Technical methods

Chromosome analysis from human skin

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Since the description of Harnden's technique for human skin culture, many modifications and simplifications have been described (Edwards, 1960; Lejeune, Turpin, and Gautier, 1960; Smulow, Rustigian, and Tye, 1961; Fox and Zeiss, 1961; Frøland, 1961; Hirschhorn and Cooper, 1961; Pariente, 1961; Ferguson, 1962; Basrur, Basrur, and Gilman, 1963). The method mainly used in this laboratory is modified from Harnden (1960) and Moorhead, Nowell, Mellman, Battips, and Hungerford (1960).

TECHNIQUE

BIOPSY A suitable region in the forearm is sterilized by swabbing with alcohol which is allowed to evaporate and does not contaminate the biopsy. An injection of Lignocaine is administered in the region from which the biopsy is to be taken, and allowed to act for a few minutes. A small piece of tissue is excised, using fine forceps and scalpel, and at once transferred to a Bijou bottle containing sterile T.C. 199 (Glaxo), and transported to the laboratory for setting up.

CULTURE All sterile work is done in an air cubicle, the atmosphere of which is sterilized by an ultra-violet lamp. The tissue is cut into pieces about 1 mm. cubed and embedded in a C.E.E.: chicken plasma clot, in a 5 cm. Carrel flask. Normally six or eight pieces are placed in one flask. Two ml. of growth medium B (Harnden) is added, the flask is flushed out with 5% CO₂ in air, stoppered with a silicone rubber bung, and placed in the incubator at 37°C. The fluid medium is changed routinely every two days but the plasma clot is not disturbed unless the explant is dislodged. Usually there is little or no growth for about three days and longer in some cases. A few fibroblast-like cells are then seen to be emerging from the explant (Fig. 1) and thereafter this outgrowth expands fairly rapidly. Mitoses can be observed in it.

When the outgrowth is judged to be adequate, usually after five to 10 days, the medium is removed and replaced by an equal amount of 0.02 % trypsin, which was previously warmed in the incubator to 37°C. After about five minutes at this temperature the cells are released from the explant, which is not disturbed by this procedure. The resulting suspension of cells is removed and centrifuged at 400 r.p.m. for five minutes. The supernatant is discarded and the pellet of cells is re-suspended in

FIG. 1. *Fibroblasts emerging from a skin explant after three days' culture.*

more growth medium and then transferred to a clean sterile flask, which is gassed with CO₂/air mixture (5% to 95%), tightly stoppered, and returned to the incubator. After a further three or four days a heavy growth of cells covers the bottom of the flask. The cells can then be subcultured for cytological examination or their number can be built up by further subculture before so doing.

Routine subcultures follow the same procedure as that outlined above for the primary explant with adequate outgrowth. Such cultures may be kept going for several months at a steady rate of growth.

When subculturing for cytological examination the cells are put into suspension as before, and a rather heavy concentration of cells placed into 6 ml. of growth medium in a 9 cm. petri dish containing a number of cover glasses attached to the bottom by means of a tiny drop of clotted plasma and C.E.E. The cells are

thoroughly mixed by gently rotating the dish, which is then placed in a sealed vessel. This is then flushed out with 5% CO₂ in air and placed in an incubator at 37°C. The atmosphere in the vessel is kept moist by having an open dish of distilled water in it.

CYTOLOGICAL PROCEDURES It was found that a considerable number of mitoses occurred about 24 hours after each subculture. Colchicine (0.005%) is added in the proportion of 0.1 ml. per millilitre of medium to the culture 22 hours after subculture. This is allowed to act for about six hours, by which time a considerable number of mitoses have accumulated. The cells on the cover slips are then treated in the way described by Rothfels and Siminovitch (1958). They are removed from the petri dishes and given 20 minutes' hypotonic treatment at 37°C. in $\frac{1}{4}$ strength Hank's solution previously warmed to the same temperature. The cover slips are then held face down in glacial acetic acid-absolute alcohol (1:3) fixative for one minute and placed gently in the same fixative in a Columbia staining dish for 30 minutes. It has been found that this modification of the Rothfels and Siminovitch technique tends to keep the cell membranes unbroken and the chromosomes therefore less likely to become scattered.

The cells are then air dried and the cover slip is inverted onto a drop of lacto-acetic orcein stain on a siliconed slide (natural orcein 2 g. in 100 ml. of a mixture of equal parts of 70% lactic acid and 99% acetic acid). When the chromosomes are sufficiently stained as seen under the microscope (approximately six hours) the cover slip and cells are floated off in 45% acetic acid, dehydrated, and permanently mounted on a fresh slide with euparal.

OBSERVATIONS

The slides are examined under oil immersion and chromosome counts are made on as many adequate metaphase figures as can be found. There are several ways for counting the chromosomes. The most commonly used method is to divide the cell into a number of sectors by eye and count the chromosomes in each sector separately, check, and then add (Ford, 1962).

KARYOTYPING

Photographs of good metaphase plates are taken and enlarged. The chromosomes are cut out of the photograph and arranged in homologous pairs in order of their descending length and the position of the centromere (Denver Congress, 1960). The photograph attached shows a karyotype in a case of Down's syndrome (Fig. 2).

