NOTE ON A STAIN APPLICABLE TO DIFFERENTIAL LEUCOCYTE COUNTS IN THE COUNTING CHAMBER.[1]

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While the Thoma-Zeiss counting chamber and its different modifications give us facilities for accurately counting red and white blood cells in a given quantity of blood, no method to my knowledge has been published which would allow us to make a differential count of the leucocytes with the same accuracy.

The usual method of counting the different kinds of leucocytes is that by stained smears. This method has the disadvantage that even with the greatest care one rarely succeeds in attaching the blood to the cover-glass or slide in a layer of perfectly even thickness. This defect produces unevenness of the stain. Moreover, there is always the possibility of some cells washing out during their passage through the several fixing and staining fluids, and, finally, artefacts are frequently unavoidable.

As we intended to examine the blood of epileptics at the Craig Colony, the consideration of these defects made it appear desirable before beginning such work to devise a method by which the counting of the red cells and a differential count of the leucocytes could be combined in one and the same procedure. In other words, I endeavored to obtain a stain of the leucocytes in the mixing pipette, so that a differential count could be made in the Thoma or similar counting chamber.

The only method that stains the leucocytes in the mixing pipette is to my knowledge the Toisson solution. This, however, only stains the white cells in a rough way, not allowing any differentiation of the same, and, in particular, not bringing out the granulations.

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In devising a differential leucocyte stain for the counting chamber, the first thing to be thought of was a fixation of the blood. The most ideal method seemed to be the one that would combine fixation and stain in the same fluid. An essential requirement was that the fixing agent should not interfere chemically or physically with the stain.

I first tried bichloride of mercury, but gave it up at once, as it formed a precipitate with one of the staining agents that I intended to use — the polychrome methylene blue of Unna — to such extent that the fluid became colorless.

Next formalin in different percentages, one and one-half, three and six per cent, was tried. These solutions either dissolved the red blood cells or made them otherwise disappear. At any rate, the erythrocytes and apparently also the leucocytes became invisible when the blood was mixed with one of these formalin solutions in the proportion of one part of blood to two hundred parts of the solution.

It occurred to me then that normal salt solution might make the red cells visible. Therefore sodium chloride was added to the formalin solution in such quantity as to make the mixture have the strength of normal salt solution. This was found to make the red cells visible in normal outlines.

Finally a three per cent formalin solution was decided on, as being the most favorable fixing agent; or, at least, it proved to give absolutely satisfactory results when a certain percentage of sodium chloride was added.

The next step was to mix the formalin-sodium chloride mixture with staining solutions. As staining agents polychrome methylene blue and eosin (yellow) were selected, which were experimented with in different concentrations. When strong concentrations of these stains were employed, in combination with such concentration of sodium chloride (plus formalin) as to give the entire mixture the strength of a normal salt solution, a crenation of the red cells was observed to take place. If the sodium chloride was now left out from the mixture, the red cells again appeared normal. But when the polychrome and eosin were reduced in strength, the red cells again became invisible, so that for weaker solutions of these stains sodium chloride had again to be added. It was thus found that sodium chloride played a very important part in the required composition of the staining fluid, and that it had to be added in a definite concentration which could vary in very narrow limits only.

Regarding the staining fluids, the following requirements have to be observed. The fluid must contain stain enough to attach itself to the stainable parts, but not be too deeply colored, so as not to obscure the lines of the scale in the counting chamber and so as not to obscure the stain of the stained structures themselves. Furthermore, it must not cause any precipitate in the blood nor any clumping of the cells.

The mixture which proved to fill all these requirements when the blood was mixed in a proportion of one to two hundred with this staining fluid was composed as follows:

1. 12 per cent aqueous solution of formalin. (Formalin Schering 12 volumes. Water enough to make up 100 volumes.)
2. 1 per cent aqueous solution of sodium chloride.
3. ½ per cent " " " eosin. (Soluble yellowish in water).
4. Unna's polychrome methylene blue.

Equal volumes of these four fluids are mixed and filtered. This gives the mixture a formalin strength of three per cent, a sodium chloride concentration of one-quarter per cent, an eosin concentration of one-eighth per cent, and a polychrome methylene blue concentration of twenty-five per cent. Care should be taken not to use too old staining solutions, particularly not a too old polychrome.

Whether the succession in which these fluids are to be mixed plays some part, I am not quite prepared to say yet. In all probability it does not. However, for the beginning it might be well to first mix 1 and 2 and then add 3 and 4.

Filtration is necessary on account of a precipitate formed between the eosin and the polychrome methylene blue.

The staining mixture itself is not very stable, and it is advisable to mix it no longer than twenty-four to forty-eight hours before use, although it may hold out a whole week. Gradually a precipitate forms which obscures the stain. While this precipitate might be removed by filtering, the latter weakens the stain. Therefore, the wisest course is to prepare the mixture just before use, or at least the same day.

I have tried to substitute the polychrome methylene blue by common methylene blue, but my experiments in this respect are still in a stage of development. However, I will say so much that in combination with the methylene blue the eosin has to be less concentrated than in combination with the polychrome; otherwise it forms precipitates or coagula in the blood, which clump the blood cells together. Furthermore, the methylene blue has to be very weak, that is, the methylene blue solution which is to take the place of the polychrome, if the volume proportions are to remain the same, must be only one-tenth per cent, at the most, and probably less strong. Whether it really will be possible to obtain the same staining results with the methylene blue in place of the polychrome, I do not know yet.

