CYTOLOGICAL INVESTIGATIONS ON CULTURES OF NORMAL HUMAN BLOOD.

By G. K. CHRUSTSCHOFF.

Assisted by E. A. BERLIN.

(*Medico-Biological Institute, Moscow.*)

(With Plates XII and XIII and Three Text-figures.)

I. Introduction.

In the year 1931, together with A. H. Andres and V. I. Iljina-Kakujewa (1), we published a preliminary communication with reference to the possibility of using leucocyte cultures of normal human blood for karyological purposes. In 1932 Haldane pointed out the necessity of an intensive study of the chromosome complex of humans and also the possibility of using cultures of the leucocytes of peripheral blood for this purpose.

The fundamental aim of our investigation was to obtain the largest possible number of cells in mitosis in cultures of normal blood cells and to use them for analysing the somatic karyotype of man.

Hitherto none of the numerous authors who have cultivated the blood cells of various animals and men have paid any special attention to mitosis.

The first to point out the presence of mitosis in cultures of leucocytes were Timofeewski and Benewolenskaja (2). These authors described how in the cultures of leucocytes of normal human blood the polyblasts originating from the lymphocytes and monocytes at a certain stage of development of the culture multiply in a mitotic manner. Somewhat later Maximov (3) showed that single mitosis may be found in polyblasts in cultures of the leucocytes of the rabbit, guinea-pig and *Macacus*. Maximov studied the problem of the multiplication of spindle-like cells and fibroblasts originating from polyblasts and states, differing from other authors, that these cells under appropriate conditions multiply exclusively in a mitotic manner. To prove this statement he gives some beautiful drawings, but nowhere refers to mass mitosis. In 1928 Caffier (4), whose work is of especial interest to those concerned with the culture of the leucocytes of normal human blood, also dwells on the question of the multiplication of the different types of cells which form in the developing culture. He gives a detailed and exhaustive description of the general

morphology of these forms, and points out that with the conditions under which he grew his cultures for 14 days he never observed mitosis. We have then the positive data for human blood of Timofeewski and Benewolenskaja, obtained in cultures grown by a special method; the positive data of Maximov for blood of other mammalia, obtained by a method similar to that of the preceding writers, and finally the negative data of Caffier, who used a different method for cultivating normal human blood. These data enable us to draw the conclusion that the presence of mitoses and also their quantity probably depend on the conditions of cultivation.

Our efforts were first directed to discovering what media would be the most effective for our purpose, and in this direction we succeeded in obtaining reliable results. In addition we attempted to find the simplest methods possible, because our final aim was not only to enlarge the possibilities of the karyological study of man, but also to work out the most convenient methods for obtaining karyological material. Consequently the central feature of our work is methodology, which we consider to have developed to a point such that it is adequate and practicable for anyone acquainted with the principles of the technique of tissue cultures. The method given in this paper differs from the method described in our preliminary communication of 1931 in that it is far simpler.

II. The technique of growing cultures.

A large number of experiments made by us after publishing our preliminary communication in 1931 showed that plentiful karyokinesis cannot be obtained in cultures of leucocytes previous to the moment when the mutation of polyblasts into stable cells begins. Therefore we paid special attention to discovering the conditions of cultivation which would lead to rapid and quantitatively greater mutation of labile forms into stable forms, and to furthering their mass multiplication in a mitotic manner.

As material for cultivation we used fragments of film coagulated on the surface of centrifuged cell elements of blood, which, as is well known, contains a great number of leucocytes. This film was obtained in the following way. The blood obtained by the puncture of a vein is collected in quantities of 8–10 c.cm. in a cooled paraffin-lined tube containing some 5–8 drops of solution of heparin in physiological salt solution; this is centrifuged for 10–12 min. at 3500–4000 revolutions. After this the transparent plasma, which usually has a yellow colour of varying

intensity depending on the subject, is drawn off. It is advisable to leave as little plasma as possible on the paste of centrifuged cell elements. After removing the plasma, 3–4 drops of embryo extract may be added to the tube, but this is not necessary. The tube must remain at room temperature, or be placed in a thermostat at 37–38° C. After some time the plasma on the surface of the cell elements coagulates. The rapidity of the coagulation is highly stimulated by embryo extract and placing in a thermostat. But neither of these, according to our observations, has any influence on the final effect. As will be seen from the above, our method of obtaining the film is similar to that accepted by the great majority of experimenters (Carrel, Timofeewski and Benewolenskaja, Maximov, Caffier). The success of the cultures largely depends on the size of the fragments taken for cultivating. We recommend transplanting fragments that are neither too small nor too large—about a square millimetre. As Maximov has shown, it is advisable to avoid those fragments of the film which are only feebly coloured or nearly white; for such fragments contain chiefly thrombocytes or blood plates and comparatively few leucocytes. In addition, as will be shown later, it is necessary to have a certain number of erythrocytes in the portion selected if we are to obtain successful cultures developing stable cells.

The cutting out of these small parts is done in Ringer solution, and must be done rapidly.

We placed selected portions in a hanging drop, using the method of Maximov, *i.e.* the small cover-glasses on which the cultures were placed were attached with Ringer solution to a large mica plate, which was placed over the cavity of a large slide. As initial medium we used a mixture of rabbit and human (usually autogenous) plasma. Such a mixture is recommended by Timofeewski and Benewolenskaja (2), and by Hirschfeld and Klee-Rawidowitsch (5). Numerous recent experiments have shown that the plasma used for our purposes must be dissolved in a certain quantity of Ringer or Tyrode solution. In this respect we agree with the method proposed by Maximov (3). The best results were obtained by a certain hypotonisation of the medium. For reasons given later we used a somewhat greater hypotonisation than that proposed by Maximov.

