

# THE INTRACELLULAR LOCALIZATION OF HEME BY A FLUORESCENCE TECHNIQUE

S. GRANICK, Ph.D., and RICHARD D. LEVERE, M.D.

From The Rockefeller Institute

## ABSTRACT

A new technique for the intracellular localization of minute amounts of heme and hemoproteins is described. The specimen is treated with 1.5 M perchloric acid in the presence of SH groups, followed by ultraviolet light irradiation in a fluorescence microscope. This fixes the proteins *in situ* and converts the heme to a porphyrin which fluoresces and is readily visualized. With this technique, hemoglobin has been demonstrated in the nuclei of avian erythrocytes, and in the nuclei of human normoblasts at an earlier stage than previously described. In addition, hemoproteins, presumably cytochromes, have been detected in the cytoplasm and nuclei of myelocytes, in thymus lymphocyte nuclei, in chick embryo liver cytoplasm, and in chick embryo somites.

The detection and localization within cells of hemoproteins such as hemoglobin, cytochromes, peroxidases, and catalases has, to date, been accomplished mainly through histochemical techniques based on the catalysis by heme of the peroxidation of benzidine, or one of its analogues, to form colored products (1). Absorption microscopy and electron opacity studies also have been employed. The technique described here represents a different approach to the localization of intracellular heme and hemoproteins. It is based on the conversion of heme to a porphyrin which fluoresces and can be readily detected with the ultraviolet light microscope. The method is simple, rapid, and sensitive, and affords rather precise resolution.

The present report is concerned with a description of this technique, its application to the study of heme in the cytoplasm and nuclei of hematic cells, and its application to other tissues.

## MATERIALS AND METHODS

### *Biological Material*

Chicken erythrocytes were obtained from adult chickens by intravenous bleeding. Chick embryo

blood was obtained from the heart, or, in very young embryos, by pricking a yolk sac blood vessel. Direct smears were made on glass slides and then air dried. Normal human bone marrow was obtained from sternal marrow aspirations, smeared on glass slides, and allowed to air dry.

Nuclei of calf thymus lymphocytes were supplied through the courtesy of Dr. V. Allfrey. These nuclei were prepared by an aqueous technique described in detail elsewhere (2).

Nuclei of chicken erythrocytes were prepared from adult chicken red cells. The cells were washed three times in 0.25 M sucrose; then 10 ml of washed red cells were resuspended in 50 ml of 0.4 M sucrose containing  $10^{-3}$  M  $\text{CaCl}_2$ . To this was added 1.5 ml of 10 per cent Triton X-100, and the mixture was then homogenized in a Waring Blendor for 1 minute. The homogenate was centrifuged at 750 *g* for 15 minutes (the supernatant solution was removed and discarded) to obtain a sediment that consisted of isolated erythrocyte nuclei and a few contaminating whole cells. The nuclei were washed several times in 0.25 M sucrose with  $10^{-3}$  M  $\text{CaCl}_2$  and finally, when the supernatant was free of hemoglobin, resuspended in this wash solution and brought to pH 7.4 with 0.2 M Tris buffer.

Chick embryo liver cells were obtained in monolayers, on glass slides, from primary cultures of these

cells grown *in vitro* by a previously described technique (3).

Chick blastoderms including embryos at the 2- to 12-somite stage of development were obtained from eggs incubated at 37°C for the appropriate length of time. After the blastoderm (and embryo) had been dissected off the vitelline membrane, it was mounted wet on a glass slide.

### Experimental Procedure

The reagent used was 1.5 M perchloric acid containing 0.2 M mercaptoethylamine hydrochloride. One hundred milliliters of this solution can be made by adding, to 87.2 ml of H<sub>2</sub>O, 4.5 gm of mercaptoethylamine·HCl and 12.8 ml of 70 per cent HClO<sub>4</sub>.

Freshly prepared air-dried or unfixed slides were flooded with 0.2 ml of 1.5 M perchloric acid containing 0.2 M mercaptoethylamine (MEA) and covered with a coverslip, and the excess solution was blotted off. The coverslip was then rimmed with paraffin to prevent drying of the specimen and examined within a few minutes. Suspensions of nuclei were mixed on slides with the reagent and then treated as above.

The specimens treated in this manner were first located and identified with phase contrast illumination using a Zeiss Ultraphot microscope. Then the light source was changed and the slide was irradiated with ultraviolet (UV) light for 10 to 200 seconds while the appearance and progressive increase in intensity of porphyrin fluorescence was observed.

The heme to porphyrin conversion under these conditions was induced by irradiation with a beam of UV light which was the same as the one used to examine for fluorescence. Any red fluorescence that might have been present when the slide was first examined would have been evidence of preformed porphyrins. No initial fluorescence was noted in any of the specimens examined.

