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Comparative cytospectrophotometry of Wright-stained erythroid cells of vertebrates

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Summary. Visible spectra of erythroid cells stained with Wright's method were studied microphotometrically. Most mature cells of vertebrates have the main peak at 520 nm (A 520) and accessory peaks at 410 nm and 600 nm (A410 and A 600), respectively. To compare the absorption at 520 nm against 410 nm and 600 nm, the relative absorption spectra were determined and effectively shown as percentage ratios (RAs) to the main peak of A 520. The A520 shifted to the shorter side of the wavelengths in birds and lower vertebrates. When the RA values of 410 and 600 nm were compared, it was observed that RA 410 of immature cells was less than that of mature cells (15%), and that immature cells of the lower RA 410 type were present in peripheral blood, as well as in bone marrow of vertebrates. Thus, photometric analysis of erythroid cells at RA 410 and RA 600 against A 520 seems to provide useful parameters for comparative studies on vertebrate erythroid cells. Six spectral types can be observed in vertebrate erythroid cells by these parameters.

Key words: Cytospectrophotometry - Wright staining - Erythroid cell

Introduction

In hematological studies, cytophotometric measurements have been carried out mostly in the determination of nucleic acids and hemoglobin of blood cells (Jope, 1949; Whitfeld, 1952; Jonxis, 1956; Sano, 1958; Jobst and Sandritter, 1965; Thorell and Raunich, 1966; Rigler et al., 1969). Little is known about the spectrophotometric property of colour development in leucocytes stained by different standard methods such as Giemsa (1904) and Wright (1902). Spectrophotometric absorptions of heme and hemoglobin derivatives have been extensively used for species classification (Wilkins and Carvalho, 1953). Erythroid cytodiagnosis, on the basis of differences in colour development between Giemsa and Wright staining, has not been explored. The method we have already reported for the classification and characterization of different types of leucocytes (Yamada et al., 1975), seems to be applicable for cytodiagnosis of vertebrate leucocytes. In the present study, the above method has been used for the cytodiagnosis of erythroid cells on their cytospectral basis and for further classification of these cells into various spectral types.

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Materials and methods

Blood samples: Two ml of blood was withdrawn by a syringe containing a drop of Anglot/ET (Nippon Shoji Co., Osaka, containing 8% EDTA and 0.001% of heparin) to prevent clotting. Venous samples were drawn from animals, such as cow (*Bos taurus,* Gmelin), pig (Sus domesticus, Brisson), guinea pig (*Calvia* cobaya, Schreber) and fowl (*Gallus* domesticus, Brisson) and from the heart of small animals, such as gecko (Gekko japonicus, Dumeril et Bibon), lizard (Eumeces latiscutatus latiscutatus, Hallowell), snake (Elaph climacophora, Boie), frog (Rana nigromaculata nigromaculata, Hallowell), tortoise (Clemmys japonica, Temminck et Schlegel), tuna (Thunnus orientalis, Temminck et Schlegel) and eel (*Anguilla* japonica, Temminck et Schlegel). To separate erythrocytes from leucocytes, the whole blood