Our cultures, as has been stated, were developed on two different plasmas, one of which (heterogenic rabbit plasma) was diluted with a Ringer solution 50 per cent. hypotonised. We obtained the hypotonisation by mixing the Ringer solution with distilled water (bidestillata) in the ratio 1 : 1.

The addition of embryo extract to the initial medium undoubtedly accelerates the process of mutation of non-granular forms from labile amoeboids into stable ones. A most important condition here is the appropriate concentration of the extract. We have come to the conclusion that the best concentration for the initial medium is 5 per cent. of extract in the medium. The embryo extract used may be either homogenic or heterogenic. We found extracts of human and chicken embryo equally suitable. It is hardly necessary to point out that the preparation of human extract, which is usually prepared from material obtained after an abortion, must be done extremely carefully. It must be remembered that portions of embryo may be polluted (as is in fact practically always the case) with maternal blood or with other secretions from the uterine mucosa.

The diluted extract may be preserved on ice for a comparatively long time in sealed tubes or carefully corked glass tubes without losing its active nature. For example, several times we used an extract that had been preserved on ice for 2 weeks with the same success as with a freshly prepared one. In this respect we entirely agree with Maximov. In analysing the problem of the factors stimulating the mutation of non-granular forms of blood into stable cells of united tissue type, we must call attention to an extremely important point. In our first communication(1) we pointed out that the number of erythrocytes in the cultivated fragment of the blood clot exercises an influence on the process of transformation. We have repeatedly observed the same thing in all the experiments made after the publication of our first communication. On the ground of these observations we advance the hypothesis that some products of the disintegration of blood cells, and especially of erythrocytes, are an essential factor stimulating and possibly determining the processes of transformation. In order to verify this hypothesis and obtain at the same time a new technical method, which was very important for us, we prepared the so-called autohemolysate in the following way. The blood clot, or more accurately the plasma coagulated on the surface of centrifuged blood elements identical with those we used for our cultures, was cut into several fragments and placed in a sterilised tube, and 3 c.c. of Ringer solution were added.

Then after soldering the tube and thoroughly sealing the stopper, the tube was placed in a thermostat for 2 days at 37° C. After this it was carefully shaken and centrifuged. We added the autohemolysate obtained in this way direct to the medium of the culture, or diluted it with the embryo extract.

In all cases we obtained a definite and uninterrupted effect accelerating the mutation of the non-granular forms that have migrated into the medium. We may therefore assume that our hypothesis was proved. We must at the same time point out that the addition of the autohemolysate to the medium only accelerates the process of mutation of labile forms into stable ones, but that it does not increase the quantity of mitosis in the culture. Appropriate periods for renewing the medium play an important part in obtaining the final result, *i.e.* a greater quantity of mitosis in the cultures. We have already pointed out in our communication that transplanting, *i.e.* cutting the culture off an old medium and placing it in a new one, does not give any results. This appears clearly if we consider the fate of the planted portion itself. The fact is, that the transplanted clot contains fewer cells in proportion as the zone of migration increases. After 2 or 3 days the central part of the transplanted portion contains only isolated cells and only the most superficial layer is rich in various forms of non-granular elements. It is evident from this that after the first transplanting these fragments become quite poor in viable cells and cannot be again replanted. Hence instead of transplanting we used a method of renewing the medium proposed by Maximov, and described by him in detail. As a medium for the renewal we always used a drop of embryo extract and a drop of autogenous plasma diluted with hypotonised Ringer solution.

The first renewal we usually made on the fourth day after planting. This period appeared to be by far the most successful for getting the maximum number of mitoses in the culture. The period of the first renewal as shown in our Summary 1 coincides with the beginning of the appearance in the cells of single or whole groups of stable cell forms. We must once more repeat our previous observation that a too early renewal of the medium may somewhat delay the process of developing stable forms. This supports the idea that the substances stimulating the mutation are products of disintegration of cell elements in the planted portions. One of the media appeared to be sufficient for our aims. The cultures, which were fixed 24 or 48 hours after this first renewal, produced a considerable number of mitoses; 48 hours is most convenient for this (see Summary 1). Numerous sets of cultures generally give a quite homogeneous result. We obtained regularly from 65 to 75 per cent. of cultures developing in the direction of stable cell forms. It is necessary to mention that whether the donator of the blood was hungry or not had no influence on the process of developing the cultures and obtaining mitoses in them, both with regard to cell material and medium (blood plasma).

SUMMARY 1.

Medium for the cultures	24 hours								
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
First group: 18 series, 630 cultures. Autogenic plasma+rabbit plasma (1 : 1)	Migration	Migration	Migration	Groups of polyblasts appear	Polyblasts dominate	Mutation of polyblasts into fibrocytes. Renewal of the medium (Protan)	Fibrocytes dominate	Fibrocytes dominate. Renewal of the medium (Protan)	Fibrocytes in a small number of cultures, isolated mitosis
Second group: 10 series, 350 cultures. Autogenic plasma+rabbit plasma, diluted by 50 % hypotonic Ringer solution (see text)	Migration	Migration	Appearance of groups of polyblasts	Polyblasts dominate Isolated fibrocytes	Polyblasts. Groups of fibrocytes. Renewal of the medium (Protan)	Fibrocytes dominate	Fibrocytes dominate. Renewal of the medium (Protan), isolated mitosis	Fibrocytes	Fibrocytes, isolated mitosis
Third group: 12 series, 480 cultures. Medium of the second group+embryo extract (chicken or human)	Migration	Groups of polyblasts	Polyblasts dominate	Forms transgressive into the fibrocytes dominate. Renewal of the medium; autogenous plasma embryo extract, isolated mitoses	Fibrocytes dominate. Increasing amount of mitoses	Fibrocytes dominate. Large amount of mitoses	The cultures were not continued		
Fourth group: 8 series, 304 cultures. Medium of the third group+autohemolysate (see text)	Polyblasts in the end of the group	Forms transgressive into fibrocytes	Fibrocytes dominate. Renewal of the medium as in third group	Fibrocytes. Small quantity of cells under mitoses	Fibrocytes. Large amount of mitoses. Renewal of the medium	Fibrocytes. Small amount of mitosis	Fibrocytes. Large amount of mitosis	The cultures were not continued	

