DIFFERENTIAL STAINING OF HUMAN CHROMOSOMES WITH QUINACRINE

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The relationship between the intensity of fluorescence of chromosome preparations and the concentration of the fluorochrome solution was studied. In a concentration below $5 \cdot 10^{-5}$ g/ml the dye can be used as immersion medium. By staining in this way the intensity and stability of the fluorescence can be increased and the differentiation of brightness along the length of the chromosomes can be improved. The method gives reliably reproducible results, it is simple in use, and is particularly convenient for rapid analysis.

Caspersson et al. [2] published a paper on the differential staining of human chromosomes with the alkylating fluorochrome quinacrine-mustard. Later, other workers obtained a similar effect when using quinacrine in its ordinary form [1, 3, 4]. Although it is not in such short supply or so toxic, ordinary quinacrine does not, however, always give satisfactory results.

The object of this investigation was to study the conditions which influence the quality of the differential staining by quinacrine and to determine the best ways of staining and microscopic examination.

EXPERIMENTAL METHOD

Experiments were carried out mainly on preparations of chromosome from human lymphocytes obtained by the standard procedure [hypotonic solution 0.075 M KCl, fixative methanol : acetic acid (3 : 1); drying without heating], and also on preparations of epithelial cells from the buccal mucosa and of human spermatozoa (using the same fixative). Quinacrine of Soviet manufacture was used chiefly for staining, and for comparison atebrin (Gurr, England) and quinacrine-mustard (Serva, West Germany) also were used. The microfluorescence apparatus consisted of the MMB-1 microscope fitted with the OSL-1 luminescent lamp and the MFNÉ-1 camera attachment. The source of radiation was a type DRSh-250 mercury lamp and filters SS-4 and ZhS-18 were used. Magnification during observation and photography was 720 and 360 respectively; objective VI 85 × 1.0; the film was KN-3 (35 mm, 90 units). Exposure was 4-8 sec, with development to nominal sensitivity. Prints were made on paper No. 4-6. Fluorimetric analysis was carried out on the SMP-05 microphotometer (Opton, West Germany) in transmitted light. Filters: BC-12 and 500, objective 40 × 1.0.

EXPERIMENTAL RESULTS

To begin with various modifications of existing techniques were analyzed. Preparations were stained in 0.5% solutions of the dye in water or in buffer for 5-15 min, washed in water or in buffer for 1-20 min, and then mounted in buffer or in water. Phosphate and citrate-phosphate buffers within the range of pH 4.0-8.0 were tested. The results of staining using water after a single distillation in a metallic still and using bidistilled water from a glass apparatus were compared.

In the next experiments the relationship between the results of staining and the concentration of the fluorochrome within the range from $5 \cdot 10^{-7}$ to $5 \cdot 10^{-3}$ g/ml was studied. It was found that in concentra-

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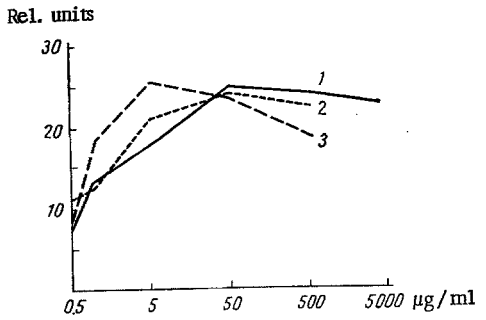

Fig. 1

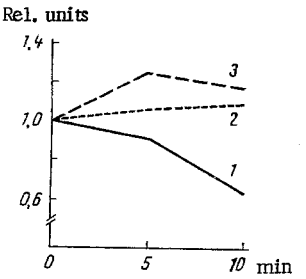

Fig. 2

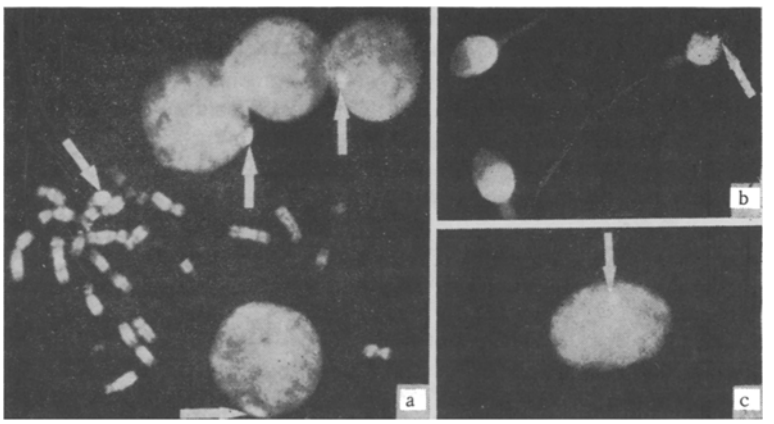

Fig. 3

Fig. 1. Intensity of fluorescence of interphase nuclei in dyes of different composition: 1) solution of fluorochrome in standard phosphate buffer, pH 7.2; 2) the same in 0.5% NaCl solution; 3) the same in a mixture of standard buffer with water (1 : 9). Abscissa, concentration of quinacrine (in μg/ml); ordinate, intensity of fluorescence (in relative units).

Fig. 2. Change in intensity of fluorescence during differentiation and staining with quinacrine solutions of different concentrations. 1) Solution of quinacrine in standard phosphate buffer, pH 7.2, concentration $5 \cdot 10^{-6}$ g/ml; 2) ditto, concentration $5 \cdot 10^{-4}$ g/ml; 3) ditto, concentration $5 \cdot 10^{-3}$ g/ml. Abscissa, time of differentiation in phosphate buffer (in min); ordinate, intensity of fluorescence, reduced to same value at beginning of differentiation (in relative units).