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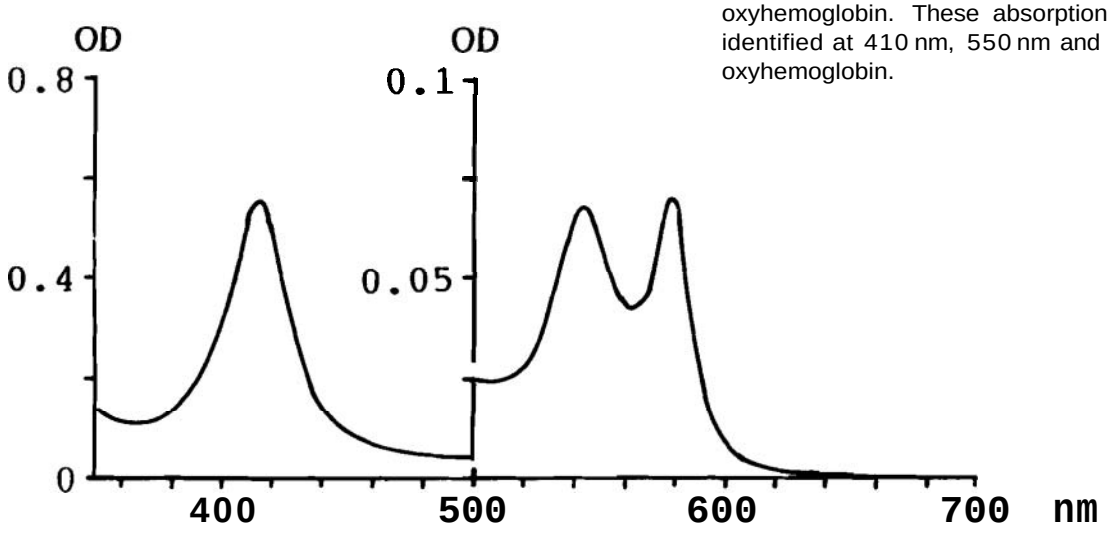


Fig. 1. Absorption spectrum of human blood oxyhemoglobin. These absorption peaks are identified at 410 nm, 550 nm and 580 nm for oxyhemoglobin.

was centrifuged at 1000 rpm for 5 minutes, and the lower layer of the pellet was diluted with four volumes of the clear supernatant blood plasma to enable erythroid cell suspension to take place.

A thin layer of blood smear was prepared on slide glass, air-dried, fixed in methanol and then treated according to our previous report (Yamada et al., 1975) and subsequently stained by Wright's method (Wright, 1902). Optimal staining was carried out by treating the specimens with 1 ml of Wright's solution (Merck) for 2 minutes, followed by 1 ml of 10 mM phosphate (pH 6.4) for 5 minutes. The rest of the procedure was carried out by routine methods (Lillie, 1948).

Cytospectrophotometry: In our previous reports on cytospectral studies of leucocytes (Yamada et al., 1975; Takeuchi and Yamada, 1976), the wavelength scanning method was used for measurement of single leucocytes on the objective plane of a microspectrophotometer.

For the present purpose, a Zeiss UMSP-1 spectrophotometer was used for the wavelength scanning with projection beams of various diameters on the objective plane (Caspersson, 1955). The relative absorption (RA) was obtained as the percentage ratio (A/A max, %) of each absorption (A) to the maximum absorption (A max). The A max was empirically chosen from the absorption near 520 nm in various erythroid cells, and A at 370 nm was fixed at 0% as described previously (Yamada et al., 1975). The RA curves of erythroid cells obtained from various vertebrates were afterwards compared.

