Histology. — *The influence of pre-treatment with or without fixation on the Sudan granulation of leucocytes and the character of phenol granulation in general.* (From the Histological Laboratory, University of Amsterdam, Dir. Prof. Dr. G. C. HERINGA.) By P. H. DE BRUYN and J. H. C. RUYTER. (Communicated by Prof. M. W. WOERDEMAN.)

(Communicated at the meeting of June 25, 1938.)

Some years ago RUYTER reported on the influence various modes of pre-treatment and fixation of the preparation had on the Sudan colourability of neutrophile leucocytes. One of his observations at that time was that use of fixatives containing sublimate took away the Sudan colourability of the granules. This action was found to be reversible: removal of the sublimate from the blood smear by a bath either in KJ or in NaCl solution caused the return of colourability. This succession of negative and positive could be repeated several times running, the intensity of the colouring diminishing gradually (Fig. 1).

From the very first the analogy to the reversible precipitation by sublimate of egg albumen, where the precipitate can be dissolved again in excess of NaCl, became apparent. This analogy became strengthened by DE BRUYN's subsequent investigations, which showed that the colourability of the leucocytes was connected with the action of naphthol, phenol and phenol compounds, treatment with the latter resulting in formation of granula, which can be stained with Sudan. This action of the phenols only takes place when the phenol solution shows a markedly alkaline reaction (optimal pH 9—10). Thus the phenomenon observed by RUYTER would be explained, if it appeared that the inhibitive influence of an acid reaction on the phenol action, resp. Sudan colouring, was the same when exercised not on the phenol action itself, but on that of the fixative fluid. The fixative used in histology, a saturated solution of sublimate, has a pH of 3.3, whereas this latter value rises to 6 when addition of NaCl causes the formation of the double salt $HgCl_2NaClHgO$.

Hence we decided to make an investigation into the influence of fixatives with verying pH upon the Sudan colouration. The consideration that sublimate is precipitated in the presence of alkali, thus rendering sufficient variation in the degree of acidity impossible, led us to choose formol as fixative. With phenol we made the following standard solutions of varying acidity (see Table I).

In order to ensure good colourability of the leucocytes with Sudan, varying amounts of naphthol were added to the Sudan solution.

The following may be said about the preparation of Sudan solutions: In order to increase the stability of the Sudan suspension a little gelatine

TABLE I. pH of various standard mixtures dissolved in pure water and in formol solution.

No.	Mixture	pH in water	pH in formol 12%
I	citric acid — Na_2HPO_4	3.0	3.30
II	„	4.0	4.32
III	„	5.0	5.25
IV	NaH_2PO_4 — Na_2HPO_4	5.91	5.85
V	„	6.98	7.15
VI	„	8.04	8.0
VII	borax — NaH_2PO_4	9.0	8.95
VIII	borax — Na_2Co_3	10.0	9.80

was added to the alcoholic solution. The gelatine was added one day after the preparation of the solution, the latter having first been filtered and boiled up again. The amount of gelatine used was 5 drops of a 5 % solution per 125 cc Sudan solution. Filtering was done through a very fine filter (SCHLEICHER and SCHÜLL 602*h*) in order to separate off the fine Sudan crystals from the solution.

The following experiments were done with the formol solutions:

A. Blood smear preparations were fixed overnight in the formol standard solutions: then they were washed and placed in sublimated Sudan to which 0.9 mg β naphthol per 125 cc solution was added [1]).

B. 8 smear preparations were fixed during one night in formol solutions of different pH, then washed and placed in a solution of sublimated Sudan III, HOLLBORN, pH ± 10 (with NaOH), to which 5 cc alcohol 40 % containing 0.5 mg naphthol was added just before use. The Sudan colouration both in experiments A and B was the stronger, the more elevated the pH of the fixative. Fixation by these solutions showing an elevated pH was none too good. The strong colouration in this experiment made it difficult to distinguish between grades in colouring. For this reason the following experiment was carried out, a smaller amount of β naphthol being used.

C. Smear preparations were fixed during one night and coloured with the following Sudan solution: sublimated Sudan III, HOLLBORN,

[1]) Cf. DE BRUYN (1938).

pH ± 10 + gelatine, to which per 100 cc 0.1 cc alcohol 40 % containing 0.1 mg naphthol was added before use.

