Kleinberger and Carl (1912) counted the erythrocytes, leucocytes and thrombocytes in the counting chamber, and then calculated their respective values from the ratios of these cells found in stained smears.

The indirect methods are obviously subject to great error for they presuppose an absolutely even distribution of the cellular elements : this rarely obtains, as Forkner (1929) and others have pointed out.

Blain (1928) was the first to introduce a direct method for the purpose of staining the leucocytes differentially so that they could be counted in the counting chamber. He utilised the vital staining properties of neutral red and prepared two solutions :- Solution 1 contained neutral red (1 : 5000), made up in Locke's solution, and solution 2 contained 12% Formalin, also made up in Locke's solution. Both solutions were adjusted to a pH of 7.4, and kept at a temperature of 39°C. while in use. The blood was first mixed with the neutral red solution, and the other solution was then added - the white cells take up the neutral red, whereas the red cells do not.

According to Shaw (1930), Blain's method when applied to human blood gives results 20% to 30% below those obtained by the acetic method. Ohlson (1935) also found lower leucocyte counts with this method than with the others which he tried, and it seems that it is chiefly the lymphocytes which are overlooked by this method. It would also appear that Blain included the thrombocytes either in the red cell or the white cell count, since he does not mention these and in a further communication (1928) already referred to he states that he identified no structures in avian blood corresponding to the blood platelets of mammals.

Coates (1928) recommends the solutions which Wright and Kinnicut (1911) used for counting platelets in

/human .....

Digitised by the University of Pretoria, Library Services, 2012

human blood. Two solutions are employed : Solution 1 contains 1 gm. brilliant cresyl blue in 300 c.c. distilled water, and solution 2 contains 1 gm. potassium cyanide in 1400 c.c. distilled water. Equal parts of the two solutions are mixed and filtered immediately before use and the resulting solution constitutes the diluting fluid. With subdued illumination, the leucocytes are said to refract the light in such a manner as to render direct counting possible.

Ohlson (1935) found the method unsatisfactory, as the distinction was not well marked between the thrombocytes and the small lymphocytes.

Kyes (1929) recommended a 2% solution of osmic acid to eliminate the possibility of red cell haemolysis and consequent liberation of nuclei.

Ohlson (1935) states that there is a danger, in this method, of injury to the cornea of the observer, and that no particular advantages were apparent in the trials made.

Forkner (1929) used a diluting fluid, containing 25 mg. neutral red in 100 c.c. of 0.9 per cent. sodium chloride solution, and states that the polymorphonuclear granulocytic cells and the monocytes can easily be differentiated from the other cells; the red cells with little or no haemoglobin, the thrombocytes and the lymphocytes remaining almost entirely unstained. The proportion of granulocytes and monocytes is then determined from a blood smear, and the total leucocyte count is calculated.

Wiseman (1930) states that with Forkner's method the difference in staining intensity of the monocytes frequently occasions inaccuracies through the missing of individual cells and that diluting fluids, which do not

/contain .....

Digitised by the University of Pretoria, Library Services, 2012

contain a fixative, often fail to preserve the erythrocytes intact. Cook and Dearstyne (1934) used this method, but made no comment on its efficacy.

Hayden (1927) used Toisson's solution, but mentions that the method was not satisfactory. Furth (1931), however, remarks that in his experience lymphocytes and thrombocytes *can* be easily differentiated in Toisson's solution. The solution has the following composition : Distilled water 160 c.c., glycerine (neutral) 30 c.c., sodium sulphate 8 gms., sodium chloride 1 gm., methyl violet 5B 0.25 gm.

Kozma (1929) also counted all three types of cells in the counting chamber, using the following solution : Sodium citrate 0.2 gm., ammonium oxalate 0.1 gm., distilled water 100. To this solution is added 8 c.c. of a one per cent. solution of eosin and 4 c.c. of a one per cent. trypanblue solution, and staining of the blood is allowed for 30 minutes before counting is commenced.

In Shaw's (1930) method, 2 solutions are used. Number 1 contains 25 mg. neutral red (Grubler), 0.9 gm. sodium chloride and 100 c.c. distilled water. Number 2 solution contains 12 mg. crystal violet, 3.8 gm. sodium citrate, 0.4 c.c. formaldehyde and 100 c.c. distilled water. Both solutions are filtered and heated to 107°F. for use; the pipette is half filled with No. 1 solution and then filled with solution No. 2. Shaw's observations were made on Homer pigeons, and all the cellular elements were differentiated by their staining reactions in the diluting fluid.