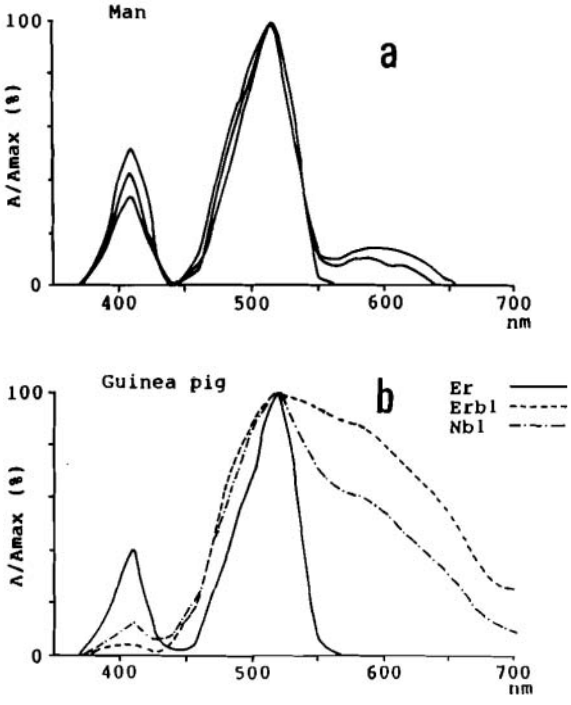


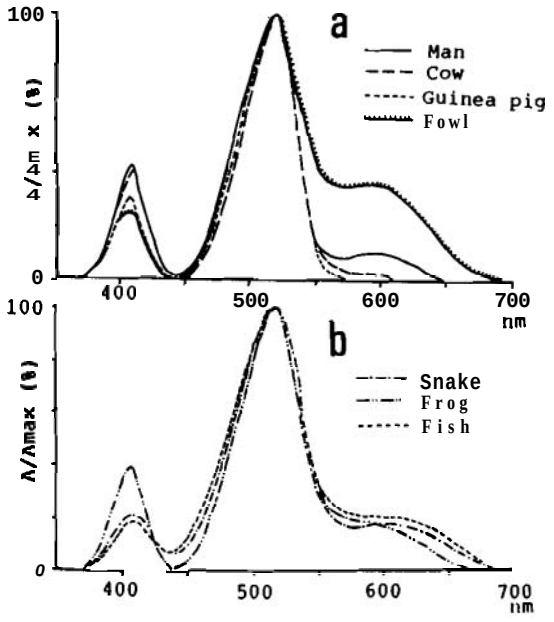
Fig. 2. Relative absorption (RA) curves of mature (Er) and immature erythrocyte from man and guinea pig. The upper figure shows RA curves of three human erythrocytes. The lower figure shows RA curves of the mature and immature erythrocytes from guinea pig bone marrow. The RA 600 of the erythroblast (Erbl) and normoblast (Nbl) is higher than that of the immature erythrocytes.

Results

7. Relative absorption (RA) curves of erythrocytes.

Vertebrate erythrocytes containing hemoglobin reveal specific absorption in the visible region of spectra (Van Assendelft, 1970). In the case of human oxyhemoglobin,

Fig. 3. Relative absorption (RA) curves of single erythrocyte found in peripheral blood of various vertebrates. In the upper figures, the RA 600 of fowl erythrocyte is markedly higher than that of mammals. The lower figures show the RA 600 values from lower vertebrates.



there were three close peaks at 410 nm (A 410), 550 nm (A 550) and 580 nm (A 580) (Fig. 1). The A 410 was ten times higher than the A 550 and A 580. Thus, A410 appears to be much more sensitive to the amount of hemoglobin than A 550 and A 580.

Visible spectra of erythrocytes in the peripheral blood were individually different in the absorption peaks at about 520 nm. When the absorption curve is represented as a percent ratio of the absorption (A) to the maximum absorption (A max), this A /A max (%) curve is called the relative absorption curve (RA). The RAs obtained from erythrocytes were compared to A 410, A 500 and A 600, respectively (Fig. 2a). At A 410, all three erythrocytes showed three different peaks. At A 500 they converged at one peak, whereas at 600 nm only two showed about 15% absorption. A high A 600 appeared bluish with Wright's staining, indicating a basophilic nature. This basophilia was more evident in the immature erythrocytes, such as the normoblast and erythroblast in the bone marrow of guinea pig (Fig. 2b). The mature erythrocyte of the guinea pig showed peak absorption at A 520, comparable to human erythrocyte.

2. Comparison of the RA curves of vertebrates.

Fig. 3 shows the RA from different animals at different spectra. In man, cow and guinea pig, a slight variation in RA could be seen at RA 410 and at RA 600, respectively. In the case of fowl, a 40% increase in absorption could be observed at RA 600. As A 600 represented the degree of basophilia, the high RA 600 in fowl was due to the presence of nucleus in erythrocytes. This phenomena can be clearly observed in the lower graph (Fig. 3b), which represents the absorption spectra from nucleated erythrocytes of lower vertebrates. It can be further seen that frog erythrocyte had a maximum absorption at A 410, whereas at A 600, all three showed more or less the same absorption pattern.