The result of the colouring could now be read off as follows.

	pH in formol 12%	Colour Result
I	3.30	negative
II	4.32	almost negative
III	5.25	weak
IV	5.85	weak
V	7.15	weak
VI	8.0	fairly good
VII	8.95	fairly good
VIII	9.80	fairly good

This table clearly shows the influence of increasing pH of the fixative on the Sudan colouring.

It is quite clear from the obtained result that the inhibitive action of the sublimate fixative on the Sudan colouration observed by RUYTER can at least partly be explained by the acidity of the sublimate solution.

Whether or not there is besides a specific action of the $HgCl_2$, is at present uncertain.

It is important to note here that the reversibility of the inhibitive action so characteristic of sublimate, is not found when other fixatives such as formol are used. After the Sudan colouring has disappeared by the action of acid formol, it does not reappear again. Possibly a further analysis of these varying actions might point the way to the study of naphthol granulation.

D. Then we tested the action of the fixative on preparations which had been exposed to the granule forming action of phenol without having been coloured by Sudan.

The phenol solution itself had to be of optimal pH (± 10).

The solution was prepared as follows: 20 % NaOH was added drop by drop to a 0.1 % water solution of phenol, until the indicator alizarine-yellow showed that the desired pH had been reached. The phenol solution thus prepared appeared capable of being kept (KOPPESCHAAR's method).

8 smear preparations fixed during one night and then washed, were placed in a 0.1 % phenol solution, pH 10.1 (with NaOH) for 5 hours and then examined under the microscope. In those fixed in solution I (pH 3.3) the granulation in the protoplasm was absent. Granulation

appeared from a pH of about 5.2 and become the better defined as the pH increased.

E. In connection with this result we decided to do another series of phenol experiments, for which a number of fixatives were prepared with regulary increasing pH from 2.5 to 7.

The buffer solutions were the following:

		pH in water	pH in formol 12%
I	citric acid — Na_2HPO_4	2.2	2.5
II	„	3.0	3.0
III	„	4.0	4.1
IV	„	5.0	4.9
V	KH_2PO_4 — Na_2HPO_4	6.24	6.0
VI	„	6.98	6.9

Three analogous phenol experiments were done with these formol solutions: smear preparations were fixed for 24 hours in the buffers, then washed and placed in a 0.1 % phenol solution pH ± 10 for about 8 hours. Then the preparations were washed and examined. Three experiments done on different days gave the following results:

Intensity of the granulation.

Sol.	pH	1 .	2	3
I	2.5	negative	negative	negative
II	3.0	„	„	„
III	4.1	some granules at the edge, no granules in the protoplasm	weak	„
IV	4.9	granulated	granulated	weak
V	6.0	„ (less than IV)	positive	„
VI	6.9	strong	strong	strong

Although the results of these experiments are not always exactly the same still they clearly show the influence of the pH of the fixative on the granulation. The change lies between a pH of 3.0 and 4.9. It is remarkable that the same pH values were found in experiment C. We may take it that this experiment proves that the degree of acidity on the fixative has

an influence on the granulation forming action of phenols. It proves not only the connection between sublimate fixation and Sudan colouration to which we concluded above, but also the connection between Sudan colouring and naphthol- (resp. phenol-) action as found by DE BRUYN.

A fortiori this explanation holds good for the inhibitive action of acid fixatives (chromic acid) on the Sudan colouration of leucocytes which RUYTER (1933) has described.

The negative action of $KMnO_4$ specially after fixation by acetone and alcohol (not formol) which can again be annulled by treatment with oxalic acid-K-sulfide (¼ % āā) solution is less easy to analyse. Remarkable too is the irreversible negative action of ultraviolet rays (which can not even be annulled by phenol with optimal pH). After treatment with a quartz lamp (Hanau) for 10 minutes (distance 60 cms) the colouring is considerably diminished. After 60 minutes it has altogether disappeared (Fig. 3). The two last mentioned means of treatment ($KMnO_4$ and ultraviolet rays) have in common that they are oxidation agents. Whether and how oxidation modifies sudanophilia is a question which must be further investigated. Just as in all the foregoing experiments, so in the above reported the neutrophile leucocytes, resp. the monocytes have been the ones in which the granulation has been observed. Eosinophiles have a much more persistent and stronger attraction for Sudan colouring matter.

