NUMEROUS ANALYSES have been made of the relationships between cell differentiation and proliferation. Light microscopy studies show that megakaryocytes specialize in two steps: first, a series of DNA syntheses leads to the final ploidy level of 8N, 16N, 32N or 64N; secondly, cytoplasm matures by losing its basophilia, acquiring granules and platelet antigens, and finally by liberating platelets. In the present study, the alternate thick and thin section technique was used to explore the relationship between "ploidization" and cytoplasmic maturation on the ultrastructural level. It was found that the synthesis of specific organelles began during the ploidization phase, and increased considerably when this process had stopped after an average of three DNA syntheses.

MATERIALS AND METHODS

Preparation of Sections

Guinea pigs weighing 300 Gm. were used. Small blocks of bone marrow were fixed in 1 per cent osmium tetroxide in 0.1 M phosphate buffer at pH 7.0 for seven minutes. Short fixation times in osmium have been shown to be adequate in many materials. These were preferred in the present study to try to minimize the nonspecific loss of transmission (NSLT) associated with this fixative. Glutaraldehyde was avoided because, with the Schiff reagent used subsequently in the Feulgen method, it introduces a diffuse staining of bone marrow cells. After fixation, the blocks of marrow were dehydrated and embedded in epon. They were then cut into series of alternate thick (about 2.5 μ) and thin (about 0.08 μ) sections with an LKB ultramicrotome (Figs. 1 and 2), care being taken to minimize the loss of material so that the megakaryocytes might be studied in totality. This cutting thus divided each megakaryocyte into 4 to 10 pairs of sections, depending on the size of the cell.

Microspectrophotometric Measurements of DNA

The thick sections of osmium-fixed material, containing about 96 per cent of the megakaryocyte nuclei, were stained by the Feulgen method. Nonspecific staining by the Schiff
Fig. 1.—Scheme of marrow sectioning, showing megakaryocyte cut into five thick and four thin sections. For clarity, thin sections magnified out of proportion to thick sections about three times.

Fig. 2.—Illustration of method. Fig. 2a.—Phase-contrast micrograph of 3-μ section of osmium-fixed megakaryocyte, stained by Feulgen method. Centrioles (ce) and one demarcation membrane (dm) are conspicuous (× 5000).

Fig. 2b.—Portion of recording of DNA measurement made on above megakaryocyte. Section of nucleus was scanned along four adjacent lines (numbered one to four), each 1-μ wide and light transmission was recorded at both 5600 and 4000 Å. Measurement at 4000 Å permits correction for 13 percent nonspecific loss of transmission created by osmium fixation and allows calculation of ploidy value, 7.9N (see methods).

Fig. 2c.—Electron micrograph of thin section contiguous to thick section in Fig. 2a., showing maturing megakaryocyte with demarcation membranes (dm), granules, four centrioles (ce), ribosomes and rough endoplasmic reticulum (× 5000).
In preliminary experiments conducted by the multiple plug method, no significant difference in mean nuclear extinction was found when diaphragms of 0.32 or 1 were used.

In Fig. 3, extinction curves of megakaryocytes are depicted. A: nucleus in bone marrow smear fixed with formol and stained by Feulgen method. B: nucleus (nu) and cytoplasm (cy) in a section of marrow following osmium fixation, epon embedding and Feulgen procedure, omitting staining by Schiff reagent. C: nucleus in a section of marrow following osmium fixation, epon embedding and complete Feulgen procedure. Cytoplasmic curve was similar to B or showed a small, negligible peak at 560 μm. Curves were plotted as logarithm extinction, using arbitrary units so that their shape might be made independent of concentration of absorbing material.

Reagent was absent or negligible. However, osmium fixation introduced a nonspecific loss of transmission (NSLT), both in the nucleus and the cytoplasm. Although this NSLT in the past did not prevent Feulgen measurements of DNA, it invalidated uncorrected scanning measurements, which cannot be limited strictly to the nucleus. The NSLT, highest at 400 μm (Fig. 3), is explained by the light scattering caused by osmium-fixed subcellular particles. The extinction spectrum over the nucleus was approximately the sum of the NSLT and the extinction of formol-fixed nuclei stained by the Feulgen method (Fig. 3). In addition, the Feulgen extinction on the latter material at 400 μm was only about seven per cent of that measured at 560 μm. Since the spectrum of the NSLT was almost the same in both cytoplasm and nucleus (Fig. 3), the Feulgen negative cytoplasm served as a “baseline” NSLT control for each nucleus measured. Consequently, the specific Feulgen extinction at 560 μm could be calculated according to the following formula

\[ F_{560} = E_{560} - \frac{E_{400}}{k} \]

in which \( E_{560} \) and \( E_{400} \) represent the measured extinction at 560 and 400 μm, respectively, and \( k \) (which varied between two and four) is the ratio of NSLT_{400} to NSLT_{560} measured in the absorbing cytoplasm (Fig. 2). Scanning microspectrophotometry was performed with the Zeiss UMSP1 operated with an Ultrafluar objective 100 ×, NA 1.25 and a condenser NA 0.3. The scanning interval and the diameter of the measuring diaphragm were 1 μm.