The different degrees of basophilia in relation to the maturity of erythroid cells of the snake are compared and represented in Fig. 4a. The erythroblast showed a lower

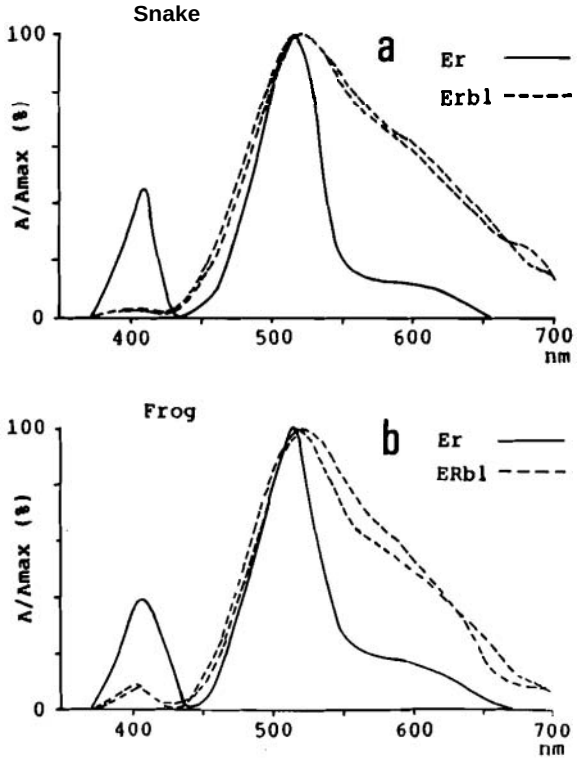


Fig. 4. Relative absorption (RA) curves of single erythroid cells in peripheral blood of snake and frog. There are varieties of the RA 600 and RA 410 in both snake and frog. the increase of RA 600 and decrease of RA 410 represent the presence of immature erythroid cell types.

absorption at A 410 and a higher one at A 600; this increase in RA was more than 60%. A similar relationship could be seen in frog erythroid cells (Fig. 4b).

3. Classification of the spectral types.

The comparative spectra studied from erythrocytes of different animals helped to categorise the animals under three parameters of wavelength maxima as (A max), RA 410 and RA 600, represented in Table 1. Under A max, the deviation of 510 nm was further classified into two groups, (1) between 510-515 nm and (11) between 516-524 nm. This was again combined with A 410 absorption of more than 16% arid less than 15%; A 600 absorption of more than 40% and less than 39%. With these combinations, six different subtypes emerged, as follows: A max in 516-524 nm, RA 410 (>16%), and RA 600 (>40%) (type I-a-H); A max in 516-524 nm, RA 410 (<15%), and RA 600 (>40%) (type I-a-L); A max in 516-524 nm, RA 410 (>16%), and RA 600 (<39%) (type 1-b-H); A rnax in 516-524 nm, RA 410 (<15%) and RA 600 (<39%) (type 1-b-L); A max 510-515 nm and RA 410 (>16%) (type II-H) and A rnax in 510-515 nm and RA 410 (<15%) (type II-L).

The erythroid cells of vertebrates can be classified in reference to the above spectral types (Table 2). Most mammals were distributed in the spectral type fixed as I-b-H. However, the spectrum of cow represented only the type I-a-H, even in matured erythrocytes. The immature type of the guinea pig bone marrow represented a shift to the type I-a-L from the type I-b-H of the adult type. In fowl blood, the erythrocytes represented the type II-H which is stained bluish, due to the presence of nucleus. In reptilia, such as gecko, lizard and snake, the spectra showed the type II-H. In amphibia, such as frog and tortoise, the spectra showed the type II-H; this was also the case with tuna and eel. In most vertebrates lower than reptilia, it was common to find immature erythroid cells in the peripheral blood. In eel, the mature erythroid cell was not only identified in the fixed type as the II-H, but also in the II-L.