The UV beam used was produced by a 200-watt high pressure mercury arc lamp, with the arc image focused on the slide to obtain maximum intensity of irradiation. The UV beam was filtered by colored glass filters that permitted transmission at 365 to 440 mμ but removed light of wavelengths longer than 440 mμ, with special precautions to exclude the region 680 to 740 mμ. A filter above the objective removed fluorescent or scattered light of wavelengths shorter than 540 mμ.

Usually, after 10 to 60 seconds of UV irradiation the red porphyrin fluorescence appears in the cellular areas containing heme or hemoproteins. The perchloric acid in the reagent fixes the proteins *in situ*. The light reaction is a photochemical reduction for which SH groups contained either in the tissue proteins or in added MEA are required. The iron of the photoexcited heme is reduced to the ferrous state and displaced from the porphyrin by two protons.

In this way the heme is converted to a porphyrin in its acidic form, which emits fluorescence at 597 to 600 and at 651 to 653 mμ.

This photochemical reaction is useful qualitatively but not quantitatively, because concomitant photochemical bleaching of the developing porphyrins also occurs. This bleaching indicates that the porphyrins formed are photocatalyzing their own reduction and destruction. The very low rate of development of heme to porphyrin conversion in 1.0 M HClO<sub>4</sub> and the high rate in 1.5 M HClO<sub>4</sub> correlate with the fact that the reaction is proportional to a power greater than 1 of the hydrogen ion activity. At higher acidities the rate of photochemical bleaching of the porphyrins is more rapid. Therefore, the concentration 1.5 M HClO<sub>4</sub> was chosen as a compromise between the rate of photochemical conversion of heme to porphyrin and the rate of photochemical bleaching of the porphyrin.

The reaction cannot be used to distinguish the heme of hemoglobin from the heme of cytochrome *c* because the porphyrins formed in both cases have the same fluorescence maxima (Table I). This result is best explained by assuming that the vinyl groups of protoporphyrin under these conditions react with SH compounds to form thioether bonds similar to the ones in cytochrome *c*. In the absence of MEA or proteins, protoporphyrin does not react in this way and its fluorescence maxima are at 604 and 659 mμ. Crystalline chicken hemoglobin reacts with HClO<sub>4</sub> to give a product with fluorescence maxima at 597 and 652 mμ, identical with those of iron protoporphyrin treated with HClO<sub>4</sub> and MEA. This result suggests that probably the SH groups of the chicken globin react with the vinyl groups. A reaction of this kind does not occur where the groups are ethyl (as in mesoporphyrin) or H (as in deuteroporphyrin or iron deuteroporphyrin).

### RESULTS

Several tissues have been examined to provide examples of the usefulness of the technique described here for the detection of heme. Heme was found in isolated nuclei of chicken erythrocytes as well as in the nuclei and cytoplasm of whole cells. In human bone marrow, heme was found in the nuclei of erythroblasts at an earlier stage than has previously been described; in myelocytes, heme appeared in small amounts in both nucleus and cytoplasm. Calf thymocyte nuclei contained traces of heme which were further identified as components of cytochromes *a* and *b*. In the chick embryo, the somite region gave a marked reaction for heme; the cytoplasm of the embryonic liver parenchyma cell was also rich in heme, in contrast to the nu-

TABLE I

*Fluorescence Maxima of Porphyrins, Iron Porphyrins, and Hemoproteins*

Proteins and red cells were treated on a slide with 1.5 M HClO<sub>4</sub> or with the HClO<sub>4</sub>-MEA reagent, as described in the text, and irradiated with ultraviolet light, and the fluorescence was scanned with a microscope spectrofluorometer (16) at a magnification of 100 to 200 times. Porphyrins were dissolved in a cuvette, in 1.5 M HClO<sub>4</sub> or in the HClO<sub>4</sub>-MEA reagent; or porphyrins were mixed with serum albumin and treated on a slide with the HClO<sub>4</sub>-MEA reagent. The porphyrins were then irradiated with UV light and the fluorescence was determined.

Compound	Solution applied		Fluorescence maxima (Standard deviation = ± 2 mμ)	
	HClO <sub>4</sub>	HClO <sub>4</sub> + MEA or HClO <sub>4</sub> + serum albumin	mμ	mμ
Protoporphyrin	+		604	659
Protoporphyrin		+	600	652
Iron protoporphyrin		+	597	652
Crystalline chicken hemoglobin	+		597	652
Human red cells	+		597	652
Human red cells		+	597	652
Cytochrome <i>c</i>		+	600	652
Hematoporphyrin	+		597	652
Mesoporphyrin	+		593	649
Mesoporphyrin		+	593	649
Deuteroporphyrin	+		592	647
Deuteroporphyrin		+	592	647
Iron deuteroporphyrin		+	592	647

cleus. Details of the results in different tissues follow.