INTERPRETATION

In healthy tissues, perpetuation of cells having chromosome abnormalities is inhibited by unknown controlling mechanisms. The incidence of aneuploidy always remains less than 2%. Tissues retain chromosome abnormalities only if these are present before birth. The only other exception is neoplasia, in which aneuploidy is frequent (Wakonig-Vaartaja, 1962), so demonstration of chromosomal aberrations in diseases of unknown

FIG. 2. Karyotype from a case of Down's syndrome (47/XY, trisomy 21) prepared from a skin fibroblast.

aetiology suggests either a congenital or a neoplastic aetiology.

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REFERENCES

- Basrur, P. K., Basrur, V. R., and Gilman, J. P. W. (1963). *Exp. Cell Res.*, 30, 229.
- Denver Congress (1960). *Amer. J. hum. Genet.*, 12, 384.
- Edwards, J. H. (1960). *Lancet*, 1, 496.
- Ferguson, J. (1962). *Med. J. Aust.*, 1, 40.

Turbidimetric method for the determination of serum globulin using the AutoAnalyzer

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An automatic procedure for the determination of globulin in serum or plasma is described. This method is based on the measurement of the turbidity which develops when serum is mixed with 27.4% sodium sulphate solution. The method gives good agreement with the salt fractionation technique (Majoor, 1946) and shows a high degree of precision.

The differential estimation of serum proteins constitutes an important fraction of the work in most chemical pathology departments and the automation of these analyses would result in a great saving of time. While there are several adequate methods available for the measurement of total protein (Stevens, 1959; Failing, Buckley, and Zak, 1960), none of the procedures proposed for the albumin estimation has gained wide acceptance.

These latter methods depend on the dye-binding capacity of albumin which has been shown to be influenced by a number of substances, *e.g.*, drugs or heparin (Niall and Owen, 1961). In our hands, none of them has given consistent results and has shown poor agreement with the salt-fractionation technique of Majoor. For these reasons, experiments were begun to find, if possible, a more satisfactory method of albumin/globulin determination.

In the course of these, it was discovered that the turbidity produced by mixing serum with 27.4% sodium sulphate varied in a linear manner with globulin content. This observation is the basis of the method to be described.

METHOD

A manifold is constructed as shown in Figure 1. Samples Received for publication 7 February 1964.

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- Ford, C. E. (1962). In *Methodology in Human Genetics*, edited by W. J. Burdette, p. 233. Holden-Day, San Francisco.
- Fox, M., and Zeiss, I. M. (1961). *Nature (Lond.)*, **192**, 1213.
- Frøland, A. (1961). *Acta path. microbiol. scand.*, **53**, 319.
- Harnden, D. G. (1960). *Brit. J. exp. Path.*, **41**, 31.
- Hirschhorn, K., and Cooper, H. L. (1961). *Amer. J. Med.*, **31**, 442.
- Lejeune, M. J., Turpin, R., and Gautier, M. (1960). *Rev. franc. Étud. clin. biol.*, **5**, 406.
- Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., and Hungerford, D. A. (1960). *Exp. Cell Res.*, **20**, 613.
- Pariente, J. (1961). *France méd.*, **24**, (2), 95.
- Rothfels, K. H., and Siminovitch, I. (1958). *Stain Technol.*, **33**, 73.
- Smulow, J. B., Rustigian, R., and Tye, M. (1961). *Proc. Soc. exp. Biol. (N.Y.)*, **106**, 757.
- Wakonig-Vaartaja, R. (1962). *Nature (Lond.)*, **193**, 144.

FIG. 1

are run at 40/hr. A 6 mm. flow cell is used with a 660 mμ filter.

It was found that, in general, blanks are unnecessary because of the high dilution involved. Occasionally in the case of a severely jaundiced or lipaemic specimen blanks may be measured by substituting saline for the sodium sulphate solution.

In this technique the peaks tend to be filled in at the tops, but their outline can easily be discerned. No difficulty has been found in obtaining consistent results, even by relatively inexperienced staff.

The reagents are 0.9% saline and 27.4% sodium sulphate, anhydrous. This reagent is kept in a wide-mouth quart size thermos flask at 37°C. This prevents the salt from crystallizing out when in use.

STANDARDS

Because of the linearity of this method (see below) and the difficulty in obtaining serum globulin, a departure from the usual method of standardization is adopted. The optical density of each peak is read off and compared with that of a serum whose globulin content has been determined by one of the standard salt-fractionation methods. This may be horse serum or one of the control sera available commercially. In our case Versatol is used. An electrophoretic analysis of this material by the Tiselius technique is available from the manufacturers on request.

To facilitate reading optical densities logarithmic ruled chart paper may be used. Alternatively, a strip of log ruled chart paper may be stuck along one of the vertical lines on an ordinary general purpose chart reader in such a way that the zero optical density lines coincide with the zero line of the recorder. The optical density of the peaks may then be read off.

CALCULATION

$$\frac{\text{Optical density of test}}{\text{Optical density of standard}} \times \text{Globulin content of standard in g./100 ml.}$$

RESULTS

The relationship between optical density and globulin content was determined by making serial dilutions from a serum whose globulin content had been assayed by fractionation with 27.4% sodium sulphate and estimation of protein with biuret reagent. Figure 2 shows that this relationship is a linear one.

The reproducibility of the method was checked by dividing samples of serum and submitting them for analysis under different names. The results of 100