Table 3 represents the RA 410 as a parameter of the amount of hemoglobin ratio to A rnax. It shows the distribution pattern of RA 410 in different animals, based on the amount of Hb. In mammals, the range varied from 25 to 60% with an average of 45%. The percentage fell to 15% in immature erythrocytes and bone marrow cells. In fowl, the absorption percentage was less than 30%. In mature erythrocytes of gecko and snake, the RA 410 was distributed at a wider range than in lizard and, moreover,

Table **1.** Spectral types of vertebrate erythroid cells.

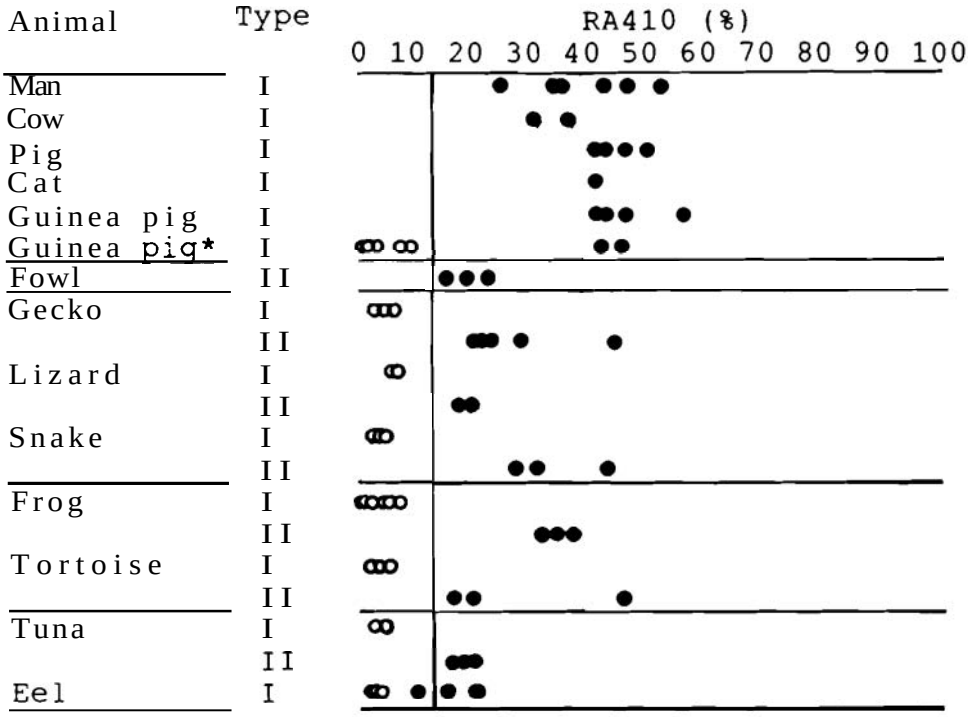
TYPE	A max (nm)	RA 410 (%)	RA 600 (%)
I-a-H	516-524	>16	>40
L		<15	>40
I-b-H		>16	<39
L		<15	<39
II-H	510-515	>16	
L		<15	

Table **2.** Relationship between spectral types and cytodiagnosis of vertebrate erythroid cells.

ANIMAL	INDIVIDUAL	SPECTRAL TYPES					
		I-a-H	I-a-L	I-b-H	I-b-L	II-H	II-L
Man	6			E			
Cow	3	E					
Pig	4			E			
Cat	1			E			
Guinea pig	4			E			
Guinea pig"	4		I	E			
Fowl	3					E	
Gecko	4		I			E	
Lizard	2		I			E	
Snake	3		I			E	
Frog	3		I			E	
Tortoise	2		I			E	
Tuna	3				I	E	
Eel	3					E	I E

