and neural ectoderm appears to be similar. Of relevance here is the demonstration of Denis (1964) that the maximum control exerted by the nucleus upon protein synthesis in *Pleurodeles* is reached immediately after neurulation.

Cytological and biochemical studies have been made by Wallace (1962) using anucleolate larvae of *Xenopus*. Larvae without nucleoli show a lesser degree of differentiation and a lesser degree of yolk utilization when comparisons are made with normal larvae at the same stage of development (confirmed by Wallace in a personal communication). This is clearly illustrated by the microphotographs of Wallace (1962) for lens, somitic tissue and pronephric tubules. The tissues of anucleolate larvae retain more yolk platelets at Stage 40 while showing less advanced cell differentiation.

A noteworthy feature of the observed intracellular utilization of yolk platelets in differentiating cells was that only a few yolk platelets were subject to utilization at any particular time while the majority of yolk platelets (except for the last stages) remained unaffected. It is possible that the number of platelets in utilization at one time would be a rough index of the cell’s metabolism directed toward differentiation, but this would involve the assumption, unjustified at present, that the duration of the various observed utilization stages remained constant for any platelet in any cell. Some of the difficulties in counting the yolk platelets per cell have been mentioned. The difficulties would be reduced in the case of studies made with amphibian cells in tissue-culture. One of the significant points about the present study, however, is that it provides information about development in intact embryos.

It should also be stressed that there are certain morphological differences in the yolk platelets being utilized when comparisons are made between different tissues within the same species, as well as between the corresponding tissues of different amphibian species. Doubtless these differences are clearest when electronmicrographs are compared, but certain of them were noticed in the present work; in particular there was the varying proportion of the utilization patterns involving the splitting of the body of the platelet into parallel discs or rodlets separated by material which stained with fast green. The lamellar envelopes demonstrated by Jurand & Selman (1964) for notochord, and which stained brown in the present work, are less prominent in certain tissues and absent in others. The low proportion of recognizable yolk-utilization figures observed within the cement gland in the present work may reflect the presence there of an unusually high
proportion of platelets which are utilized by a pathway which is not readily observed by the present staining method. Perry & Waddington, in unpublished electron-microscope observations on the cement gland of *Xenopus*, have observed yolk-platelet utilization figures in which the main-body component is apparently reduced by irregular fragmentation. It is not known if these differences in the morphological changes associated with yolk utilization are a reflection of the action of different enzyme systems or whether they are a consequence of the different demands of diverse cell types upon a food reserve of standard composition.

Among other approaches that have been made to the general problem of how yolk protein is converted into tissue protein, there is the study of the amino acids which may collect in cells as a result of the degradation of yolk protein but before they can be utilized in protein synthesis (Deuchar, 1963b). Deuchar (1963a) has also made preliminary experiments with radio-isotope labelled amino acids to try to trace them between yolk and tissue protein. Others have studied enzymes which may be involved in yolk breakdown (reviewed by Deuchar, 1962). These include phosphoprotein phosphatases (as in Flickinger, 1956, 1960), proteases like cathepsins (Deuchar, 1958) and peptidases.

**SUMMARY**

1. *Xenopus* embryos were fixed in Smith’s fixative, embedded in paraffin wax, serially sectioned, stained with safranin and fast green and examined by light microscopy. By this method various zones within the yolk platelets were recognized as colour differences. These zones correspond to regions of different ultrastructure which have been recognized by electron microscopy, both for normal yolk platelets and for the case of yolk platelets which are being utilized in the course of embryonic development.

2. The crystalline central core of the normal yolk platelet was safranin-positive. The granular cap-shaped borders stained with fast green. A low proportion of the platelets were ‘twinned’.

3. For considerable numbers of endodermal cells lining the archenteron of normal neurulae, all yolk platelets within the same cell were observed to be at the same stage of yolk utilization. The platelets showed alternating stripes of fast-green and safranin-positive material. At a later stage the yolk platelets stained entirely with fast green. These cells subsequently degenerate into the lumen of the archenteron.

