A second cylindrical lens (A) of slightly longer focal length than lens B is placed between the tube screen and the camera, with its axis crossing that of lens B at a right angle. The position of lens A is adjusted to focus the width of the tracing (analogous to the width of the slit in light-slit recording) and is not critical in adjustment. Lens B is not altered in any way and focusses the fineness of the hairline. Incidentally, the use of the camera with its cylindrical lens possesses the advantage of permitting various other phenomena to be simultaneously recorded by conventional rather than special methods. The number of other phenomena which may be so recorded is limited solely by the width of the photosensitive paper.

So simple is the arrangement and so lacking in critical adjustment that a test tube of the proper diameter filled with clear fluid can be used for lens A with satisfactory results, providing the excursion of the dot is not great enough to introduce spherical aberration.

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The Occurrence of Protoporphyrin in the Reticulocytes.

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Van den Bergh and Hyman[1] described the regular occurrence of protoporphyrin in the erythrocytes. This was proven to be preexistent and not simply derived from hemoglobin during the process of extraction. Subsequently, protoporphyrin was noted in megaloblasts and erythroblasts of the embryonic bone marrow, and of pernicious anemia marrow, by Borst and Königsdörffer.[2] By means of perfusion experiments, Van den Bergh, Grotepass and Revers[3] demonstrated that the surviving liver is capable of converting proto- into coproporphyrin. This led them to suggest that the erythrocyte protoporphyrin might be parent to the coproporphyrin of the

[1] Van den Bergh, A. A. H., and Hyman, A. J., *Deutsch. Med. Wchnschr.*, 1928, **54**, 1492.

[2] Borst, M., and Königsdörffer, H., Untersuchungen über Porphyrie, S. Hirzel, Leipzig, 223, 1929.

[3] Van den Bergh, A. A. H., Grotepass, W., and Revers, F. E., *Klin. Wchnschr.*, 1932, **11**, 1534.

bile, feces, and urine. They evidently assumed that this was coproporphyrin-III, corresponding in configuration to aetioporphyrin-III, hence to hemoglobin. However, coproporphyrin-I has been isolated from normal urine,[4, 10] bile and feces,[5] in increased amounts from the feces of patients with hemolytic jaundice,[6] and pernicious anemia,[7] as well as in most pathological urines having an increased porphyrin content.[4,5,8,9] Coproporphyrin I is not a hemoglobin derivative; the existing evidence[5, 6, 7] indicates that increased amounts of this substance are excreted when there is increased hemopoietic activity. Since the largest amounts have been encountered in hemolytic jaundice, a disease in which active regeneration is constantly manifested by an increase in reticulocytes, the possibility was suggested that the erythrocyte protoporphyrin of Van den Bergh resides in the reticulated cells. The fact noted by Key[11] that reticulocytes are lighter than other erythrocytes, permitted investigation of this question. By centrifuging various samples of blood of different reticulocyte content, upper and lower fractions containing many or few reticulocytes have been obtained; these were subjected individually to Van den Bergh's extraction procedure.[1, 3] The final solutions from each fraction, in equal volumes (2-10 cc.) of 5% HCl, or of ethyl acetate, were directly compared as to intensity of red fluorescence in Wood's light.[1] The light source consisted of a mercury arc lamp.* In this way it was found that the upper fractions regularly yielded a much more intense fluorescence than the lower. This is shown in Table I. The values in the column "amount extracted" indicate the actual fractions of the sample which were employed; thus, in the first instance the upper and lower thirds were extracted while the middle third was discarded. The values given in parentheses represent the amount of packed cells (one-half hour at 3000 r.p.m.) in the fraction. In instances 8-15 inclusive, the measured volume of cells recorded was taken from the top for purpose of comparison with cells from the bottom while the bulk of cells between was discarded. In a number of the experiments, the more fluorescent solution was diluted until the intensity of fluorescence was approximately the same in both solutions. For example, the solu-

4 Watson, C. J., *J. Clin. Investig.*, 1936, **15**, 327.

5 *Ibid.*, in press.

6 *Ibid.*, 1935, **14**, 110.

7 *Ibid.*, 1935, **14**, 116.

8 *Ibid.*, 1935, **14**, 106.

9 Dobriner, K., *J. Biol. Chem.*, 1936, **113**, 1.

10 Hoerburger, W., Inaug. Diss., Erlangen, 1933.

11 Key, J. A., *Arch. Int. Med.*, 1921, **28**, 511.

* Hanovia Analytic Model lamp, with Corning filter No. 587 (3200-4000 A.).