*Chicken Erythrocytes*

In 1951 the presence of hemoglobin in the nuclei of bird erythrocytes was unequivocally demonstrated by Stern *et al.* (4), who isolated the nuclei by Behren's non-aqueous technique. Subsequent studies on erythroblasts of reptiles, birds, and mammals utilizing the benzidine stain (5, 6), microspectroscopy (7), and electron opacity (8) have confirmed the presence of hemoglobin in erythrocyte nuclei.

Fig. 1 illustrates the presence of heme in both cytoplasm and nucleus of adult chicken erythrocytes, following their treatment with perchloric acid and UV irradiation. The fluorescence, presumably due to hemoglobin, is most intense in the nuclei of these cells and in a perinuclear area which seems to correspond to the nuclear membrane. This marked perinuclear concentration of hemoglobin was also observed by O'Brien (6), using an

ortho dianisidine stain. It is possible that this perinuclear and marginal concentration of hemoglobin is a fixation artifact. The nuclear fluorescence appears in a "checkerboard" pattern similar to that seen in chromatin stained with Wright's stain. This type of distribution suggests that the hemoglobin is closely associated with the DNA and lends some support to the hypothesis of Tooze and Davies (9) which suggests that, in erythroblasts, hemoglobin may act like a histone to combine with DNA and thereby act to terminate its own synthesis. On the other hand, the fluorescence in the cytoplasm due to the hemoglobin is fairly uniformly distributed except in one or two negative areas corresponding to persistent reticulum.

The temporal appearance of the induced fluorescence is illustrated in Figs. 2 *a* to 2 *c*. Fifteen seconds after the initiation of UV irradiation a positive reaction is first visible (see arrows) in the nucleus and the outer cell border (Fig. 2 *a*). This peripheral area of early, intense fluorescence is too broad to represent merely the cell membrane. It

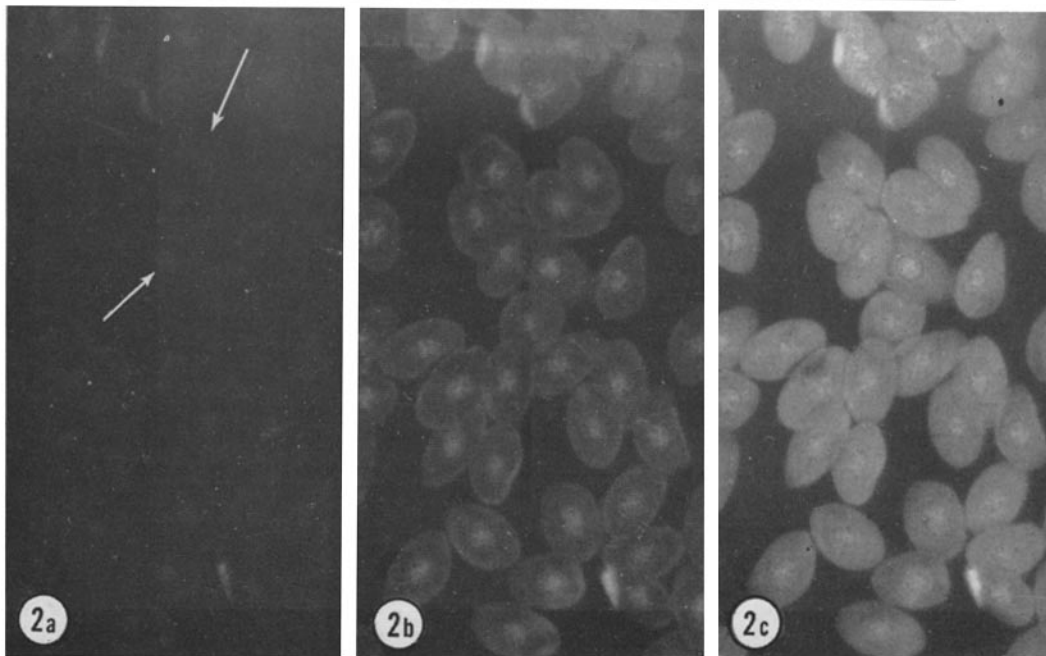
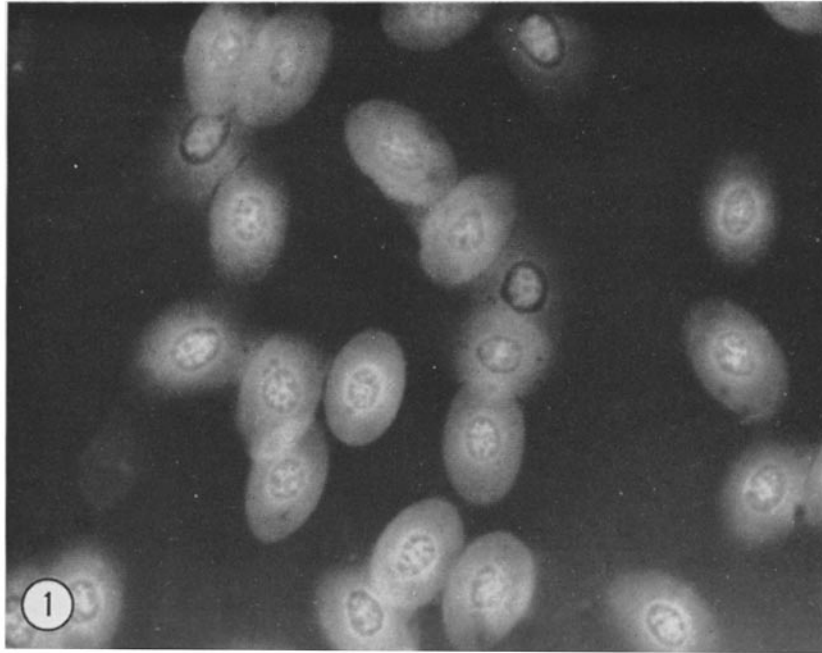