Palmer and Biely (1935) consider Shaw's method satisfactory for the enumeration of the leucocytes in fowl blood, but Ohlson (1935) is of opinion that it is no easier to distinguish the thrombocytes by this method than in

/Blain's .....

Digitised by the University of Pretoria, Library Services, 2012

Blain's or in Toisson's solution. However, he does not state to what extent he considers the discrimination of the thrombocytes possible by Blain's method or in Toisson's solution, but Shaw himself thinks that the thrombocytes cannot be distinguished with certainty from lymphocytes in Toisson's solution, and according to Forkner (1929) the thrombocytes may be confused with the lymphocytes in Blain's method.

Wiseman (1930) recommends the use of a solution having the following formula : 95 c.c. Ringer's solution 5 c.c. formalin and 50 mg. phloxine. This solution stains the granulocytes a bright red in contrast with the other cells which stain much less brilliantly.

The total leucocyte count is then obtained by calculation of the percentage of these cells in the differential count. Wiseman states that maximum staining is obtained within one hour, but suggests that where time is not a factor of importance less phloxine should be used as more dilute solutions (3 mg. dye in 100 c.c. solution) stain the granulocytes almost specifically and the contrast is then at its best, but then a time interval of at least 3 hours is required.

Magath and Higgins (1934) consider that in none of the direct methods are the leucocytes stained in such a manner as to be always distinguishable from the erythrocytes.

Emmel (1935) counted the white cells in a 0.85 per cent. sodium chloride solution tinted with gentian violet, but he says : "There is often difficulty in differentiating the lymphoid precursors of erythrocytes, young erythrocytes, and occasionally basophilic erythroblasts when counting erythrocytes and leucocytes in the same chamber."

From this review of the methods advocated for avian leucocyte counting, it will be evident that, as there is no

/unanimity .....

Digitised by the University of Pretoria, Library Services, 2012

unanimity of opinion about them, the writer had to select one most suitable according to his own observations. After trying them all, he reached the following conclusion : By none of the direct methods could the leucocytes in ostrich blood be satisfactorily distinguished in the counting chamber from haemocytoblasts, basophil erythroblasts red cell nuclei or from the thrombocytes.

The methods of Blain and Shaw are also impracticable when counts have to be made from adult ostriches, which cannot be brought into or very near a laboratory, for the diluting fluids have to be at body temperature when mixed with the blood and when counts are being made. When the blood has to be collected eight miles away from the laboratory, as had to be done in this case - for there were no facilities for keeping the ostriches nearer - it is very difficult to conform to such conditions.

The writer's observations on the counting of the leucocytes of ostrich blood in Forkner's solution coincide with Wiseman's on fowl blood.

Wiseman's solution is preferred for leucocyte counting in ostrich blood for the following reasons : The granules in the granulocytes stain in such a distinctive manner, that these cells can be readily identified. This counting fluid is particularly suitable for counting white cells in ostrich blood, since ostrich blood contains a high percentage of granulocytes. The cells are stained satisfactorily within a short time and they are preserved for a long time. Counts made weeks later agreed, within limits of counting errors, with those made shortly after mixing. The counts can, therefore, be made at leisure at any convenient time. Since the staining qualities of the solution do not deteriorate, it can be prepared in large quantity if the bottle is kept well corked - to prevent

/evaporation .....

Digitised by the University of Pretoria, Library Services, 2012

evaporation of the formalin - and shaken every time before use.

Ohlson (1935), who made a comparative study (on fowl blood) of various methods in order to gauge their accuracy for both erythrocyte and leucocyte counts, found that Blain's method gave the most consistent results for leucocytes, Wiseman's method being placed third. He favours Wiseman's solution for use when dealing with pathological blood, stating that all the direct methods failed with the blood of an erythroleucotic chicken - the immature erythrocytes being confused with leucocytes - whereas in Wiseman's solution all the haemoglobin - bearing cells are stained in a characteristic manner; also that Wiseman's technique gave the lowest coefficient of variation in the erythrocyte counts and that it showed a tendency to give higher red cell counts. He also remarks: "An explanation for the tendency of the Wiseman method to give higher erythrocyte and leucocyte values is lacking. The erythrocytes are fixed and more distinctly stained than with the other methods." (The writer used Wiseman's solution as diluent for erythroleucotic fowl blood but also in this solution basophil erythroblasts and lymphoid haemoblasts could not be differentiated from non-granular leucocytes.)