If we take a survey of the above mentioned observations, then the conclusion we draw is, that granulation in the leucocytes colourable by Sudan must be formations containing hydrophile colloids (albumens) and lipoids, on the condition of which depends their ability to take up Sudan colouring matter, this condition being influenced by certain pre-treatments of the smears.

It is interesting to add a few observations which seem to show that the same influence which acts inside the cell on the granules as shown by their colourability, seems also to affect the surface of the cell, causing changes which affect and are affected by the colouring solution. In studying Sudan granulation, RUYTER noticed that when the colouring was strong, one often had the impression that there were two kinds of granulation: 1. a fine granulation which regularly filled up the body of the cell; 2. a coarser one which seemed to bulge out on the surface of the cell (fig. 2). These last mentioned granules (so-called outside granules) proved capable of being removed from the cell surface by intense washing; the granulation inside the cell remaining unaltered. The granules could be removed from the cell surfaces by the micromanipulator. What was the cause of this deposition of colouring matter on the cell surface? The formation of these drops is not simply a matter of a deposition of floating Sudan drops upon the cell surface. After hours of observation we never saw such an occurrence, nor a deposition of floating Sudan drops on the micromanipulator needle or on eventual pollutions in the solution of colouring matter.

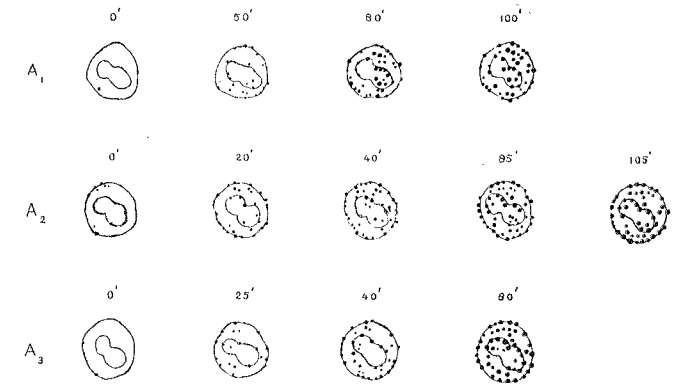


Fig. 4A.

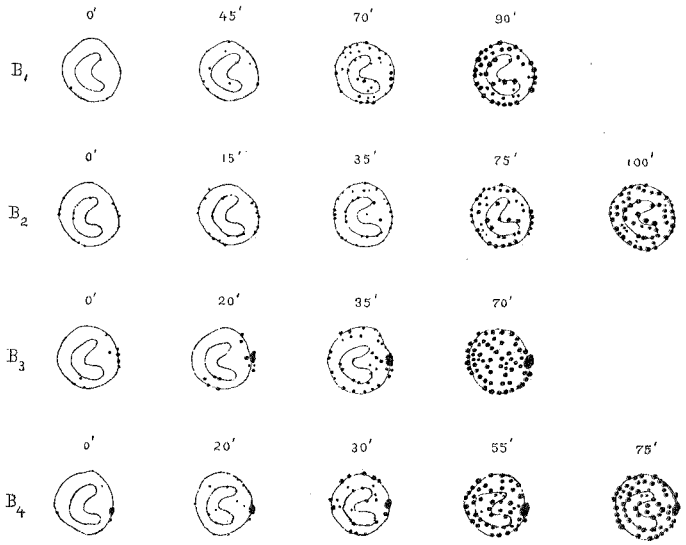


Fig. 4B.

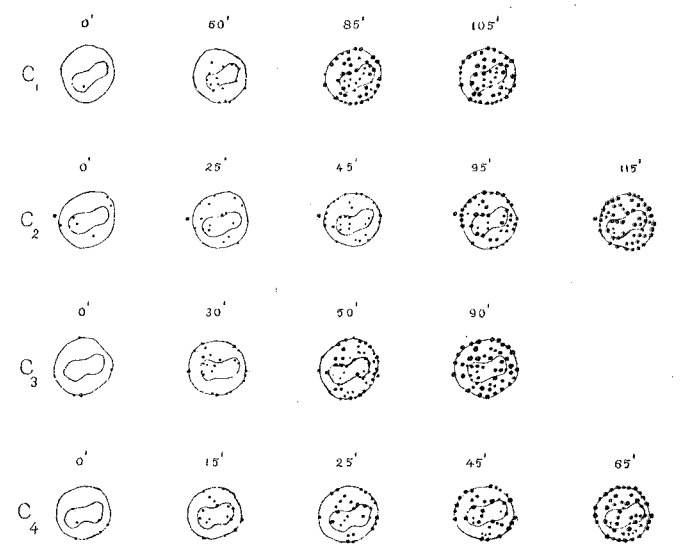


Fig. 4C.