There has been some discussion of the necessity of diluting the plasma and of general hypotonisation of the medium. It seems to us that by diluting the plasma and subsequently obtaining clots in it, a considerably less close fibrin net is obtained. In the coagulation of a non-diluted plasma this is not generally a mechanical factor directly affecting the cells. It secures a more equal and rapid diffusion into the medium of those elements which gather in the fragment itself as a result of the autolytic processes which take place in it. With regard to the part played by these elements we have already spoken.

With reference to hypotonisation the following suppositions are possible. There is no doubt that the degree of hypotonisation, found at the moment of planting, decreases from the time of placing the medium on the slide to the time of coagulation of the medium and of sealing the culture in an hermetically closed cavity. Hence the culture appears to develop in a medium very weakly hypotonised. This weak hypotonisation presumably plays the part of changing the physical properties of the surviving cells and stimulates the change in a certain direction. We must assume that the degree of hypotonisation decreases still more and perhaps it amounts to isotonicity as the autolytic process advances.

The last supposition, however, requires careful verification.

III. THE GENERAL MORPHOLOGY OF CULTURES.

The general morphology of cultures of human leucocytes has been frequently described by numerous authors. The most exhaustive and exact data are to be found in the works of Timofeewski and Benewolenskaja (2), and Caffier (4). Our observations completely confirm their data and we therefore limit ourselves to a short description, dwelling chiefly on those points which have a relation to our fundamental problem.

The fragments planted in the cultures in the great majority of cases contain all the white elements of blood in their normal proportions.

A few minutes after the planting of the culture an intensive migration of the leucocytes into the medium begins. Until the end of the first 24 hours the planted fragment is surrounded by a fairly large zone of migration; this is usually 1·5–2 times larger than the fragment. During the first 24 hours we recognise here all the characteristic elements of blood. Usually the most peripheral zone is occupied by degenerating neutrophiles. The zone nearest to the implant is densely filled with lymphocytes, monocytes and single eosinophiles. All these elements are in a state of amoeboid movement.

The general rate of migration of all the elements depends directly on the composition and physical conditions of the medium. For example, the greatest migration was observed in groups 3 and 4 of our experiments (see Summary 1), in which the plasma used as initial medium was always diluted by hypotonic Ringer solution. The cultures developing in an undiluted plasma always gave a considerably smaller migration.

Sections of the cultures made it possible to observe the fate of the fragment itself, and we never observed cell divisions in it. During the first 24 hours there is a fairly rapid unification of the fragments of cell elements. By the third or fourth day only groups of degenerated erythrocytes and separate strongly hypertrophied non-granular elements (also with evident signs of degeneration) remained in the central part of the fragment planted. In distinction from the central zone of the implant, the periphery remains from the second day densely filled with non-granular forms.

During the first 24 hours the great majority of the non-granular cells (lymphocytes and monocytes) begin to increase in volume, and small granular ingredients become clearly visible in their cytoplasm.

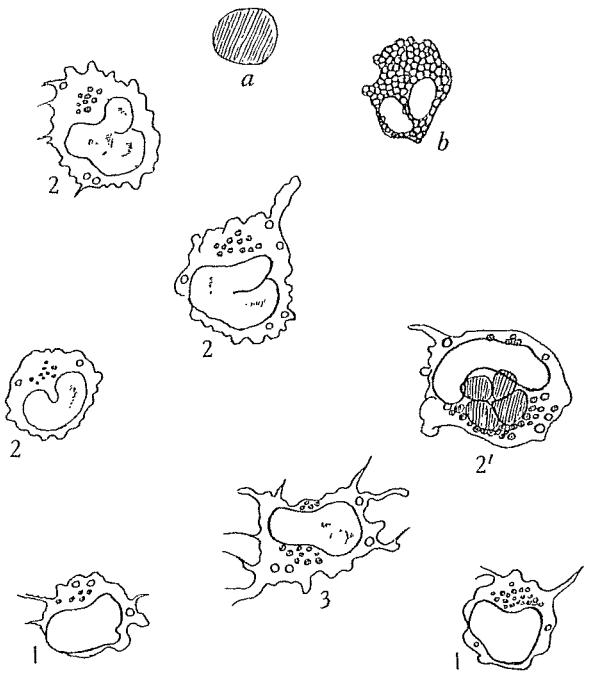
Vital staining with a very weak solution of a neutral dye (neutral Red 1 : 10,000 in Ringer solution) makes it possible to follow quite clearly under the microscope the changes that occur in the non-granular cells during the first 24 hours. Text-fig. 1 shows the cells of a 24-hour culture in group 4 of our experiments. The cells, greatly enlarged in volume, contain a considerable amount of fine granulation which stains well with N.R. and isolated fat particles. It is characteristic that the grains which can be stained with N.R. are always situated apart from the fat. This agrees with what Maximov(3) described for other Mammalia. Usually the N.R. grains are grouped near the nucleus, while the fat drops are in the peripheral fragments of the cytoplasm (Text-figs. 1, 2).