Wiseman's solution was used in making both the erythrocyte and the leucocyte counts, but the solution was slightly modified by substituting 0.9 per cent sodium chloride solution containing 20 mg. sodium bicarbonate per 100 c.c. for Ringer's solution. The modified Wiseman diluting fluid, therefore, has the following formula: Phloxine 50 mg., sodium bicarbonate 20 mg., sodium chloride 0.9 gm., formalin 5 c.c., distilled water 95 c.c.

This solution proved as satisfactory as that containing Ringer's solution and its preparation entailed less work. (Locke's solution is isotonic for ostrich blood and in Wiseman's solution it is also a suitable substitute for Ringer's solution). Staining was allowed for at least two hours before the cells were counted.

The....

Digitised by the University of Pretoria, Library Services, 2012

The staining qualities of the phloxine are affected by the concentration of sodium bicarbonate in the diluent. If 50 mg. sodium bicarbonate are used instead of 20 mg. in the modified Wiseman solution, then all the cells, except the granulocytes, stain only faintly, and if staining of the granulocytes is allowed for at least four hours the contrast between the bright red granulocytes and the other cells is very marked. It is possible to recognise unstained granulocytes, but counting is greatly facilitated when the cells are well stained.

Turbidity may appear in an old sample of diluent, but this can be easily removed by adding a small quantity of sodium bicarbonate.

A difficulty with all the methods is that solutions sufficiently concentrated to preclude a large counting error cannot be used. With all the methods the erythrocytes are preserved and when the red cell count is somewhat high it is necessary to use dilutions of 1 in 200.

However, in normal ostrich blood - though the red cells are comparatively large - counts can be made with ease in dilutions of 1 in 100. Dilutions of 1 in 50 are also suitable but it was customary also to count the red cells in the same chamber and in this concentration accurate counting is difficult and, if prolonged, a severe strain on the observer's eyes as the cells are then close together. But as the numerical error resultant on multiplication is halved by using the 1 in 50 dilution it may be used with advantage when the intention is to count the granulocytes only, for the erythrocytes are not so close together as to obscure the granulocytes.

It was customary always to have ready for use a number of bottles each having a capacity of a little over 100 c.c. and containing 99 c.c. of the diluent, for the

dilutions.....

Digitised by the University of Pretoria, Library Services, 2012

dilutions were effected by using 1 c.c. blood to 99 diluent. Only pipettes and burettes were used for which certificates of accuracy had been issued by the United States Bureau of Standards.

Another drawback which the writer has not seen mentioned by previous workers is the marked tendency which the leucocytes - both in ostrich blood and also in fowl blood - show to clump after the blood has been drawn. The intention at first was to collect the ostrich blood in an anticoagulant and to make the subsequent dilutions when convenient, as Neser (1923) did. But it was found that, unless the blood is mixed with the diluting fluid immediately after it has been drawn, clumping of the white cells and particularly of the heterophiles is so marked as to vitiate the count; as many as 20 or more leucocytes may be together, usually intermixed with thrombocytes. When the mixing takes place immediately after the blood has been drawn, clumping is usually absent or only slight.

The best results were obtained when a red cell pipette was used, for then the interval between the drawing of the blood and the mixing was reduced to a minimum, but using a pipette on the intractable ostrich is usually so difficult that it may be considered impracticable.

It was ascertained that clumping will also take place in fowl blood if there is some delay in filling the pipette, or if the blood is first received into an anticoagulant. Many diluting fluid besides Wiseman's and Wiseman's (modified) were tried, namely, Hayem's, Toisson's, Tyrode's, Locke's, Ringer's, 0.9 per cent. sodium chloride and also the bird's own plasma, but clumping was no less marked in any of them.

The bottle containing the blood and the diluting

fluid.....

Digitised by the University of Pretoria, Library Services, 2012

fluid was usually shaken for about three minutes before the fluid was introduced into the chamber, but even shaking for longer periods had no effect in reducing the clumping; on the contrary it often increased it. The fluid was also shaken with beads, but this also proved ineffective. Blood, allowed to stand for half an hour, was added to a 1 per cent. solution of acetic acid, but clumping was marked. Human, equine and bovine blood, similarly treated, did not show agglutination, so that this quality seems to be characteristic only of avian blood.

All the anticoagulants mentioned under "Preventing Coagulation of the Blood" were tried but without success in overcoming this disadvantage. The mixing had, therefore, to be always made at the place of bleeding. When only the red cells have to be counted the blood may be diluted hours later if kept cool in the interim.