FIGURE 1 Fluorescence photograph of adult chicken erythrocytes after treatment with perchloric acid and UV light irradiation. Red fluorescence is recorded as white.  $\times 2000$ .

FIGURE 2 Temporal appearance of fluorescence in adult chicken erythrocytes after treatment with perchloric acid reagent and UV light. Figs. 2 *a*, 2 *b*, and 2 *c* represent the reaction at 15, 40, and 60 seconds, respectively, after the initiation of UV light irradiation. Positive reaction is first visible in nucleus and outer cell border (arrows).  $\times 1000$ .

corresponds more closely to the "marginal band" of avian erythrocytes. This structure, which has been identified as a fibrillar network in the electron microscope by Fawcett and Witebsky (10), is postulated to form a support for the cell membrane. At 40 seconds (Fig. 2 *b*), the nucleus and cell border still give the most intense reaction, but definite fluorescence is now apparent in the cytoplasm. After 60 seconds (Fig. 2 *c*), there is no longer a difference in degree of fluorescence reaction between the cytoplasm and the cell border; however, the nucleus remains most intensely fluorescent. The porphyrin fluorescence begins to fade 3 to 5 minutes after it reaches its peak intensity. The intensity of the induced fluorescence in any particular area appears to be directly related to the concentration of heme in that site.

The distribution of fluorescence in cells of the primitive erythroid line (Fig. 3) is similar to that seen in the definitive erythrocytes. The primitive cells in this smear were obtained from the blood of a 5-day old chick embryo. The pattern of induced fluorescence differs from that in the mature erythrocytes only in showing a somewhat greater number of negative-reacting areas in the cytoplasm. This is no doubt due to the presence of sizable amounts of RNA and non-hemoproteins in the cytoplasm of this immature cell type, as revealed by the marked cytoplasmic basophilia seen with Wright's stain.

The perchloric acid-ultraviolet light-induced fluorescence in isolated adult chicken erythrocyte nuclei is shown in Fig. 4. This positive reaction in the isolated nuclei eliminates the possibility that a fixation artifact produces the nuclear reaction in intact red cells. It also indicates that the heme-chromatin attachment remains even after aqueous isolation of the nuclei.

#### *Normal Human Bone Marrow*

The presence of heme (presumably in hemoglobin) in the nuclei of orthochromic normoblasts (*on*) as demonstrated by this perchloric acid-UV technique is illustrated in Fig. 5 *b*. In this cell type the fluorescence is almost equally distributed between cytoplasm and nucleus, with a somewhat greater reaction in the former. Unlike the nucleated chicken erythrocyte, the orthochromic normoblast shows no evidence of a more intense reaction at the cell border or nuclear membrane. Though it is not well demonstrated in the photographs, the nuclear fluorescence is not homo-

geneously distributed. Instead, it is similar in pattern to that seen in the nucleus of the chicken red cell. The mature non-nucleated red cells present in the figure exhibit greater fluorescence than do their nucleated counterparts because of their higher concentration of hemoglobin. In the mature erythrocytes the fluorescence is homogeneously distributed over the cell except for a faint central area corresponding to the "central area of pallor." In addition, in these cells the fluorescence does not first appear at the cell margin as is seen in chicken erythrocytes. The polymorphonuclear leukocyte (*pmn*) seen in Fig. 5 *b* exhibits relatively faint fluorescence which cannot be localized.

An earlier stage of erythroid maturation is represented by the two polychromic normoblasts (*pn*) from human bone marrow shown in Figs. 6 *a* and 6 *b*. The fluorescence is fainter than in the orthochromic normoblast, but again there is a fairly uniform distribution between nucleus and cytoplasm. Fluorescence in the two polymorphonuclear leukocytes shown in this photograph is barely perceptible.