Among the cells described there occur isolated cells with phagocyte-like parts. In most cases the erythrocytes appear to be phagocyte-like. For these reasons we include these forms either among the polyblasts of Maximov or among the macrophages.

On further cultivation (2, 3, 4 days) the polyblasts undergo a further change. They increase in volume, and there appears a clear division of the cytoplasm into a dense inferior layer (endoplasm) and a thin peripheral transparent and homogeneous layer (ectoplasm). At the same time fairly clearly defined shoots appear on the surface of the ectoplasm, sometimes as swellings, sometimes as margins; these are the formations that Carrel described as "undulating membranes".

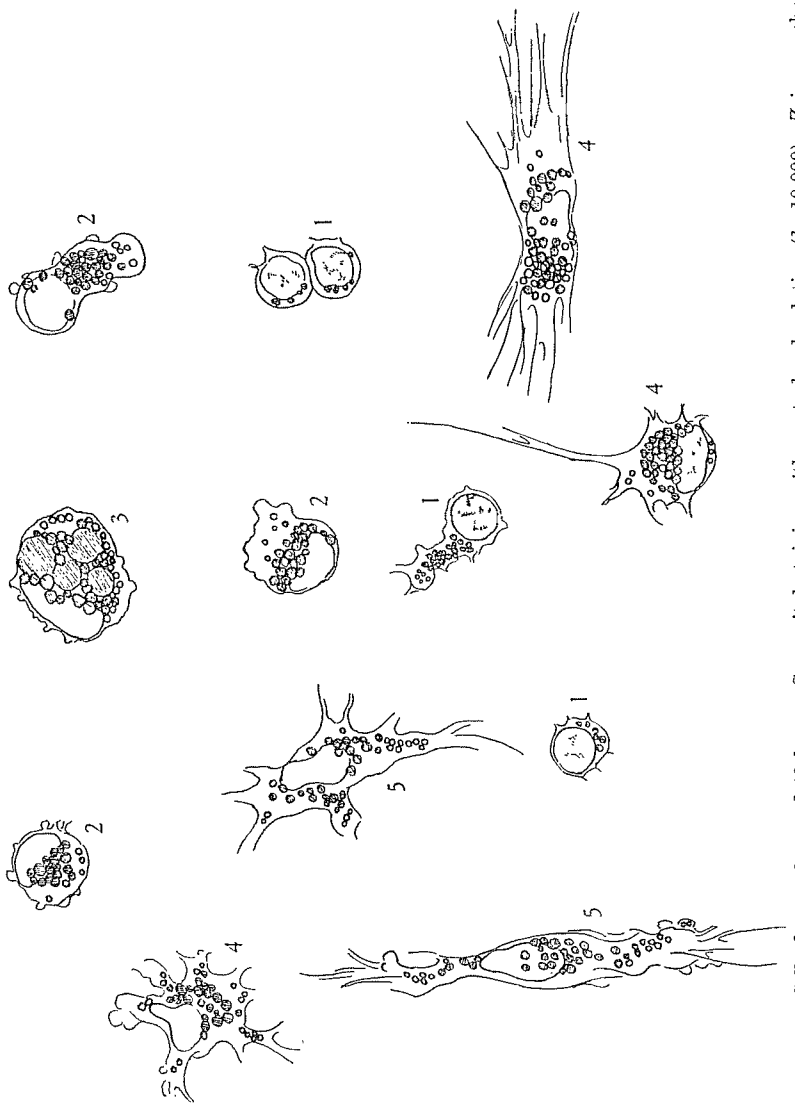
The polyblasts—macrophages, encountering one another in the process of migration—unite with the shoots and form connected groups of cells.

Vital staining shows a considerable increase of N.R. granulation. Not only does the number of grains increase, but also the dimensions of the grains themselves (Text-fig. 2). With the increase of the "segregation apparatus" there also takes place an increase of the number of



Text-fig. 1. Cells from culture of 24 hours. Supravital staining with neutral red solution (1 : 10,000). Zeiss apochr. 2 mm. oc. ×10, camera lucida. *a*, erythrocyte; *b*, eosinophile leucocyte; *1*, lymphocyte; *2*, monocyte; *2'*, *3*, polyblast.

fat particles. The relative position of N.R. grains and fat drops remains the same as in the previous stages. The N.R. granules are always included in the endoplasm, while the fat drops can be detected in the ectoplasm (Text-fig. 2). A very characteristic quality of a polyblast is a sharp osmiophilism of its cytoplasm. This is especially noticeable on the preparation after a fixation of 24 hours in Flemming solution (Plate XII, fig. 2). A clearly defined osmiophilism of the whole cytoplasm and especially of the endoplasm shows special constituents undoubtedly connected with the specific change. All these changes in the non-



Text-fig. 2. Cells from culture of 48 hours. Supravital staining with neutral red solution (1 : 10,000). Zeiss apochr. 2 mm. oc. comp. ×4, camera lucida. *1*, lymphocytes; *2*, *3*, polyblasts; *4*, polyblasts transforming into fibrocyte-like cells; *5*, fibrocyte-like cells.

granular cells are connected with a gradual change of structure in the nucleus. The nucleus of a lymphocyte and monocyte gradually loses its irregular contours, the small lumps of chromatin grow smaller and are distributed more regularly over the whole volume of the nucleus (Plate XII, figs. 1 and 2).