The formation of these drops shows a close analogy to the formation of granules colourable by Sudan inside the protoplasm as is shown by the following considerations:

1. Under the inhibitive action of ultraviolet rays the formation of the surface drops stops pari passu along with the colourability of the inside drops or granules (fig. 3);

2. Three cells taken from a preparation coloured in Sudan, were cleaned of the Sudan drops on their surface by the micromanipulator; continuous observation showed the new appearance of these surface drops. From the accompanying figures (Fig. 4) it appears that the distribution of the newly formed drops is remarkably similar to that of those first seen and then removed. This is scarcely otherwise explainable than by supposing that there are inhomogenities of the surface layer at those points, which must be supposed to be comparable to the granulations inside the cell, as follows from the analogous conducts under the influence of ultraviolet rays. All the observations reported here point to a physico-chemical character of the granulation formation in the leucocytes by phenol.

EXPLANATION OF THE FIGURES.

Fig. 1.

Effect of alternating treatment of neutrophile leucocytes with $HgCl_2$ and Lugol's solution. Sequence of illustrations; from upper left hand corner to the right, from upper right hand corner to second row left, etc.

Upper left hand corner: Result after fixation by a saturated aqueous solution of $HgCl_2$ followed by treatment with Lugol's solution.

Top row, second from the left: Result of repeated immersion in mercury chloride solutions.

Top row third from the left: Result after $HgCl_2$ — Lugol — $HgCl_2$ — Lugol, etc.; thus the bottom right hand picture shows the result after the eighth immersion in $HgCl_2$ solution.

Fig. 2.

Neutrophile leucocyte, monocyte and lymphocyte. Fixation in 12% neutral formalin (24 hours). Coloured by ROMEIS' Sudan solution.

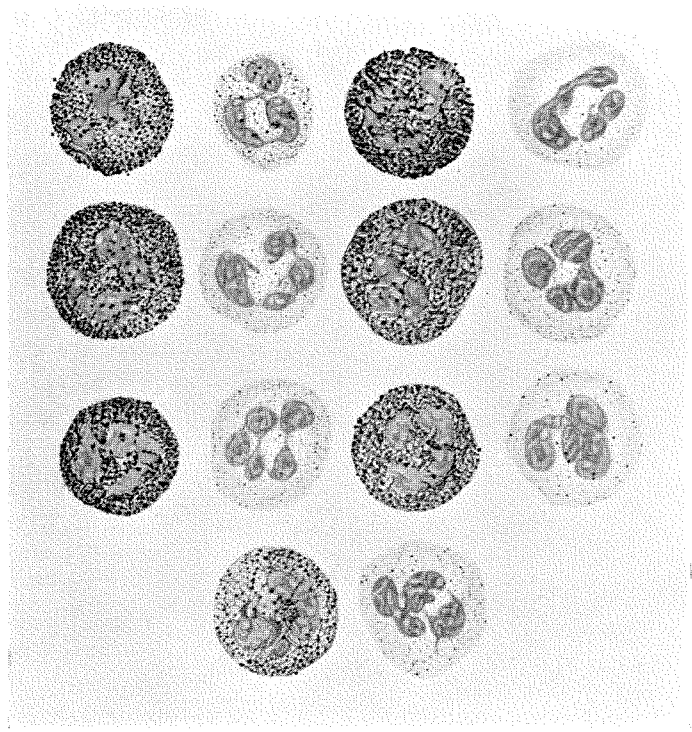
Fig. 3.

Neutrophile leucocytes. Fixation in formol alcohol 30 minutes. Coloured by standardized Sudan solution 18 hours.

I. before treatment with ultraviolet rays;
II. after 10 minutes' treatment with ultraviolet rays
III. after 30 minutes' treatment with ultraviolet rays } (Hanau lamp), 60 cms distance.

Fig. 4.