The total DNA of each megakaryocyte was calculated by adding up the amounts measured for each section, and the ploidy value was determined from similar measurements made on orthochromatic normoblasts, known to be diploid.

**Autoradiographic Studies**

Labeling of DNA-synthesizing megakaryocytes was carried out in vitro as described by Rubini et al. Blocks of guinea-pig marrow were incubated for one hour at 37°C in a

*In preliminary experiments conducted by the multiple plug method, no significant difference in mean nuclear extinction was found when diaphragms of 0.32 μ or 1 μ were used.
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Fig. 4.—Histogram of ploidy values of guinea-pig megakaryocytes, using above-described technique.

labeling solution consisting of 1 ml. of fetal calf serum and 20 μCi. of thymidine (methyl-3H), specific activity 15C/mM. (CEN, Mol, Belgium). After washing with fetal calf serum, the blocks were then fixed with a six per cent glutaraldehyde solution in phosphate buffer at pH 7.0, postfixed for 40 min. in one per cent osmium tetroxide in phosphate buffer and finally embedded in epon.

Alternate thick and thin sections were cut, as described above. The thick sections were placed on a glass slide, covered with Kodak AR 10 stripping film and exposed for five weeks. Subsequently, the slides were developed for six minutes in Kodak D170 developer and fixed for six minutes in thiosulfate fixer. Finally, they were mounted and examined in phase contrast microscopy.

Electron Microscopy

The thin sections were stained by uranyl acetate and lead citrate, and examined under a Siemens or a Hitachi electron microscope, operated at 80 or 75 KV, to assess the stage of the cell cytoplasmic maturation. Low magnification photographs of the thick sections were used to correlate the microspectrophotometric or autoradiographic findings on each megakaryocyte with its electron microscopic characteristics (Figs. 2 and 5).

RESULTS

Relationship between DNA synthesizing ability and cytoplasmic maturation

Ultrastructure of megakaryocytes during the ploidization phase. “Immature megakaryocytes” were studied by analysis of both DNA synthesizing and mitotic cells. Megakaryocytes incorporating thymidine had, in addition to the

*Immature megakaryocytes correspond to megakaryoblasts in Bessis’ nomenclature, and not to basophile megakaryocytes, which are non-DNA synthesizing cells. The term immature was used here, since it was felt that the name megakaryoblast should be reserved to cells younger than those described in this study. Maturing megakaryocytes corresponded to Bessis’ basophile and granular megakaryocytes.
high nuclear–cytoplasmic ratio, two distinctive characteristics. (1) In the majority of sections, the nucleus was either U shaped (Fig. 5) or ring shaped (Figs. 6a, 6b), and partially encircled a central cytoplasmic area which communicated with the rest of the cytoplasm. This aspect suggested that the nucleus of immature cells roughly assumes the shape of a round mass interpenetrated by cytoplasmic extensions. In sections cut through the top of the nucleus (Fig. 6c) and possibly also in the youngest cells, the central cytoplasmic zone was much reduced in size. (2) Although immaturity was evident from the abundance of ribosomes and polyribosomes, well developed nucleoli, and the rather diffuse nuclear pattern (Figs. 5, 6), a small number of the specific organelles were already formed in most of the immature megakaryocytes. The central cytoplasmic zone contained a large Golgi apparatus, often extending into the outer cytoplasm (Figs. 5, 6), and known to be synthesizing the early granules (Fig. 5; Ref. 29). Microtubules appeared to originate in the vicinity of the centrioles (Fig. 5) and, in other pictures they were visible at the periphery of the cytoplasm, accompanied by myofibrils. Demarcation membranes were also found in immature cells. In addition to these organelles, immature megakaryo-

Fig. 5.—Immature megakaryocyte synthesizing DNA. 5a: Autoradiogram of thick section of marrow incubated for one hour in \(^{3}H\) thymidine showing unlabeled (u) and labeled (L) megakaryocytes. Basic fuchsin staining, phase contrast (× 1000). 5b: Thin section corresponding to 5a. 5c: Golgi zone of same cell showing granule formation. Arrow points to microtubule presumably originating near centriole ce. Glutaraldehyde-osmium fixation (× 7000).
Fig. 6.—Sections through immature megakaryocytes. A, B: Showing central cytoplasmic zone partially encircled by nuclear lobes. Autoradiography of adjacent thick section demonstrated thymidine incorporation. Glutaraldehyde and osmium fixation × approximately 3500. C: Section through top of nucleus. Ploidy value is indicated. Osmium fixation (× 7300).