An early stage normoblast (*bn*) in telophase is shown in Fig. 7. It is difficult to determine with phase-contrast microscopy to what level of development this cell should be assigned. However, the ability to undergo mitosis excludes stages past the very early polychromic normoblast. Even at this early stage of maturation, a period of intense cytoplasmic basophilia, the heme reaction in the cytoplasm is relatively marked as compared with that in the metamyelocyte (*meta*). Using microspectrophotometry, Thorell (11) reported that in basophilic normoblasts globin was detectable before the appearance of heme. Contrariwise, the present study indicates that significant amounts of heme are present at this stage of development, either as free heme or as hemoglobin, and in greater amounts than might be expected for cytochromes alone. It is of interest that the nuclear area in this cell in mitosis is devoid of any appreciable amount of heme. However, in congenital erythropoietic porphyria, Schmid *et al.* (12) found benzidine-positive inclusions in normoblast nuclei and also observed that some of these nuclei contained a fluorescent porphyrin.

The distribution of heme in an eosinophilic myelocyte (*em*) is shown in Fig. 8 *b*. The reaction is faint, but definite, in the cytoplasmic granules and nucleus. Neutrophilic myelocytes (*myel*) and

metamyelocytes (*meta*) also give a faintly positive reaction of equal intensity in the cytoplasm and nucleus (Fig. 9 *b*).

### *Thymocyte Nuclei*

In order further to evaluate the presence of hemoproteins in the nuclei of non-erythroid hematic cells, nuclei isolated from calf thymus lymphocytes were studied. Fig. 10 shows a large cluster of these nuclei exhibiting a definitely positive reaction after treatment with perchloric acid and UV light. In isolated single nuclei the reaction is too faint to be recorded and can only be discerned when the nuclei are viewed in clusters. On the basis of present knowledge of nuclear metabolism, it is most logical to assume that the hemoprotein responsible for the nuclear fluorescence is a cytochrome. Studies from the laboratory of Mirsky and Allfrey (13, 14) have shown that thymocyte nuclei can carry out oxidative phosphorylation. Hematin enzymes should be expected to play an important role in these reactions. Recent observations in this laboratory (15) utilizing difference spectra of isolated thymocyte nuclei suggest the presence in this cell fraction of a *b* type cytochrome and also possibly of an *a* type cytochrome.

### *Chick Blastoderms and Embryos*

The actively proliferating areas of the developing embryo exhibit fluorescence when treated with perchloric acid and irradiated with UV light. The

6-somite embryo depicted in Fig. 11 shows the most intense fluorescence reaction in the somite region. The marked heme reaction in the embryonic tissue would appear to be due to a high cytochrome content in this rapidly metabolizing tissue. In the surrounding blastoderm, fluorescence is noted in the blood islands which are just lateral and posterior to the embryo. The fluorescence in this hematopoietic tissue is greater than that in the embryo, probably because of beginning hemoglobin synthesis. It should be noted that at this stage of development benzidine stains for heme are still negative.

### *Fetal Chick Liver Cells*

The fetal liver cells shown in Fig. 12 demonstrate the perchloric acid-UV-induced fluorescence that is almost exclusively localized in the cytoplasm, indicating its relatively high content of cytochromes. The nuclei, by comparison, fluoresce only slightly.

Further application of this perchloric acid-UV-induced fluorescence technique for the detection of heme in cells has recently been made by Krassner (17). He was able to demonstrate "cytochromes" only in culture forms of hemoflagellates, not in *in vivo* forms.

A preliminary account of the method reported here has been presented in *Fed. Proc.*, 1964, **23**, 222.

This work was supported in part by United States Public Health Service Grant GM-04922.

*Received for publication, October 27, 1964.*

---

FIGURE 3 Perchloric acid-UV light-induced fluorescence of erythrocytes of primitive cell line obtained from 5-day chick embryo.  $\times 2000$ .