TABLE I.

Source of blood and reticulocytes	% before centrifuging	Amt. extracted after centrifuging		Reticulocyte % after centrifuging		Relative intensity of red fluorescence in final solution	
		Upper	Lower	Upper	Lower	Upper	Lower
1. Pernicious anemia (after peak of retic. response to liver extr.)	5.0	1/3	1/3	9.0	3.8	2	1
2. Famil. hemol. jaundice	8.3	1/3 (3.8cc.)	1/3 (3.8cc.)	14.3	3.0	5	1
3. Normal	1.2	1/4 (15cc.)	1/4 (15cc.)	3.0	0.2	Moderate	Absent
4. Famil. hemol. jaundice	19.5	1/3 (2.1cc.)	1/3 (2.1cc.)	29.0	13.2	3	1
5. " " "	20.0	1/3 (5.2cc.)	1/3 (5.2cc.)	30.0	9.0	Moderate	Faint
6. " " "	16.6	1/3 (3.6cc.)	1/3 (3.6cc.)	22.0	3.5	4	1
7. Hypertension	1.1	1/4 (25cc.)	1/4 (25cc.)	4.1	0.5	Moderate	Absent
8. †Coronary disease	2.0	5.5cc. (200cc.*)	5.5cc.	10.0	0.6	3.5	1
9. † " "	2.0	5.5cc. (45cc.*)	5.5cc.	8.8	0.3	Moderate	Faint
10. †Normal	1.2	5cc. (100cc.*)	5cc.	5.0	0.44	"	"
11. †Polycythemia vera.	1.6	6.1cc. (100cc.*)	8.6cc.	3.8	0.9	"	"
12. Pernicious anemia (after peak of retic. response to liver extr.)	9.0	1.2cc. (12.5cc.*)	1.2cc.	14.0	1.2	"	"
13. Pernicious anemia (after liver therapy)	31.0	2.5cc. (40cc.*)	2.5cc.	68.0	1.0	Intense	"
14. †Pernicious anemia (after liver therapy)	30.0	1.6cc. (38cc.*)	1.6cc.	80-85'	1.8	"	"
15. Pernicious anemia (after liver therapy)		2.5cc. (48cc.*)	2.5cc.	8.5'	0.7	Moderate	Absent

*Total amount of blood from which respective fractions were obtained.

†In these instances the upper and lower fractions as first obtained were centrifuged again; the amounts noted were then taken from the top of the upper, and bottom of the lower fractions.

tion from the upper fraction in case No. 2 was roughly 5 times as fluorescent as that from the lower fraction. In other of the experiments the intensity of fluorescence is simply described as intense, moderate, faint or absent.

It should be noted that greater red cell concentration (incident to packing) was present in the lower fractions; thus in cases 9 and 10 the amounts noted contained 10,760,000 and 11,200,000 R.B.C. per cmm., respectively, in the upper fractions, but 15,040,000, and 15,400,000 in the lower. The difference in fluorescence between upper and lower fractions was much more marked in instances where the initial reticulocyte counts were high, as in cases of hemolytic jaundice, or of pernicious anemia at the peak of reticulocyte response to liver therapy. The most marked difference in reticulocytes and fluorescence in the two fractions was observed in case 14.

By utilizing the same volume of packed erythrocytes (obtained by centrifuging at 2500 r.p.m. for one-half hour) it was possible to observe a marked increase in erythrocyte-protoporphyrin during the peak of reticulocyte response produced by liver therapy in pernicious anemia, with subsequent marked diminution as the count returned to normal:

a.	11-9-36	Retic.	3.6%	1.4cc.	cells, no fluorescence
b.	11-11-36	"	38.0%	1.4 "	" intense fluorescence
c.	11-23-36	"	1.7%	1.4 "	" very faint fluorescence

The most striking proof of the porphyrin content of the reticulocytes was obtained by administering phenylhydrazine to a rabbit; this was given either subcutaneously or intraperitoneally as follows: 1st day, 0.1 gm.; 3rd day, 0.1 gm.; 5th day, 0.1 gm.; 7th day, 0.2 gm.

On the eighth day the hemoglobin had fallen to 4.1 gm. per 100 cc., the erythrocytes to 1,670,000 per cmm. At this time the reticulocytes numbered 100% of the erythrocytes; after staining with brilliant cresyl blue no red blood cells could be seen, which did not contain at least a moderate amount of reticulated substance; the majority were heavily reticulated. Only occasional nucleated erythrocytes were noted. 0.48 cc. of packed cells from this blood were extracted according to Van den Bergh's method. The red fluorescence of the final 5% HCl solution (8 cc.) was the most intense that has yet been observed in solutions obtained from erythrocytes. The fluorescence of the final solution from 3.8 cc. of packed cells from another rabbit whose blood contained but 7% reticulocytes, was relatively very faint.