Megakaryocytes contained many mitochondria and profiles of rough endoplasmic reticulum (Fig. 5). 17.5 per cent of megakaryocytes had the cytological characteristics described above.

In mitotic megakaryocytes, granules, (Fig. 7), demarcation membranes and microtubules were similarly visible.28

Ultrastructure of megakaryocytes after the ploidization phase. Maturing megakaryocytes represented the majority (67.5%) of the recognizable elements of the series. Although in some sections, the central cytoplasmic zone partially encircled by the nucleus was still visible, it appeared less frequently with maturation. Cytoplasmic development in these cells involves, in addition to extensive granule synthesis, formation of large amounts of demarcation membranes.
Fig. 7.—Immature megakaryocyte in ana-telophase of mitosis, showing Golgi apparatus and early granules. Glutaraldehyde osmium fixation. Electron micrograph (×6200). Insert: phase-contrast picture of adjacent thick section (×7470).

(Figs. 8, 9), which eventually delineate the future platelet territories (Fig. 9c). Since the formation of membranes is restricted to the intermediate part of the cytoplasm, the latter becomes divided into three concentric zones. The inner zone has an immature aspect similar to that of younger cells (Fig. 9a). The outer zone contains myofibrils and microtubules. With progressive maturation, nucleoli and ribosomes present thus far, disappeared, while mitochondria became smaller and less numerous, and membrane formation and zonation became more conspicuous.

Thrombopoietic megakaryocytes were generally pycnotic cells, with a greatly enlarged and bubbling marginal zone (Fig. 10). Newly formed platelets, still attached to the megakaryocyte, could be seen protruding into the vessel (Figs. 10, 9d). In some cases the released platelets bore a hypertrophied peripheral zone contrasting with the near absence of this organelle in circulating platelets. Pycnotic nuclei surrounded by a narrow band of cytoplasm (Fig. 11), presumably remnants of thrombopoietic cells, were also included in this group which represented 15 per cent of the total megakaryocytes.

Relationship between Polyploidy and Cytoplasmic Maturation

The ploidy values reached peaks of frequency at 8N, 16N and 32N (Fig. 4). The proportion of these three classes was 20 per cent, 67.5 per cent and 12.5 per cent, respectively. No 4N megakaryocyte could be recognized. In the 32N and the 16N group, the coefficient of variation between individual mega-
Fig. 8.—Similar cytoplasmic organelle development in maturing megakaryocytes of different ploidy value. Osmium fixation (× 5700).

karyocyte DNA content was 9 and 10 per cent, respectively (excluding immature cells).

Figure 12 shows a diagram of megakaryocyte maturation versus ploidy. For each cell the state of cytoplasmic maturation and the degree of ploidy have
Fig. 10.—Thrombopoietic megakaryocyte and five platelets (pl) protruding into vessel. Black arrows in top of figure indicate vessel limits. Lobes of megakaryocyte nucleus labeled nu. Note bubbling (bub) of hypertrophied peripheral zone. (See Fig. 9d). Osmium fixation (× 3100).

Fig. 9.—Successive stages of cytoplasmic specialization in three maturing (a, b, c) and one thrombopoietic (d) megakaryocyte. a, b, c show development of demarcation membranes (dm) at different ploidy levels. d newly formed platelet protruding through vessel wall (v) visible on both sides of platelet (see Fig. 10). Osmium fixation: (a) (× 8100), (b) (× 74300), (c) (× 29600), (d) (× 9700).
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been determined. Immature cells, able to synthesize DNA, were found at ploidy levels 8N to 23N. Maturing and thrombopoietic megakaryocytes which had completed ploidyization could be encountered at each of the three ploidy levels 8N, 16N or 32N. As an illustration of this observation, Figure 8 shows two megakaryocytes which have a nearly identical submicroscopic appearance. One is a 16N, the other a 32N cell.

Study of thrombopoietic megakaryocytes was of particular interest since few analyses had been made on this stage before. Despite the fact that microphotometric error was relatively larger on the condensed, pycnotic nuclei of thrombopoietic cells, it was clear that thrombopoiesis could be observed at all three ploidy levels (Figs. 10, 11, 12).

DISCUSSION

The Method

The technique of alternate thick and thin sectioning has been previously used to combine electron microscopy and autoradiography. The present study shows that it also makes it possible to measure the relative amount of a cellular substance, i.e., DNA in relation to the cell’s ultrastructure, thus permitting the combination of quantitative cytochemistry and electron microscopy. The validity of this procedure is demonstrated by the following facts: Megakaryocyte nuclei of guinea-pigs could be grouped in three ploidy classes, namely 8N, 16N and 32N (Fig. 4); the proportion of these three classes was 20 per cent, 67.5 per cent and 12.5 per cent, respectively, to be compared with very similar values obtained by conventional microspectrophotometry in guinea-
pigs\(^{12}\) and rats\(^{13}\); among the 16N and 32N classes, the coefficient of variation of DNA content was 9 or 10 per cent. This variation consists mainly of technical errors, since biological dispersion around the class modal value is probably very small.\(^{32}\) Therefore, the precision of the method is sufficient to exclude an error of one ploidy class.