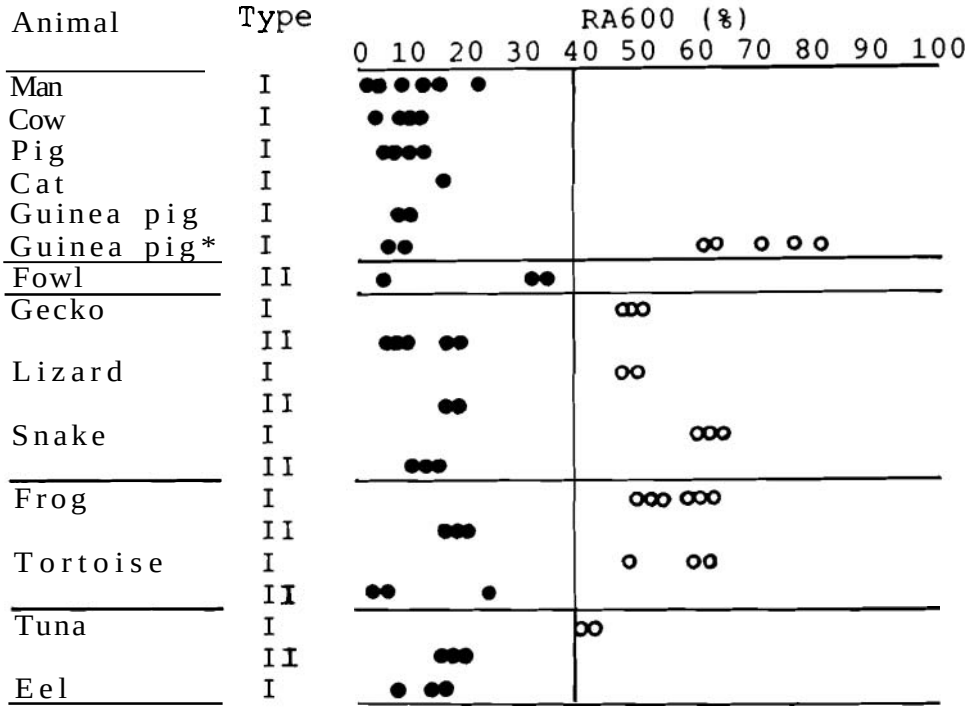
E: erythrocyte, I: immature erythrocyte, ": bone marrow.

Table 3. Distribution of RA 410 in vertebrate erythroid cells.



●: erythrocyte, o: immature erythrocyte, *: bone marrow

Table 4. Distribution of RA 600 in vertebrate erythroid cells



●: erythrocyte, o: immature erythrocyte, *: bone marrow

very low values (less than 15%, identified as immature types) were mixed. All immature types represented type 1. In tuna and eel, the RA 410 value was less than in fowl, the percentage distribution being around 20%. The lowest values, of less than 10%, were also obtained in immature erythroid cells of tuna and eel. Comparison of RA 410 among vertebrates showed a demarcation line at 15%, situating all the immature peripheral erythroid cells and bone marrow erythrocytes below the 15% level.

The other parameter, the RA 600, was considered to be due to basophilia in the nucleated cells. In mammals which have non-nucleated mature erythrocytes, the RA 600 was concentrated in an average value of 10% and ranged from zero to 23% (Table 4). Against these low values of mature cells, immature cells of the bone marrow showed a value higher than 60%, as in guinea pig. Thus, the RA 600 seems to be a reasonable parameter to identify the maturity of erythrocytes. In the mature cells of lower vertebrates, the RA 600 was rather higher than that of mammals and it ranged around 20% in average. It ranged up to 50% or more in immature cells identified in reptiles and amphibia. However, the highest value was obtained in immature cells of guinea pig bone marrow. The RA 600 of mature cells of tuna and eel represented about 20% in average, but increased to 40% in immature cells. From these findings, a clear border line could be put at the 40% level, below which most immature cells of vertebrates disperse.