Participation of leukocytes or platelets in the production of fluor-

escence in the upper fractions was excluded by a separate extraction of the platelet-leukocyte coat; no fluorescence was demonstrable, although the extract of the underlying, upper strata of red blood cells, containing most of the reticulocytes in the sample, exhibited definite red fluorescence (carried out in case 11). The upper ⅓ (3.6 cc. packed cells) of a blood sample from a patient with pneumonia,who exhibited leukocytosis of 38,000, was extracted without any attempt to remove the buffy coat. The final 5% HCl solution was compared with that from the upper ⅓ (2.1 cc. packed cells) of case 4 (hemolytic jaundice; leukocytes 12,500). In the latter the reticulocytes were 29%, in the former 5%; the fluorescence from the hemolytic jaundice cells was obviously more intense. This constitutes further evidence that the leukocytes are not concerned in the production of fluorescence.

As a result of these findings it is believed that the reticulocytes contain most, if not all, of the protoporphyrin—which Van den Bergh noted in the erythrocytes.† Whether this is in reality the parent substance of the coproporphyrin-I of bile, feces, and urine remains to be determined. Certain findings described in a separate communication[5] support this possibility. A probable lack of strict correlation between reticulocyte percentage and amount of protoporphyrin may account for the failure to identify reticulocytes with "fluorescytes";[12, 13] nevertheless, Müller-Neff[14] has recently concluded that the latter are younger cells, and that an increase in their number indicates increased erythropoietic activity.

Protoporphyrin and brilliant cresyl blue have been found to be mutually precipitable; the addition of a very dilute (almost colorless) solution of crystalline protoporphyrin (from hemin IX), in phosphate buffered physiological saline having a pH of 7.4, to an equal amount of a 0.25% solution of brilliant cresyl blue in physiological saline, is followed by gradual precipitation of a dark blue, almost black substance. At first this appears in a finely divided, nearly imperceptible form; after several minutes the aggregates gradually become larger and sink slowly to the bottom of the tube. This precipitate is not crystalline; microscopically, it is quite similar in appearance to the material seen in reticulocytes stained with

† In the present investigation it was noted that the porphyrin obtained from the red cells has protoporphyrin characteristics, *i. e.*, that it is chloroform soluble and may be extracted from 5% HCl by chloroform; the amounts obtained have not been large enough for spectroscopic identification.

12 Keller and Seggel, *Fol. Haematol.*, 1934, **52**, 241.

13 Seggel, K., *Ibid.*, 1936, **54**, 374.

14 Müller-Neff, H., *Ibid.*, 1936, **56**, 18.

brilliant cresyl blue. Washing the precipitate with physiological saline or water removes but very little of either dye or porphyrin. After solution of the washed precipitate in glacial acetic acid and subsequent mixture with water and ether in a separatory funnel, the protoporphyrin is found in the ether and the brilliant cresyl blue in the aqueous fraction. This behavior, considered with relation to the weak acidic character of protoporphyrin and the basic nature of brilliant cresyl blue, suggests that the precipitate is a salt, and that the acetic acid in dissolving the precipitate, has replaced the porphyrin.

Protoporphyrin behaves similarly toward other basic dyes which have been used in staining reticulocytes, *viz.,* Nile blue sulphate, Janus green B, and methylene blue. Of interest is the observation that the precipitate with methylene blue is much smaller in amount, and appears more slowly; this dye was formerly used in staining reticulocytes, but is much less efficient than brilliant cresyl blue. Wright's stain does not differentiate the reticulated cells. No precipitation occurred in mixtures of this stain and a dilute solution of protoporphyrin. In each of the above instances where a dye was tested with protoporphyrin, control solutions of each substance were observed for the same period; no precipitation occurred. A dilute solution of coproporphyrin (I) has been similarly treated with brilliant cresyl blue without resulting precipitation. These various observations suggest a relationship between protoporphyrin and the supravital staining of the reticulocytes with basic dyes.

Summary. The protoporphyrin which Van den Bergh and Hyman discovered in the red blood cells, has been found to reside chiefly, if not solely, in the reticulocytes. Brilliant cresyl blue and protoporphyrin are mutually precipitable; whether this reaction is responsible for the supravital staining of reticulocytes remains to be determined.