Forkner (1929) mentions the rapidity with which coagulation takes place in the blood of the fowl as one of the difficulties in securing an accurate count of the white cells. The writer experienced no difficulty in filling the pipettes when making counts of fowl blood, as the blood never coagulated before it could be properly diluted, but it was observed that, when the dilution of fowl blood did not take place immediately, clumping was at times also marked. It may have been this that was responsible for the difficulty which Forkner experienced, rather than that fowl blood coagulates too soon.

At first a Burker counting chamber was used, but later it was replaced by a Levy-Hausser counting chamber, guaranteed to be within the tolerance for accuracy established by the United States Bureau of Standards. This counter,

which.....

Digitised by the University of Pretoria, Library Services, 2012

which is recommended by Kolmer and Boerner (1931), has features which make counting much easier and which are not found with some other counters.

The total number of granulocytes in at least four of the entire ruled areas, viz., in 3.6 c.mm. of the mixture, was always counted and so were all the red cells in at least 0.12 c.mm. Errors due to smallness of samples were thus reduced to a minimum. The counting of the red cells was spread over four of the entire ruled areas, the two rectangular areas, each measuring 3 mm. x 0.05 mm. and crossing each other in the centre of the ruled area, being used, i.e., the cells, in 0.03 c.mm. of the mixture were counted in each chamber. In this way a better average count check was ensured.

The difference between 2 sets of red cell counts thus made seldom varied by more than 5 per cent. but differences up to 10 per cent. were noted in spite of the utmost care in making the preparations as recommended by Neser (1923) and others to ensure accurate counting, Marked differences were sometimes obtained when smaller samples were counted or when the count was spread over two ruled areas only.

All the diluting fluids mentioned on page 82 were tried to see whether more regular distribution of the red cells could be obtained but none of them proved to be better than the diluent used.

Neser's method of using an ordinary pipette drawn from glass tubing for filling the counting chamber was adopted for ostrich blood. The point should be cut in

Digitised by the University of Pretoria, Library Services, 2012

such a way that the fluid flows readily in between the two surfaces but without flooding the chamber when the forefinger is slightly withdrawn from the upper end of the pipette.

It was found most convenient to use a 6 x ocular and an 8 mm. lens and a strong white light for illumination.

The leucocyte cell counts are given in tables 3 - 10 and a statistical analysis of the data is shown in table 19. The results show that the mean leucocyte count of all the results from the normal birds (1 - 5) was 21,050 $\pm$ 419 with a range of 12,326 to 32,273 (standard deviation 3482; coefficient of variability 16.5 per cent.).

It will be seen that the coefficient of variability is much higher than that for the erythrocytes. Palmer and Biely (1935), who made leucocyte counts of 100 normal S.C. white Leghorn hens, reported a coefficient of variability of 20.77 while, according to these writers, the leucocyte counts made by Cook and Dearstyne (1934) on 75 normal Rhode Island Red and Barred Plymouth Rock fowls show a coefficient of variability of 55.09.

The average leucocyte count for bird No.6 is comparatively high and so is the coefficient of variability of the results from this bird. It

~~It~~ therefore appears as if the leucocyte count was affected by the defect from which the bird suffered.

The clinically healthy birds (7 - 17) showed a mean leucocyte count which is significantly lower than that of the normal birds and the coefficient of variability of their counts is comparatively high. The results from

the....

Digitised by the University of Pretoria, Library Services, 2012

the worm-infested birds (18 - 22) fluctuate considerably between the figures 8,697 and 30,033.

As in the case of the erythrocyte counts, the leucocyte counts cannot be correlated with the age or the sex of the birds.

Hayem (1879) recorded for the ostrich a leucocyte count of 9,000 per c.mm. (table 1).

DIFFERENTIAL COUNTS.

In the differential counts at least 400 cells were always counted in each case and the counts were recorded on sheets as were used by Neser (1923).

Table 20 shows sixteen differential counts. These were obtained from the same smear prepared from ostrich blood by counting 100 leucocytes each time. A statistical analysis of these counts which can be regarded as typical gives the following results for example in respect of the lymphocytes and the heterophiles: By counting 100, 200, 300 and 400 leucocytes the chances are 20:1 that the errors associated with the lymphocyte counts are respectively 8.0, 5.1, 4.6 and 4.0; the errors associated with the heterophile counts are respectively 11.2, 8.0, 6.4 and 5.6. It is evident that by counting 400 leucocytes, fairly accurate results may be expected.

Table 20.....

Digitised by the University of Pretoria, Library Services, 2012

TABLE 19.

Statistical analysis of leucocyte counts.