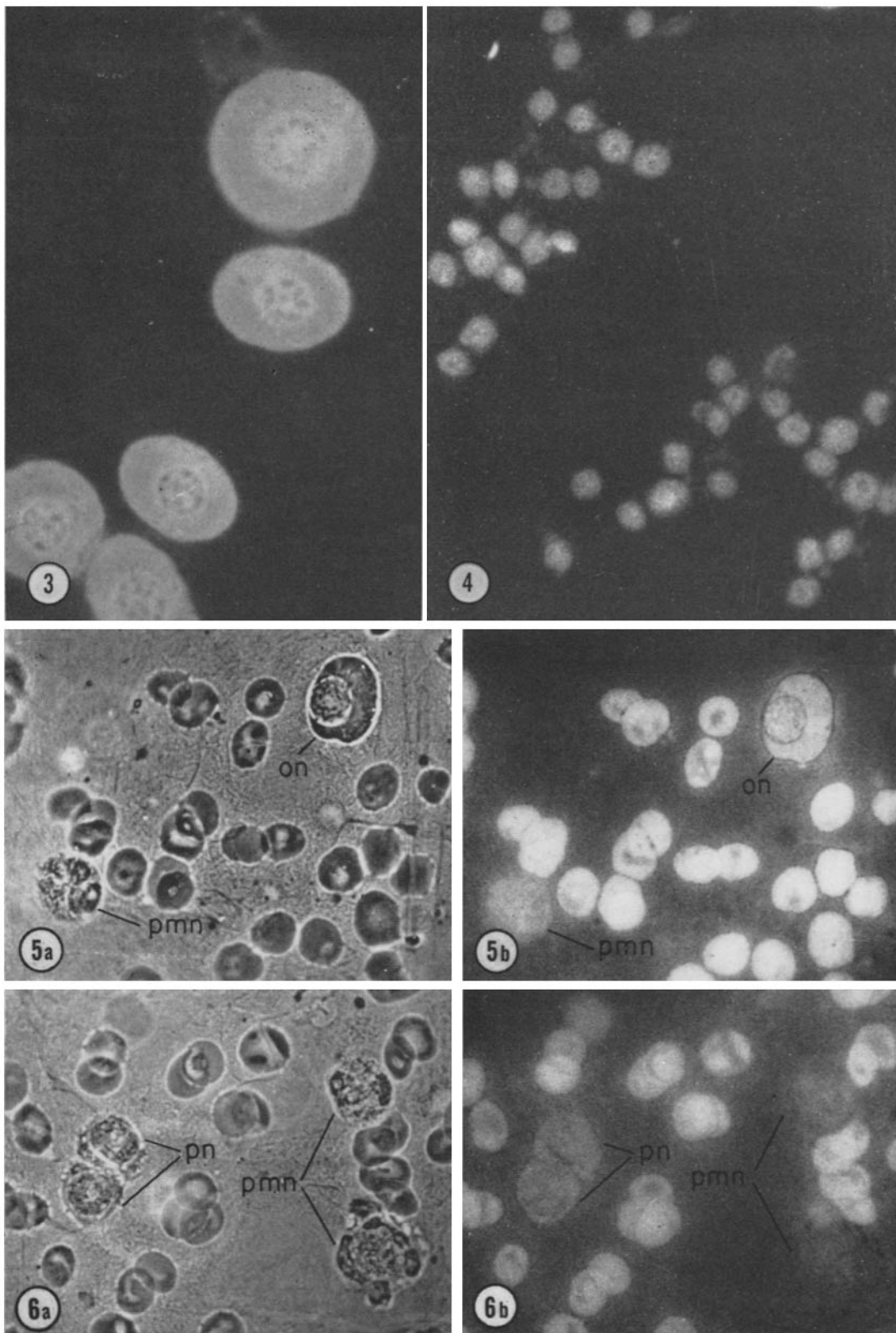
FIGURE 4 Perchloric acid-UV light-induced porphyrin fluorescence in isolated chicken erythrocyte nuclei.  $\times 1000$ .

FIGURE 5 Fig. 5 *a*. Phase-contrast photograph of a smear of human bone marrow containing an orthochromic normoblast (*on*) and a polymorphonuclear leukocyte (*pmn*) among many mature erythrocytes.  $\times 1000$ .

Fig. 5 *b*. Fluorescence photograph of the field shown in Fig. 5 *a* after treatment with  $\text{HClO}_4$ -MEA reagent and UV light.  $\times 1000$ .

FIGURE 6 Fig. 6 *a*. Phase-contrast photograph of a smear of normal human bone marrow containing two polychromic normoblasts (*pn*) and two polymorphonuclear leukocytes (*pmn*).  $\times 1000$ .

Fig. 6 *b*. Fluorescence photograph of the field depicted in Fig. 6 *a* after induction with perchloric acid and UV light.  $\times 1000$ .