Three neutrophiles (A, B and C). Fixation in formol alcohol, 30 minutes. Coloured by standardized Sudan solution 18 hours. Inside granules do not appear in the picture. Three (A) or four (B and C) experiments have been carried out with each of the leucocytes. First (A_1, B_1, C_1), the outside granules have been removed with the manipulator. The horizontal rows show the reappearance of these granules (time in minutes). Then (A_2, B_2, C_2) they are again removed; after reappearance the removal is repeated two (A) or three times (B and C). It will be seen that in a given leucocyte the localisation of the granules remains practically the same throughout the experiment.



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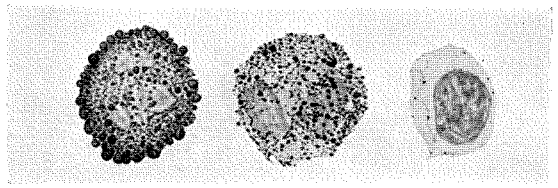

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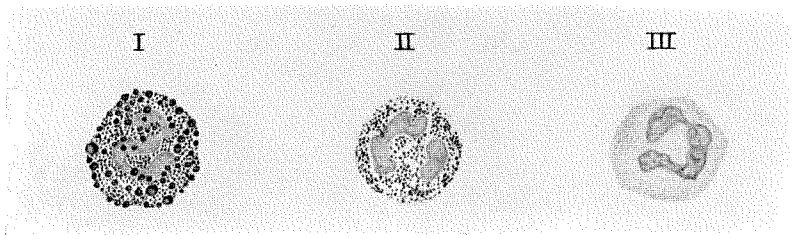

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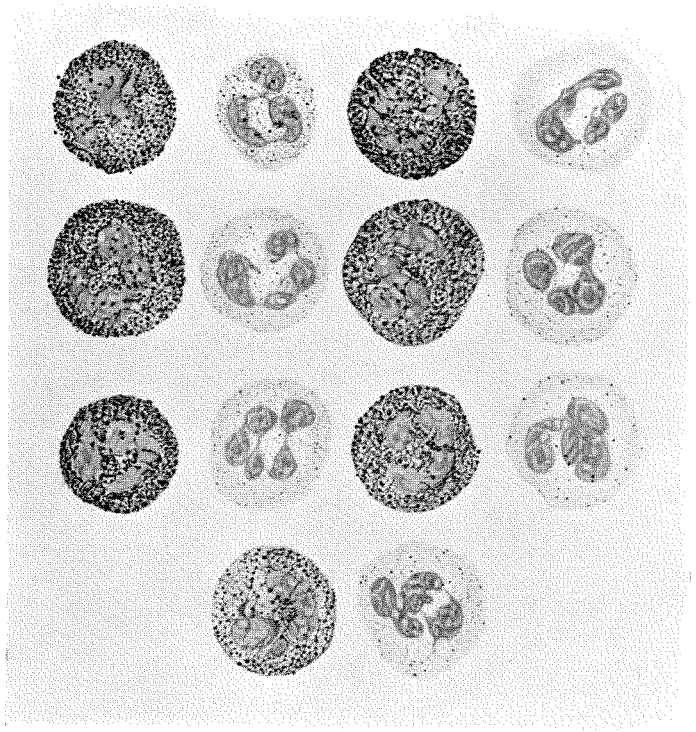

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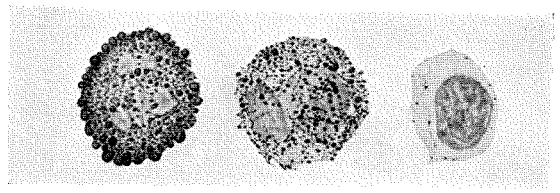

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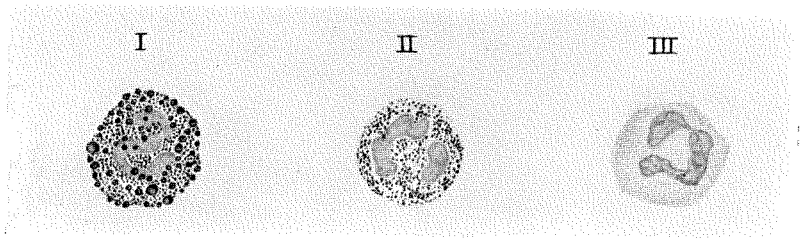

Fig. 3.

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