Usually at the end of the third 24 hours more and more cells with several shoots, generally elongated or in the form of a star, are situated in groups (Plate XII, fig. 1). With the change of form the polyblasts lose the characteristic difference between ecto- and endoplasm. Nevertheless a whole series of cells with offshoots preserve very thin films on the ends or sides of the offshoots—remnants of the undulating membrane (Plate XII, fig. 1, *a*). The mutations described are connected with a gradual loss of osmiophilism. All sorts of coarse ingredients, remnants of the phagocyted portions (Plate XII, fig. 2, *2*), appear in the cytoplasm of many cells having offshoots. This appearance, and the presence of the remnants of the undulating membrane, show clearly that the cells having shoots originate from the polyblasts. The structure of the nucleus confirms this (Plate XII, fig. 1).

The cells with offshoots, which are undoubtedly stable, immobile cells, connect themselves by their offshoots into syncytial nets and form whole groups of tissue complexes (Plate XII, fig. 1). After vital staining we succeeded in discovering that the N.R. granulation in the cells with offshoots becomes more diffused in the cell (Text-fig. 2; Plate XII, fig. 2). Nevertheless the fat particles continue to remain separated from the N.R. granula. This less compact and diffused distribution of N.R. granulation corresponds with the loss of a clearly marked differentiation of cytoplasm into endo- and ectoplasm.

In the greater part of our cultures during the fourth 24 hours, a considerable number of spindle-like cells was observed among the complexes of the cells with offshoots. The cells, by reason of a whole series of cytoplasmatic and nuclear structures, approach very nearly to the usual stable cells of connective tissue, *i.e.* fibrocytes, and by this term we shall call them in what follows and also in our summary. The fibrocytes undoubtedly originate from offshoot polyblasts, or more exactly from the transitional forms of the previous stages in the development of the culture. On the basis of the analysis of these transitional forms we may indicate the following process of mutation. Gradually the greater part of the offshoots disappears, while two usually remain at the poles. All vestiges of the undulating membrane and the visible ectoplasmatic border disappear. At the same time the osmiophilism of the protoplasm

vanishes and the nucleus acquires the typical structure of a fibrocyte—a thin film over the nucleus, fine chromatin grains, diffused through the whole nucleus, and two or three large distinct nucleoli. But even at this stage of development the spindle-like cells often preserve distinct traces of their origin, grains, phagocyte-like particles, etc. (Plate XII, fig. 2, *1*). All these changes in the non-granular forms occur sporadically in the migration zone. In cultures 4 days old we may find only niduses of tissue complexes on the border of the fragment itself and on the periphery of the migration zone. In a great number of cases the changes occurred so regularly that the cultures ultimately resembled a well-grown culture of connective tissue (Plate XII, fig. 2).

In order to obtain the maximal number of cell elements the fourth 24 hours is the most appropriate period for the renewal of the medium. About this time there are considerable niduses of spindle-like fibrocytes in the culture, although other forms of polyblasts (rounded and with offshoots) are present in considerable number. The renewal of the medium and the insertion of new material favouring mutations somewhat delays the rate of new mass migrations. This corresponds with a whole range of data obtained lately in connection with the study of the conditions of the differentiation and the multiplication of the cells in tissue cultures. In the selection of the time for the renewal of the medium, following the method of Chlopin(6) we tried to postpone the "final" differentiation, and to conserve in this way the greater part of the cells capable of reproduction. Our hypothesis appeared to be valid (see Summary 1). Hence the cell forms in the zone of migration which appeared in the fifth and sixth 24 hours (*i.e.* in 24–48 hours after the renewal of the medium) appeared to be approximately the same as before the renewal of the medium, *i.e.* in the fourth 24 hours (Plate XII, fig. 2). But there is no doubt that the process of mutation, though delayed, continued. This is indicated by the fact that more and more "ripe" forms of fibrocytes appeared in the migration zone in the fifth and sixth 24 hours. A hardly noticeable osmiophilism of the cytoplasm and the presence of the thread-tonofibrils lying along the cells is characteristic in a large number of cells (Plate XII, fig. 2, *1*). In the sixth 24 hours these particular forms begin gradually to dominate in the cultures. The fifth and the sixth 24 hours were the periods in the life of the cultures when we could discern the first mass mitosis (see Summary 1).

IV. CELL DIVISION.

It is not the task of the present article to analyse in detail the somatic karyotype of man, nor to describe exhaustively the morphological details of the mitosis encountered in the cultures. We are especially interested in the chromatin figure and in the chromosomes from the point of view of the possible use of our cultures as material for karyological investigations. Therefore we shall limit our description to a series of typical figures that give a general idea of the material. Cell divisions, as shown in Summary 1, appear in the cultures in great number after the first renewal of the medium, *i.e.* in the fifth 24 hours of the life of the cultures. Previous to this time we found only rare and isolated mitosis in lymphocytes of average size. These cells under mitoses are extremely minute, in consequence of the extremely densely situated small and large chromosomes; their chromatic figure in spite of the best fixation was not fit for analysis. In addition, in these segregating lymphocytes we never met with plates that were situated parallel to the plane of the covering glass, and therefore in the complete preparation of the cultures these mitoses are completely unfit for our aims. It is evident from the aforesaid that cultures less than 5 days' old are an unsuitable material for karyological investigations. On the other hand, after the first 24 hours of the renewal of the medium our cultures are adequate to all the demands of modern karyology. The maximum of mitoses in the cultures appears in the 48 hours after the renewal of the medium. Therefore special attention was paid to these cultures, and all of the mitoses to be described belong to cultures fixed at 6 days' old, *i.e.* 48 hours after the first renewal of the medium. This time proved to be the most suitable because more than half of the cells under mitosis had entered the metaphase stage (see Summary 2), which is the most appropriate for karyological analysis. This shows the existence of a certain rhythm of cell divisions in the

SUMMARY 2.