4. Twelve tissues were studied up to the stage at which the larvae feed. The cap-shaped green borders of normal yolk platelets are absorbed at the earliest stages of cell differentiation. Subsequently, in individual yolk platelets, the safranin-positive material is replaced by fast-green staining material, beginning at the edges. Particularly in notochord, but also in somitic tissue and caudal epidermis, a brown zone is developed round the edges of yolk platelets. The
brown zone corresponds to the lamellar zone observed by electron microscopy. Only a few yolk platelets were utilized at one time within any differentiating cell. Graphs have been constructed to show the percentage of yolk-platelets in the process of utilization at particular developmental stages for eleven representative tissues: trunk and caudal epidermis, neural ectoderm, myotome, notochord, pronephric tubules, parachordal cartilage, myocardium, liver, pharynx, oesophagus and anterior intestine.

5. Extraction tests and tests with known substances indicated that anionic chromium in Smith's fixative played an essential part in the safranin staining of yolk platelets. It is further suggested that the phosphate groups of phosvitin may be involved. Certain nucleoli stained with safranin.

6. Yolk utilization was observed by the present method in the follicular atresia of large oocytes of Xenopus and in certain embryonic amphibian cells in tissue-culture. The changes in staining which accompanied yolk absorption in these cases were similar to those described for normal embryonic development.

RÉSUMÉ

Etude, avec une méthode de coloration à la safranine, de l'utilisation des plaquettes vitellines par les tissus d'embryons de Xenopus

1. Des embryons de Xenopus ont été fixés au liquide de Smith, enrobés dans la paraffine, débités en coupes sériees, colorés à la safranine et au vert solide et examinés en microscopie ordinaire. A l'aide de cette méthode, des differences de couleur permettent de distinguer diverses zones à l'intérieur des plaquettes vitellines. Ces zones correspondent à des régions d'ultrastructure différente qui ont été reconnues au microscope électronique, à la fois dans les plaquettes vitellines normales et dans celles qui sont utilisées au cours du développement embryonnaire.

2. Le noyau cristallin central de la plaquette normale est safranine-positif. Les contours granuleux en forme de calotte sont colorés au vert solide. Une faible proportion des plaquettes sont 'jumelées'.

3. Dans de grands nombres de cellules endodermiques limitant l'archentéron de neurulas normales, toutes les plaquettes vitellines à l'intérieur de la même cellule se trouvaient au même stade d'utilisation du vitellus. Les plaquettes présentaient des bandes alternées de matériel coloré au vert solide et à la safranine. A un stade ultérieur, les plaquettes vitellines se sont colorées entièrement au vert solide. Ces cellules dégénerèrent par la suite dans la lumière de l'archentéron.

4. Douze tissus ont été étudiés jusqu'au stade de la prise de nourriture. Les contours verts, en forme de calotte, des plaquettes vitellines normales sont absorbés aux stades les plus précoces de la différenciation cellulaire. Ensuite, dans des plaquettes prises individuellement, le matériel safranine-positif est remplacé par du matériel colorable au vert solide, en commençant par les bords. Dans la notochorde en particulier, mais aussi dans le tissu somitique et l'épiderme
caudal, une zone brune se développe autour des bords des plaquettes vitellines. La zone brune correspond à la zone lamellaire observée en microscopie électronique. Un petit nombre de plaquettes seulement se trouve utilisé en même temps dans une cellule en cours de différenciation. Des graphiques ont été établis pour montrer le pourcentage de plaquettes vitellines en cours d'utilisation à des stades précis du développement, pour onze tissus représentatifs: épiderme troncal et caudal, ectoderme neural, myotomes, notochorde, tubules prônéphriques cartilage parachordal, myocarde, foie, pharynx, œsophage et intestin antérieur.

5. Des tests d'extraction et des tests à l'aide de substances connues ont indiqué que le chrome anionique du fixateur de Smith jouait un rôle essentiel dans la coloration à la safranine des plaquettes vitellines. On suggère en outre que les groupes phosphate de la phosvitine pourraient y être impliqués. Certains nucléoles se sont colorés à la safranine.

6. L'utilisation du vitellus a été observée avec cette méthode au cours de l'atresie folliculaire de gros oocytes de *Xenopus* et dans certaines cellules embryonnaires d'Amphibien en culture de tissus. Les changements de coloration qui accompagnaient l'absorption du vitellus dans ces cas étaient semblables à ceux qui ont été décrits dans le développement embryonnaire normal.

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