Fig. 3. Examples of fluorescent staining by the method described in the text: a) interphase nuclei and fragment of metaphase plate from a patient with lengthened Y-chromosome (blood culture); b) spermatozoa and c) interphase nucleus of epithelial cell from the buccal mucosa (scraping) of a man with normal karyotype; arrows mark brightly fluorescent area of the Y-chromosome.

tions below $5 \cdot 10^{-5}$ g/ml the intrinsic fluorescence of the dye solution in the layer under the coverslip creates virtually no harmful background. With such low concentrations it was possible to analyze the preparations directly in the fluorochrome as medium. This often repeated variant of the staining technique had many valuable advantages: the brightness of fluorescence was much greater than by staining followed by differentiation, the photoresistance was several times greater, there was no deposition of the dye as droplets surrounding the stained structures. The brightness of fluorescence reached a maximum after 2-3 min and thereafter remained unchanged. The effect was independent of the quality of the water and could be reliably reproduced even if tap water was used. By visual assessment the quality of staining was unaffected by changes in the concentration of the dye within the range from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-6}$ g/ml. Better results

were obtained if the pH of the dye was 6.5-7.5, in solutions with a low salt concentration (1 part of buffer to 10-20 parts of water). However, it was observed in several preparations that these solutions give a marked hypotonic effect: the chromosomes and interphase nuclei swell and are deformed. This undesirable artifact was eliminated if dye was made up in buffer of nominal molarity or in 0.5-1% NaCl solution, although with an increase in the salt concentration the brightness and photoresistance of the dye were somewhat reduced.

These observations were verified by quantitative analysis. The preparation was stained successively in increasing concentrations of quinacrine (from $5 \cdot 10^{-7}$ to $5 \cdot 10^{-3}$ g/ml) and each time the brightness of fluorescence of the same object (an interphase nucleus) was determined. With concentration of $5 \cdot 10^{-4}$ g/ml or higher the preparation was dipped quickly in the same solvent as was used to prepare the dye before measurement. The initial analysis was carried out on a working fluorescent system using incident light. The brightness of fluorescence for each concentration was calculated as the reciprocal of the exposure required to obtain photographic negatives of the same optical density. This fairly rough method nevertheless was adequate to show that with a change in concentration of several thousand times the intensity of fluorescence changed by not more than twice. These results were confirmed by more accurate measurements on the SMP-05 microphotometer. The relationship between the intensity of fluorescence and the fluorochrome concentration and composition of the solvent is shown in Fig. 1. In all these cases pH was 7.2. Graphs showing the change in relative brightness during washing out of the dye after staining the specimen in a solution of quinacrine in phosphate buffer in concentrations of $5 \cdot 10^{-6}$, $5 \cdot 10^{-4}$, and $5 \cdot 10^{-3}$ g/ml are given in Fig. 2. The first point corresponds to the measurement made immediately after a short rinsing, and the subsequent points correspond to repeated measurements after incubation in the buffer for 5 and 10 min. Comparative measurements of the brightness of staining with quinacrine solution in a concentration of $5 \cdot 10^{-4}$ g/ml in 0.5 and 1.5% NaCl solution in phosphate buffer, pH 7.2, and in a mixture of the same buffer with water in the ratio of 1 : 9 showed that the ratio between these brightnesses was 1 : 0.87 : 0.95 : 1.1 respectively.

On the basis of the results of these observations two alternative staining methods were chosen for practical purposes. In the first, the simplest and most convenient for rapid analysis, the dye is made up in tap water. If a hypotonic effect takes place, the dye is diluted with 0.5-1% NaCl. In both cases the pH is close to 7.0-7.3. The quinacrine concentration is approximately $5 \cdot 10^{-6}$ g/ml, with permissible deviations of 3-5 times on either side. Staining is carried out for 2-3 min after which, without washing off the dye, the preparation is covered with a cover slip so that no air bubbles form beneath it. To keep the preparation for a long time, the dye is removed in alcohols of increasing concentration.

A clearer image was obtained by the use of the water-immersion VI 85 × 1.0 objective with correction. Oil immersion objectives (MI 90 × 1.25 and planachromat 100 × 1.25, Zeiss, East Germany) gave inferior results, even when the water-containing layer beneath the coverslip was minimal. Photomicrographs of specimens of human tissues stained and photographed as described above are given in Fig. 3.

The results thus show that staining can reach saturation point even if the dye is very highly diluted. There is a wide plateau in the concentration range from a few units to hundreds of micrograms per millimeter, and there is even a slight decline in fluorescence with an increase in the fluorochrome concentration. It can accordingly be concluded that in the ordinary technique overstaining takes place at first, and this is followed by differentiation of the specimen. Nevertheless, the end result in this case is worse than if quinacrine solutions of low concentration are used. As regards the clarity of the differential staining, these observations show that it depends more on the properties of the specimen than on the method of staining. Preparations obtained in different experiments or after different times of keeping give different effects under the same conditions. Clear differentiation along the length of all the chromosomes of the set is comparatively rarely observed. In the typical case bright fluorescence of characteristic areas of individual chromosomes is seen (the distal part of the Y-chromosome, the short arms of some acrocentrics, areas near the centromere in chromosomes 3 and 5, and so on), coupled with very slight gradations of brightness along the length of the chromosomes as a whole. After an analysis of specimens of this type we tried staining them with quinacrine-mustard by the method of Caspersson et al. [2] but did not obtain any improvement in the picture.

LITERATURE CITED

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