Quantity of cells under mitosis in seven arbitrarily chosen series of cultures.

No. of series	Prophase	Metaphase	Anaphase	Telophase	Total No.
13	4	8 (3)	3	3	18
16	2	11 (2)	1	1	15
21	1	7 (2)	—	2	10
24	11	33 (13)	2	14	60
25	12	20 (9)	3	4	39
27	17	51 (24)	8	15	91
	50	143 (62) 56% (43%)	19	40	252

The number of metaphases situated under an angle or perpendicular to the plane of the coverglass are included in brackets.

cultures, the more so since the metaphase according to all present data is the shortest period of the karyokinesis. The existence of a rhythm in the cell divisions is also shown by the fact that in the great majority of cases cultures of the same series, fixed at the same time, gave an identical ratio of karyokinetic stages. This circumstance leads to the necessity of arranging exact intervals between the maxima of all divisions for obtaining a maximal number of cells under mitosis in a definite stage. Our experiments show that these intervals repeat themselves in 8–10 hours, if we desire a maximum of metaphases.

For the study of mitoses, after having tried several methods, we chose the following: the cultures were fixed in Flemming solution with a reduced quantity of osmium and acetic acid (1 per cent. of chromic acid, 16 c.cm.; 2 per cent. osmic acid, 1 c.cm.; 2–3 drops of a strong acetic acid).

In this fixative the cultures were left for 12–14 hours at room temperature.

The time elapsing from the moment of taking the culture from the thermostat to plunging it into the fixative must be minimal (5–10 sec.); the culture must not get cold. The material is better in proportion to the rapidity of fixation. After fixation follows washing in running water for 24 hours. Then, during the day, spirits of increasing strength are used, and for the night a spirit of 96 per cent. is used.

We tried to use in addition to Flemming solution other fixatives recommended for karyological work (Bouin, Bouin-Allen, Levitsky, Helly). None of these gave as good results as the above variation of Flemming solution.

The lessened quantity of osmium in the fixative used by us plays a very important part, since, in spite of prolonged fixation, the cultures appear feebly impregnated with osmium, and it is possible to produce a complete preparation without previous bleaching, which is not favourable for the staining we use. Complete preparations of cultures for karyological purposes have quite definite advantages over sections.

The best results were obtained by staining by the method of Feulgen-Rosenbek. After keeping in 96 per cent. spirit, the cultures were rapidly transferred through spirits of diminishing strengths to distilled water, where they were kept for about an hour. Then a hydrolysation for 30 min. at 60° C. was undertaken. After hydrolysis they were submerged in cold hydrochloric acid, rapidly washed in distilled water, and placed in a solution of fuchsin-sulphuric acid for 2–4 hours at room temperature in complete darkness. Thereafter, without washing, the cultures were plunged in sulphur water for 10–15 min., where they usually acquired

a rose colour. Next a washing in running water follows for several hours—during this time the colour becomes still more intense. The removal of the water we accomplished by the method of Fisher, by means of alcohol, acetone and acetone-xylol. It is necessary to point out that the intensity of the colour of the stable and dividing nuclei depends on the rapidity of the transfer through the alcohol before the staining and after it. The staining is in inverse proportion to the period the material is kept in the acid.

None of the stains usually used for the staining of the complete cultures gave us results approaching those obtained by the method of Feulgen. In particular, the iron haematoxylin of Heidenhain is almost completely unsuitable because of the unusual difficulty in the differentiation of the complete culture.

We observed approximately the same number of cells under mitosis in polyblasts with offshoots and in rounded ones, as also in the fibrocytes. In each of these cells we observed typical peculiarities of chromatin figure. In the greater part of the polyblasts, the chromosomes in the metaphase are situated in the form of a corona (Plate XIII, fig. 1). The ends of the long chromosomes usually stand out quite distinctly from the common exterior circumference of the whole corona (Plate XIII, fig. 1, *c*, *d*). In some figures the small and middle-sized chromosomes are situated apart from the whole mass of chromosomes in the centre. The corona of chromosomes is not always closed; there may be a rupture on one side (Plate XIII, fig. 1, *b*, *c*), or several such ruptures, and then the single chromosomes, appearing as if they had fallen backward from the whole chromatin figure, are clearly distinguishable (Plate XIII, fig. 1, *a*). These ruptures remind us of the defects in the chromatin figure, which are described in the myelocytes of leucaemic blood by Andres and Shiwago (7). But this is only a superficial resemblance. We do not find here those typical anomalies which are characteristic of the chromosomes of an atypical mitosis in leucaemic blood. All the chromosomes are clearly separated and distinctly constructed, there is no conglomerated chromosome mass among them, and we therefore consider this mitosis to be quite normal and typical for polyblasts. We are inclined to consider the figures with an unclosed corona as the earliest stages of the metaphase, or more exactly as a transition stage from the prophase to the metaphase. These figures belong thus apparently to the polyblasts, which have in the passive state a somewhat polymorphous nucleus. In the prophases of such polyblasts we observe a quite unequal distribution of the chromosomes; in one portion of the nucleus they are extremely inter-

twined, in another portion of it they are situated quite separately. This irregular distribution of the chromosomes in the prophase completely corresponds to the appearance of a metaphase with a ruptured corona.

