Technical methods

Application of the Feulgen reaction to smears in exfoliative cytology

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The routine examination of smears in exfoliative cytology is based upon subjective assessment of morphological features, a practice now firmly established for its clinical value in the diagnosis of malignant disease. Advances in routine practices depend on preliminary research, and amongst the newer techniques of interest to cytologists are those of cytochemistry. These may be either qualitative or quantitative in character, and offer the prospect of an objective method of evaluation.

The presence of enlarged or hyperchromatic nuclei is a common feature of neoplasms, believed to reflect an increase in the overall level of nuclear metabolism. There is scope for research studies into the significance of this and other characteristics which may contribute to improvements in clinical cytology. The amount of material available is usually limited, however, and the possibility of carrying out more than one type of examination on a specimen is worth exploring. There are occasions when a smear proves difficult to evaluate owing to the presence of atypical cells, and in such cases it would be of interest to apply objective methods of examination. If the cells of unusual type are abundant, a second slide, hitherto unstained, may be used with the reasonable certainty of finding others of this class. It may happen, however, that only one slide is available, or, alternatively, that only a very small number of cells of unusual interest are present. In these circumstances it would clearly be of advantage if slides stained for routine examination could be restained by a cytochemical method for further study.

The metabolism of deoxyribonucleic acid (D.N.A.) is of current interest and methods are available for its quantitative estimation. Deoxyribonucleic acid is now generally accepted as the chemical carrier of genetic information from a cell to its progeny at the time of division. Since the characteristics which distinguish malignant cells from their normal counterparts are transmitted to their descendent cell populations, it is clearly important to acquire information about the D.N.A. content not only of cells which are obviously malignant but also of those of doubtful status from fields commonly described as precancerous. It would be of interest, therefore, to determine whether quantitative methods of estimating D.N.A. could be applied to smears after these have been stained by routine techniques for morphological examination.

Smears of normal human buccal cells were made on clean slides, and wet-fixed in absolute methyl alcohol. These were divided into two groups. Smears of the first group were stained immediately by the Feulgen technique according to Stowell (1945). Those of the second group were stained first by the Papanicolaou technique using EA50, then destained by washing in tap water for one hour followed by standing in 70% ethyl alcohol for as long as necessary to remove the cytoplasmic stain completely. The Feulgen technique was then applied; this led to the disappearance of the nuclear Papanicolaou stain during hydrolysis.

Quantitative measurements of the binding of Feulgen stain by individual nuclei were made by reading its degree of absorption at 546 $m\mu$. This was carried out on a micro-densitometer of the scanning and integrating type (Deeley, 1955)¹ as in previous investigations (Meek, 1961a, 1961b, 1962a, 1962b; Meek and Sparshott, 1961). One hundred intact nuclei were measured in each group.

The average value of the nuclear content of D.N.A., as indicated by Feulgen staining and expressed in arbitrary units, is shown in the Table.

TABLE

Stain	Mean Value	S.D.
Feulgen	37.9	4.0
Papanicolaou-Feulgen	39.4	3.3

Washing in tap-water removed the eosin from the cytoplasm almost completely in isolated cells, but the light green proved to be more resistant. Both stains disappeared in 70% alcohol; this took place within a few minutes in the case of scattered cells in the thin parts of the smears but up to four hours was needed for dense clumps. However, owing to the requirements of micro-densitometry, the latter were often unsuitable for measurement since it is essential for the nuclei to be separate and surrounded by an area of unstained background; this eliminates contiguous or overlapping nuclei.

It was found that the nuclear stain remaining from the Papanicolaou method was rapidly removed during hydrolysis.

CONCLUSION

The results show no significant difference between the two groups of smears, indicating that the Papanicolaou stain can be removed completely without loss of Feulgen-staining material.

¹Made by Messrs. Barr and Stroud, Anniesland, Glasgow, W.3.

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The determination of 3-methoxy 4-hydroxy mandelic acid in urine

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A simple colorimetric method for the determination of 3-methoxy 4-hydroxy mandelic acid (V.M.A.) in urine has recently been described by Woiwood and Knight (1961). In this method, urine, after acidification and saturation with sodium chloride, is extracted with ether and the extracted V.M.A. removed into aqueous solution with dilute alkali. After coupling with diazotized para-nitro aniline the azo derivative is extracted into chloroform from which it is re-extracted with sodium hydroxide as a red solution.

Trials in this laboratory have confirmed the suitability of this method for routine use, but we have found it helpful to introduce the following modifications into the technique:—

1 Ethyl acetate is substituted for ether as the first extracting solvent, two successive 10 ml. portions being employed.

2 The combined ethyl acetate extracts are washed with 2 ml. distilled water.

3 Extraction into aqueous solution is brought about by shaking with 25, 15, and 10 ml. portions of 0.01 M phosphate buffer, pH 7.6 (50 ml. 0.2 M KH_2PO_4 + 42.7 ml. 0.2 M NaOH, diluted 1 : 20 with distilled water).

The use of ethyl acetate is safer than ether in the routine laboratory, and the smaller volume of organic solvent makes subsequent handling easier. It was found that variable amounts of acid were being carried over in this first extraction; the washing step was found to remove 60 to 70% of this. The use of a buffer at pH 7.6 for the subsequent extraction avoided any large swing in pH during this step, together with the need for any final adjustment of the pH of the combined extract.

STANDARD CURVE

We have prepared a standard curve giving absolute optical density values for the azo-derivative of V.M.A. in alkaline solution, in order to calculate overall recoveries of quantities of V.M.A. taken right through the procedure. It is shown in Fig. 1 and is prepared as set out on page 389.

The optical density of the red solutions was measured on the Hilger Uvispek spectrophotometer at 510 m μ using 1 cm. cells.

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