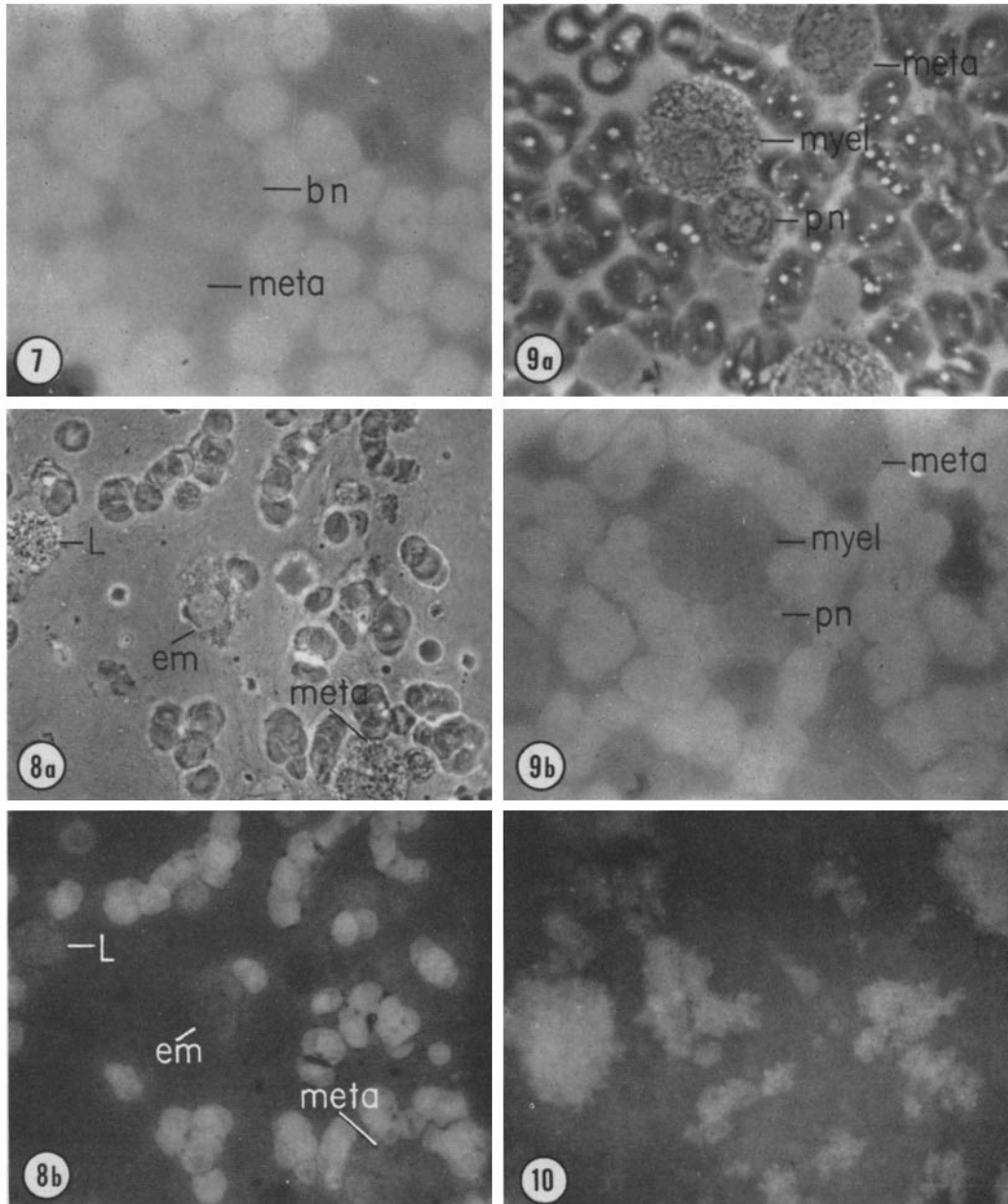


FIGURE 7 Perchloric acid-UV light-induced porphyrin fluorescence in a basophilic normoblast (*bn*) in mitosis. Just below this cell is a neutrophilic metamyelocyte (*meta*). Normal human marrow.  $\times 620$ .

FIGURE 8 Fig. 8 *a*. Phase-contrast photograph of human bone marrow smear. This field contains an eosinophilic metamyelocyte (*em*), a neutrophilic metamyelocyte (*meta*), and a large lymphocyte (*L*).  $\times 620$ .

Fig. 8 *b*. Fluorescence photograph of field shown in Fig. 8 *a* after treatment with the  $\text{HClO}_4$ -MEA reagent and UV light. There is faint fluorescence in both the cytoplasm and the nucleus of the eosinophilic myelocyte (*em*). The neutrophilic metamyelocyte and the lymphocyte also exhibit faint, but non-localizable, fluorescence.  $\times 620$ .