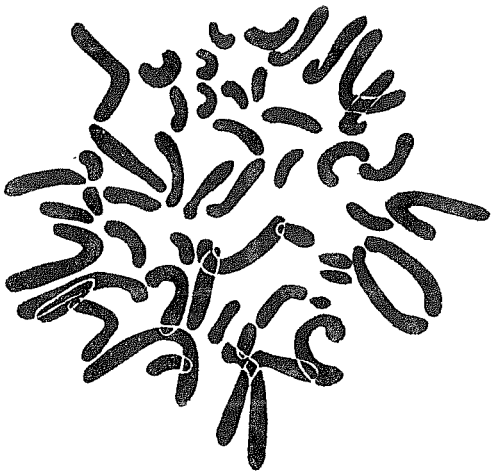
The corona-like figures in the polyblasts are not quite suitable for drawing and for counting the chromosomes. The chromosomes are situated in them generally quite close together, lying on one another in different planes. Only a very good fixation and a sufficiently selective, but at the same time sufficiently transparent staining, makes it possible to analyse the figure. Nevertheless they may be used as corroborative material to others where no doubt exists, such as one described below.

Polyblasts with a "corona-like" chromatin figure usually occur in the depths of the plasma clot or on the inside of a newly developed tissue in the zone of migration. We found other figures in metaphases nearer to the surface of the cover-glass or directly under it. These figures are regular plates with more or less regular distribution of chromosomes in one plane. They have, in common with the corona-like figures, only the peculiarities that nearly all the chromosomes are situated on the periphery of the plate. The greater part of the medium-sized and small chromosomes are located in the centre (Plate XII, fig. 3). Such figures are the most suitable for drawing and analysing. In Plate XII, fig. 3 is given a microphotograph of one of the best figures of this type. The drawing (Text-fig. 3) and the photographs show that all the chromosomes are quite definitely distinguishable with a minimum of single elements lying on them. These drawings were made at different times by three observers, who did not differ even by a single chromosome in their counting of them. There appeared to be 52 chromosomes. The cell belongs to an entirely normal man aged 37. How must we explain the distinct difference in the chromatic figures of the same type of cells? In connection with the fact that the last type of plate is always discerned immediately under the cover-glass, it seems to us that the location of the chromosomes depends on mechanical factors, which influence the whole of the cell. In connection with a lamina-like location of the chromosomes, the whole cell always appeared enlarged in its diameter and flattened (Plate XII, fig. 3). These are two typical figures characteristic of the metaphase in the polyblasts.

The metaphases in the fibrocytes are characterised by a somewhat different location of the chromosomes and also by the character of the components themselves (Plate XIII, fig. 2). In these figures there appears a quite regular location of differently sized chromosomes, which is typical for higher vertebrates and has several times been described in birds and

mammals. Large and variously curved chromosomes are found in the peripheral zone, while all of the centre is occupied by small stick-shaped, bean-shaped, and oval ones (Plate XIII, fig. 2). By no means rare are figures in which the pairing of the chromosomes appears unusually clearly (Plate XIII, fig. 2, *b*). The figures in the fibrocytes are always more distinct and larger as compared with most figures in the polyblasts. But we find the same types of chromosomes in both species of cells, though the large chromosomes in the fibrocytes at the metaphase stage are always longer and thinner.

Different subjects, showing other features characteristic for a human chromosome, may have clearly noticeable differences in the length and



Text-fig. 3. Drawing of mitosis represented in Plate XII, fig. 3; 52 chromosomes. Normal blood, 6-day culture.

breadth of the chromosome. For example, in the cases we observed, several times one subject differed fairly distinctly from two others by much longer chromosomes in the polyblasts as well as in the fibrocytes. We do not agree with the opinion of Hence (8) that one never succeeds in seeing metaphases from the pole. As shown in Summary 2 we obtained 57 per cent. metaphases, the lamina of which were situated parallel to the covering glass. We consider the indications given on this question by Caffier (9) and Kemp (10) to be quite correct.

In conclusion we may point out that in none of the numerous series of our cultures did we discover any noticeable quantity of cells under mitosis similar to those found in large quantities by Caffier (11) in

mesenchyme cultures of the human lung. We noticed only isolated cases of abortive mitoses, belonging to cells which were evidently perishing.

V. Conclusions.

1. In the cultures of leucocytes of normal human blood under definite conditions of cultivation there appear periods of mass multiplication of cells in mitotic fashion.

2. The mass multiplication is observed in the polyblasts (macrophages) at the stage of their mutation into stable cells, and in the fibrocytes originating in haemocultures from polyblasts.

3. For obtaining the maximal quantity of mitoses the best time for fixation is the second 24 hours after the renewal of the medium, this latter being performed not later than the end of the fourth 24 hours after the planting of the culture.

4. After appropriate fixation, the figures of divisions may be used for karyological analysis.

5. The method proposed enlarges the possibilities of the investigation of the human karyotype, and enables us to undertake the karyological investigation of any anthropogenetical or medical phenomena.

REFERENCES.

(1) Chrustschoff, G., Andres, A. and Iljina-Kakujewa, W. (1931). "Kulturen von Blutleukozyten als Methode zum Studium des menschlichen Karyotypus (Vorläufige Mitteilung)." *Anat. Anz.* **73**, H. 6/8.

(2) Timofeewski, A. and Benewolenskaja (1928). "Über Leukozytenkulturen des Menschenblutes *in vitro*." *Arch. exp. Zellforsch.* **5**.

(3) Maximov, A. (1928). "Cultures of blood leucocytes. From lymphocyte and monocyte to connective tissue." *Ibid.* **5**.

(4) Caffier, P. (1928). "Die prospektiven Potenzen des normalen Menschenbluts." *Ibid.* **6**.

(5) Hirschfeld and Klee-Rawidowitsch, E. (1928). "Untersuchungen über die Genese der Blutmakrophagen und verwandter Zellformen, sowie ihr Verhalten in den—*in vitro*—Kulturen. I. Normales und leukämisches Menschenblut." *Z. Krebsforsch.* **27**, H. 1/2.