The stain can be used also in a solution of one part of blood with one hundred parts of the stain, but not with as good results as in the dilution of one of blood to two hundred parts of the stain. For lesser dilutions (for instance, one part of blood with ten to twenty parts of stain), the staining fluid cannot be used in the composition above described.

Just in what manner the proportions of the components would have to be modified, or the components themselves altered to make the stain available for such dilutions, is a subject for further investigations.

For such dilution as one to ten or one to twenty it might become necessary to make the red cells invisible, and the chemico-physical change then produced might create an unsurmountable obstacle to a successful differential leucocyte stain.

As to the results of the stain here described, *i.e.*, of the differential leucocyte stain as applicable to dilutions of one part of blood with two hundred (or one hundred) parts of stain, they were unusually brilliant.

The stain makes its appearance gradually. If the room is not too chilly, room temperature is sufficient, but the staining is hastened by moderate heat, preferably body temperature. The pipette is placed either in the incubator or under the tongue. In this case the stain is almost at its height within from fifteen to thirty minutes. At any rate, it gives then absolutely satisfactory results. It is still present and very beautiful after one and a half hours, and probably considerably longer, but just how much longer is not yet determined.

The red cells appear a very pale yellow. The white cells show a beautiful stain of the nuclei, protoplasm, and granulations. The protoplasm as a rule appears a pale blue, the neutrophile granulations a dark violet, and the eosinophile granulations a bright red. The nuclei differ in color in the different kinds of leucocytes. In the polymorphonuclear cells they are all purple. The outlines of the protoplasm are very distinct in most cells, which latter appear in different shapes, although I have not been able to see ameboid movements.

The lines of the scale in the counting chamber are absolutely unobscured and there is no precipitate.

The objection has been raised by some of the colleagues to whom I demonstrated the stain that, with dilutions of one to two hundred or even one to one hundred only, a very limited number of leucocytes could be counted in the counting chamber at one sitting, that is, in one specimen. This holds true for many of the counting chambers in use. There is no reason, however, why the ruled space of the counting chamber should not be so extended as to occupy the greater part of the entire central disc.

Fig. 1 demonstrates such an arrangement, enlarged ten times in diameter.

The ruled space contains here forty-eight large squares, enclosed by double lines and measuring one square millimeter each, in addition to eight small squares, which latter are one-fourth the size of the large ones. The square contents of the entire ruled space is, therefore, fifty square millimeters, a very convenient number for calculations.

If we assume the cubic millimeter to contain 10,000 leucocytes, the fifty square millimeters of the counting chamber would represent $\frac{10000}{10} \times \frac{50}{200}$, *i.e.*, 250 leucocytes if the blood

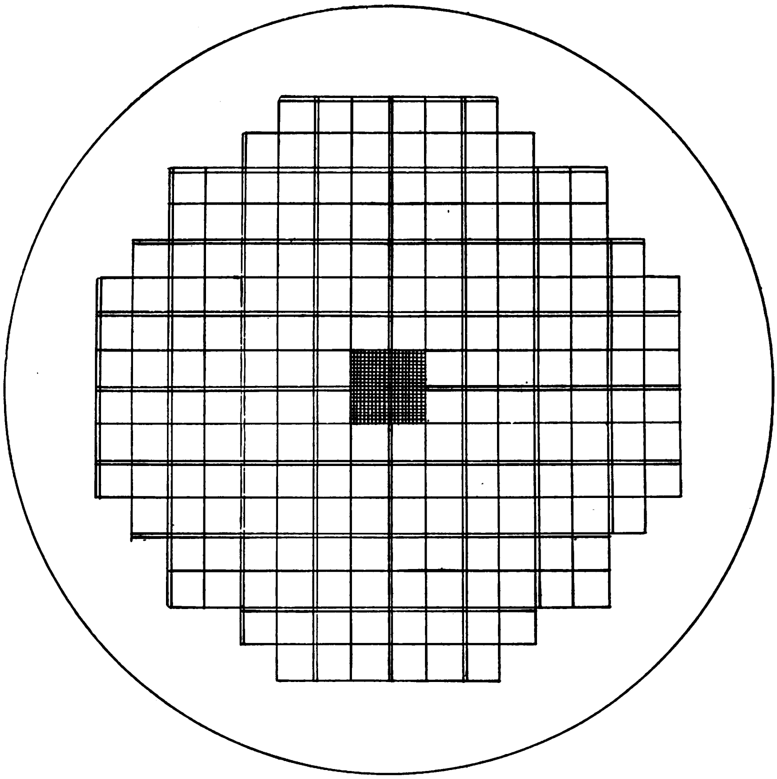

FIG. 1. — Magnified ten times in diameter.

was diluted one to hundred, and this number of leucocytes could be doubled, — made 500, — by using a dilution of one to one hundred instead. This certainly would suffice to make the method practical. However, the dilution of one to two hundred is preferable, giving more accurate staining results.

As indicated in the figure, the central square (one square millimeter in size) could be divided into 400 squares, just as in the Thoma counting chamber, so as to make it applicable for red blood counts at the same sitting.

[The writer is in correspondence with Leitz and other firms, with a view of having such a counting chamber constructed.]