Discussion

Previous studies (Yamada et al., 1975; Takeuchi and Yamada, 1976) have shown Wright's method (Wright, 1902), in combination with cytospectral analysis, to be one of the most suitable methods in leucocyte typing (Yamada et al., 1976; Takeuchi and Yamada, 1976). In these studies, the background absorption was kept minimal when blood cell smears were subjected to Wright's method at pH 6.4, fixed spectra being used throughout. For the purpose, a relative absorption (RA) curve, was used with the absorption maxima (A max) being fixed at 100% and A 370 at zero %. The RA curve proved suitable even for erythroid cells of lower vertebrates containing nuclei.

In the present study, we have attempted to identify immature erythroid cells from mature erythrocytes in circulation. In mammals, the erythrocytes are anucleate cells. In vertebrates lower than fowl, even mature cells are nucleated (Forkner, 1929; Bloom and Fawcett, 1975). This was clarified by the spectral change in the RA. Furthermore, basophilia overlapped in immature cells during the formation of hemoglobin (Yamada, 1985). Details of hemoglobin formation in a single cell have been studied by many authors (Thorell, 1947; Thorell, 1955; Seno, 1957; Matioli and Thorell, 1963). Most hemoglobin synthesis begins in the reticulocytes and reaches a maximum in the early normocytes (Nakao et al., 1957; Seno et al., 1957; Sano, 1958). The relative increase of hemoglobin, known as erythropoiesis (Maximow, 1937; London et al., 1950; Jonxis, 1956), was expressed in the value of RA 410 which was regarded as a parameter of hemoglobin amount to the A max. It was reasonable to estimate the relative amount of hemoglobin in such immature cells (Fig. 2) as the normoblast and erythroblast of guinea pig and cow (Yamada et al., 1975). This relation was compared among erythroid cells of peripheral blood in various animals. In peripheral blood lower than reptiles, the RA 410 lower than 15% was often mixed up with a higher RA 410 of the erythroid cells. The distribution of RA 410 seemed to be wider in erythroid cells of lower vertebrates and widest in those of fishes. Consequently, the RA 600, which is a parameter of basophilia, was higher than 40% in erythroid cells of vertebrates lower than reptiles. These findings indicate that immature erythroid cells are mixed even in the peripheral blood of lower vertebrates, as previously reported elsewhere (Dawson, 1933; Jordan, 1938). Thus in lower vertebrates, the appearance of immature cells is accompanied by higher basophilia and less hemoglobin (Jordan, 1919; Dawson, 1933; Barret, 1936; Jordan, 1938; Buthie, 1939; Catton, 1951; Andrew, 1965).

The specific absorption of hemoglobin was measured and distinguished from the other absorption to identify growing erythroid cells. There were marked differences between normoblasts and normocytes. Value of A 410 was higher in normoblasts than in normocytes, and value of A600 was much higher in erythroblasts than in normocytes. These differences have been previously demonstrated by us in the nucleate erythroid cells found in the bone marrow embedded in bovine heart (Yamada et al., 1975), in which A 600 was regarded as a parameter of cytoplasmic basophilia. The A 600 value helped in distinguishing young erythroid cells from normoblasts. It was found that, in lower vertebrates, immature erythroid cells coexisted in the peripheral blood along with mature erythroid cells. This type of relationship has already been suggested in fish species (Dawson, 1933; Duthie, 1939; Tomonaga et al., 1973).

In conclusion, erythroid cell types were classified by spectral analysis according to the amount of hemoglobin and basophilia. In the previous reports in combination with Giemsa and Wright stainings, these kinds of qualitative studies on the polychromatic colour of erythroid cells were lacking. As we have indicated in the present study, it is striking to identify the spectral types even by a few parameters. It is clear that most erythroid cells of lower vertebrates are much more basophilic in the same relative parameter as A 600 in contrast to the maximum absorbance at about 520 nm which relates to eosin.

Thus, these findngs are useful to identify by spectral analysis the erythroid cell types of various vertebrates stained by a standard technique such as the Wright's method.

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