(6) Chlopin, N. (1931). "Studien über Gewebekulturen in artfremdem Blutplasma. V. Das Verhalten und die Verwandlungen des menschlichen Mesenchyms im Explantat." *Arch. exp. Zellforsch.* **12**.

(7) Andres, A. and Shiwago, P. (1933). "Karyologische Studien an myeloischer Leukämie des Menschen." *Folia haematol.* **49**, H. 1/3.

(8) Hence, A. (1929). "Comparison of mitosis in chick tissue-cultures and in sectioned embryos." *Biol. Bull.* **50**, No. 2.

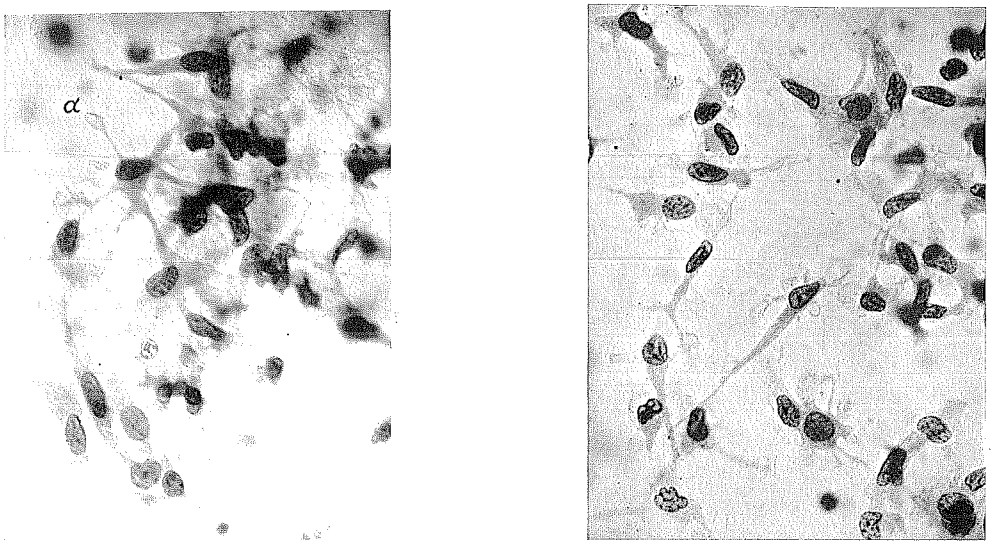


Fig. 1.

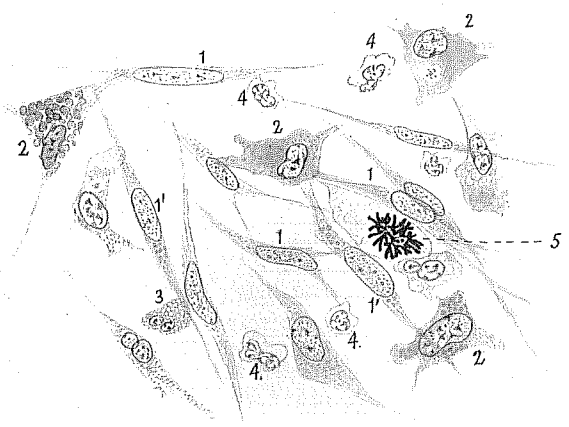


Fig. 2.

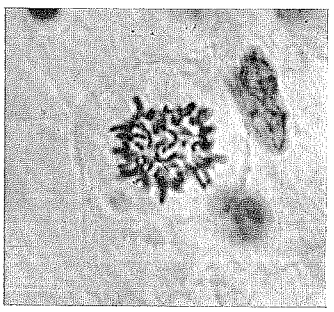


Fig. 3.

Fig. 1.

Fig. 2.

(9) CAFFIER, P. (1930). "Über das Verhältnis der Zellachse zur Achse der Teilungsspindel bei der Mitose menschlicher Zellen *in vitro*." *Arch. exp. Zellforsch.* **10**, H. 2.

(10) KEMP, T. (1929). "Über das Verhalten der Chromosome in den somatischen Zellen des Menschen." *Z. mikr.-anat. Forsch.* **16**.

(11) CAFFIER, P. (1932). "Chromosomenstudien am menschlichen embryonalen Gewebe. Ein Beitrag zum Problem des Wachstums von Embryo und Tumor." *Z. Geburtsh. Gynäk.* **101**.

EXPLANATION OF PLATES XII AND XIII.

PLATE XII.

Fig. 1. Cells from 3-day culture. Fixation Bouin-Allen, haematoxylin Carazzi. Zeiss apochr. 4 mm. oc. comp. ×4, microphotograph.

Fig. 2. Cells from 4-day culture. Flemming, Feulgen staining. Zeiss apochr. 2 mm. oc. comp. ×4, camera lucida. *1*, fibrocytes; *1'*, *2*, polyblasts; *3*, eosinophil leucocyte; *4*, lymphocytes; *5*, mitosis in fibrocyte.

Fig. 3. Mitosis in polyblast. 6-day culture. Flemming, Feulgen staining. Zeiss apochr. 2 mm. oc. comp. ×15, microphotograph.

PLATE XIII.

Fig. 1. Mitosis in polyblasts. 6-day culture. Flemming, Feulgen staining. Zeiss apochr. 2 mm. oc. comp. ×10, microphotograph.

Fig. 2. Mitosis in fibrocytes. 6-day culture. Flemming, Feulgen staining. Zeiss apochr. 2 mm. oc. comp. ×10, microphotograph.