**Pattern of Megakaryocyte Maturation**

The present study proposes a middle way between two opposite conceptions of megakaryocyte maturation and shows that ploidization and development of cytoplasmic organelles are neither strictly parallel nor consecutive processes. Contrary to light microscopy autoradiography studies, and in partial agreement with the studies of Kinosita,\(^{33}\) ultrastructural analyses detect some early formation of specific organelles in immature megakaryocytes (Figs. 5, 7). The reasons for this discrepancy are to be found not only in the superior resolving power of the electron microscope, but also in the particular localization of the early granules. Whereas in imprints the nucleus appears solid, in sections, it shows several lobes which partially encircle a central cytoplasmic zone containing most of the Golgi apparatus and the early granules. The central cytoplasmic zone communicates with the outer cytoplasm (Figs. 5, 6). Granule formation is not the only detectable sign of cytoplasmic maturation in immature cells; demarcation membranes, microtubules and myofibrils also appear during the ploidization phase. In addition, Odell et al. have shown that all recognizable cells of the thrombocytic series incorporate \(^{35}\)S into chondroitin sulfate,\(^{34,35}\) the acid
mucopolysaccharide presumably responsible for the alcian blue staining of demarcation membranes.27,34

The length of the plloidization phase can be inferred from ploidy measurements on cells such as maturing and thrombopoietic megakaryocytes which never synthesize DNA. These cells were found mostly in the 16N group and much less frequently in the 8N and 32N groups27 (Fig. 12). Similar findings were made in light microscopy by de Leval,9 and Odell and Jackson.10 Thus, the common conclusion of light and electron microscopy studies is that the plloidization phase comprises, on the average, 3 ± 0.5 (1 S.D.) genome duplications. This finding suggests a possible mechanism for the regulation of platelet production.37,38

The end of plloidization is marked by several cytological changes. The nucleus is transformed into an irregularly segmented mass, a modification best observed in imprints,15 but evident in sections also. The Golgi apparatus gradually ceases to be encircled by the nucleus. The nuclear size remains constant15 as cytoplasmic volume increases, thereby decreasing the nuclear-cytoplasmic ratio. Finally a massive synthesis of demarcation membranes and granules occurs and the latter become visible in light microscopy.9,16 At this stage it is possible to demonstrate platelet antigens15 among which is thrombosthenin, present in granules and membranes.36

It is thus apparent that organelle formation is initiated in immature megakaryocytes and reaches a peak after the plloidization phase. This pattern of megakaryocyte maturation bears some resemblance to specialization of the other marrow series. Granulocytes as well as megakaryocytes begin synthesizing granules in the Golgi apparatus during the phase of genome multiplication. In both series, nuclear segmentation accompanies the end of this phase. In addition, both erythrocytic and megakaryocytic series, after nuclear pycnosis, produce an enucleate element, the metabolic activities of which have many similarities.37

**Summary**

The thick and thin section technique was used to study guinea-pig megakaryocytes by electron microscopy, combined with either autoradiography or cytophotometric determination of DNA. Megakaryocytes engaged in plloidization already showed granule formation, which began in the Golgi apparatus partially encircled by the nucleus. The plloidization phase stopped at the 8N, 16N or 32N level, after an average of 3 ± 0.5 (1 S.D.) genome duplications. Microtubules, myofilaments and demarcation membranes were also present in the immature megakaryocytes but organelles were formed in increasing amounts in non DNA-synthesizing cells. Platelet liberation, possible at the 8N, 16N or 32N stage, occurred in non DNA-synthesizing cells, principally at the 16N level.

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1 The origin of demarcation membranes has been a matter of controversy, since these have been considered either as smooth endoplasmic reticulum29,37 or cell membrane derivatives. It should be pointed out that, not only cell membranes, but also such a derivative of the smooth endoplasmic reticulum as muscle sarcoplasmic reticulum is lined with acid mucopolysaccharides, functions as a calcium extrusion pump and, is in close proximity to contractile elements. References pertinent to this problem are given by Statland, B. E., Heagan, B.M., and White, J. G.: Uptake of calcium by platelet relaxing factor. Nature 233:521, 1929.
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DNA Metabolism and Development of Organelles in Guinea-Pig Megakaryocytes: A Combined Ultrastructural, Autoradiographic and Cytophotometric Study

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