Bird No.[1]	Sex	No. of counts	Minimum count per c.mm.	Maximum count per c.mm.	Mean count per c.mm.	Standard ~~error~~ of the mean.	Standard deviation.	Coefficient of variability.
1	Male	15	12326	29255	21985	846	3279	14.9
2	"	15	13157	24751	19859	829	3210	16.1
3	"	12	19436	31303	22708	655	2267	9.9
4	Female	15	14157	32273	20733	974	3773	18.1
5	"	13	15880	27495	20179	798	2875	14.2
1,2 and 3	Males	42	12326	31303	21432	489	3172	14.8
4 and 5	Females	28	14157	32273	20476	736	2892	19.0
1 to 5	Males & Females	70	12326	32273	21050	419	3482	16.5
6	Female	14	17422	45372	29562	2441	9130	30.8
7 to 11	Males	5	8409	16146	13125	1439	3166	24.1
12 to 17	Females	6	7868	24570	14066	2449	5878	41.7
7 to 17	Males & Females	11	7868	24570	13638	1264	4172	30.5
18 to 22	Males & Females	6	8697	30033	17210	2803	6729	39.0

1) For particulars re birds see pages 9-11.

Digitised by the University of Pretoria, Library Services, 2012

TABLE 20.

Differential Counts from same smear prepared from ostrich blood.

	Per cent.				
	Lymphocytes	Monocytes	Heterophiles	Eosinephiles	Basophiles
	31	5	46	7	11
	38	7	41	4	10
	32	5	47	3	13
	26	1	52	7	14
	28	3	52	6	11
	26	5	56	6	7
	28	1	59	5	7
	27	5	55	5	8
	20	4	61	4	11
	30	4	49	8	9
	26	3	56	6	9
	22	5	59	11	3
	30	6	45	8	11
	29	5	48	1	17
	32	2	46	5	15
	25	5	53	7	10
Av.	28.1	4.1	51.5	5.7	10.3

The differential counts were always made diagonally across the smear, and an Ehrlich eye-piece was used for limiting the field, thus rendering critical observation of the cells at the edges of the field possible.

The differential counts are tabulated in tables 3 - 10 and statistical analyses are shown in tables 21 - 25. Examination of the results shows that the lymphocyte counts of the normal birds (1 - 5) varied from 19.2 to 37.0 per cent, averaging 26.8 ± 0.5 per cent. (standard deviation 4.1; coefficient of variability 15.3 per cent.).

The average lymphocyte count of birds 1 - 5 is significantly higher than that of birds 7 - 17.

The monocyte counts of all the results from birds 1 - 5 varied from 0.5 to 8.5 per cent., averaging 3.0 ± 0.3 per cent. (standard deviation 1.2; coefficient of variability 40.0 per cent.). Though the counts show high variability the averages do not differ much.

The.../

Digitised by the University of Pretoria, Library Services, 2012

The heterophile counts shown by birds 1 - 5 varied from 40.5 to 78.5 per cent., averaging 59.1 ± 0.9 per cent. (standard deviation 7.7; coefficient of variability 13.0 per cent.). This average differs appreciably from that obtained with the results from bird No.6 and from the average shown by birds 7 to 17.

The eosinophile counts of birds 1 - 5 varied from 0 to 19.5 per cent., averaging 6.3 ± 0.5 per cent. (standard deviation 4.0; coefficient of variability 63.5 per cent). These cells show the highest variability.

The averages for birds 5 and 6 are exceptionally low while the average figure for birds 7 - 17 is exceptionally high compared with the mean count shown by birds 1 - 5. As stated on page 11 it is most likely that all the birds Nos. 7 - 17 were worm-infested and the high eosinophile counts of these birds may possibly be correlated with verminosis; however, some of the worm-infested birds (18 - 22) showed comparatively low eosinophile counts.

The basophile counts of birds 1 - 5 varied from 1.0 to 10.5 per cent., averaging 4.7 ± 0.3 per cent. (standard deviation 2.5; coefficient of variability 53.2 per cent.). This average does not differ appreciably from that shown by birds 7 - 17. The few differential counts on the worm-infested birds (18-22) were most erratic.

THROMBOCYTE COUNTS.

The thrombocytes were counted by the method employed by Magath and Higgins (1934). The ratio between these and the leucocytes was computed from the number of thrombocytes enumerated each time 200 leucocytes were counted in the smear.

As the thrombocytes are very unevenly distributed the ratio obtained is only approximate, even when 200 leucocytes have been counted. ~~It is evident that~~ The method is

far..../

Digitised by the University of Pretoria, Library Services, 2012