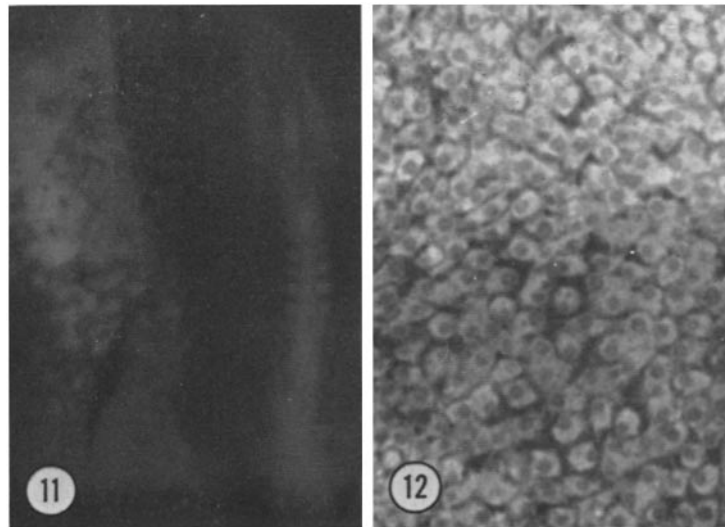


FIGURE 11 Perchloric acid-UV light-induced fluorescence in a 6-somite chick embryo. Fluorescence is also present in the developing blood islands which are lateral and posterior to the embryo.  $\times 25$ .

FIGURE 12 Fetal chick liver cells grown *in vitro* on a coverslip. The perchloric acid-UV light-induced porphyrin fluorescence shown in the figure is almost exclusively localized in the cytoplasm.  $\times 160$ .

---

FIGURE 9 Fig. 9 a. Phase-contrast photograph of human marrow smear. Present in this field are a neutrophilic metamyelocyte (*meta*), a neutrophilic myelocyte (*myel*), and a polychromic normoblast (*pn*).  $\times 620$ .

Fig. 9 b. Fluorescence photograph of the field shown in Fig. 9 a after treatment with the  $\text{HClO}_4$  MEA reagent and UV light. Faint, non-localizable fluorescence is present in the myeloid cells and is of much lesser intensity than that seen in the normoblast (*pn*).  $\times 620$ .

FIGURE 10 Perchloric acid-UV light-induced porphyrin fluorescence in clumps of isolated thymocyte nuclei.  $\times 150$ .

## REFERENCES

1. FLATMARK, T., A histochemical technique for the demonstration of cytochrome *c*, *Nature*, 1963, **200**, 1112.
2. ALLFREY, V. G., The isolation of subcellular components, in *The Cell*, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1959, **1**, 193.
3. GRANICK, S., Induction of the synthesis of  $\delta$ -aminolevulinic acid synthetase in liver parenchyma cells in culture by chemicals that induce acute porphyria, *J. Biol. Chem.*, 1963, **238**, PC 2248.
4. STERN, H., ALLFREY, V. G., MIRSKY, A. E., and SAETREN, H., Some enzymes of isolated nuclei, *J. Gen. Physiol.*, 1951, **35**, 559.
5. DE CARVALHO, S., Cytochimie de l'hémoglobine. Demonstration de l'existence de cette substance dans les noyaux des erythrocytes, *Compt. rend. Soc. biol.*, 1951, **145**, 607.
6. O'BRIEN, B. R. A., The presence of haemoglobins within the nucleus of the embryonic chick erythroblast, *Exp. Cell Research*, 1960, **21**, 226.
7. WILKINS, M. H. F., and DE CARVALHO, S., The violet light microscope estimation of haem in living cells, *Blood*, 1953, **8**, 944.
8. DAVIES, H. G., Structure in nucleated erythrocytes, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 671.
9. TOOZE, J., and DAVIES, H. G., The occurrence and possible significance of haemoglobin in the chromosomal regions of mature erythrocyte nuclei of the newt *Triturus cristatus cristatus*, *J. Cell Biol.*, 1963, **16**, 501.
10. FAWCETT, D. W., and WITEBSKY, F., Observations on the ultrastructure of nucleated erythrocytes and thrombocytes with particular reference to the structural basis of their discoidal shape, *Z. Zellforsch u. mikr. Anat.*, 1964, **62**, 785.
11. THORELL, B., Studies on the formation of cellular substances during blood cell formation, *Acta Med. Scand., Suppl.*, 1947, **200**.
12. SCHMID, R., SCHWARTZ, S., and SUNDBERG, R. D., Erythropoietic (congenital) porphyria; a rare abnormality of the normoblasts, *Blood*, 1955, **10**, 416.
13. ALLFREY, V. G., MIRSKY, A. E., and OSAWA, S., Protein synthesis in isolated cell nuclei, *Nature*, 1955, **176**, 1042.
14. ALLFREY, V. G., and MIRSKY, A. E., The role of deoxyribonucleic acid and other polynucleotides in ATP synthesis by isolated cell nuclei, *Proc. Nat. Acad. Sc.*, 1957, **43**, 589.
15. LEVERE, R. D., and GRANICK, S., preliminary observations.
16. GRANICK, S., and MAUZERALL, D., unpublished observations.
17. KRASSNER, S. M., Comparative enzymatic studies of culture and spleen forms of *Leishmania*, *Proc. 1st Internat. Congr. Parasitol.*, Rome, in press.