to endanger proteins or other substances which are sensitive to acid.

The authors wish to record their thanks to the late Sir Norman Haworth, F.R.S., and to Prof. M. Stacey, F.R.S.,

for their interest in this work. One of us (A. J. S.) is indebted to the University of Birmingham for the award of a Research Scholarship.

REFERENCES

- Gilbert, G. A. & Swallow, A. J. (1950). *Biochem. J.* **47**, 502.
Manegold, E. (1940). *Z. Ver. deutsch. Ing. Verfahrenstechnik*, no. 4, 124; no. 5, 145; no. 6, 176.
Meyer, K. H. & Sievers, J.-F. (1936). *Helv. chim. Acta*, **19**, 649.
Schwarz, R. & Huf, E. (1932). *Z. anorg. Chem.* **203**, 188.
Teorell, T. (1935). *Proc. Soc. exp. Biol., N.Y.*, **33**, 282.

The Cytological Interpretation of the Feulgen Reaction

BY EDGAR STEDMAN AND ELLEN STEDMAN
Department of Biochemistry, University of Edinburgh

(Received 13 May 1949)

The chemical test for deoxyribosenucleic acid discovered by Feulgen (1914) and applied by Feulgen & Rossenbeck (1924) under the name of nucleal stain to histological sections has served many useful purposes in the hands both of its originator and of subsequent workers. It now finds much favour as a staining process for cytological preparations containing dividing cells, partly, no doubt, because of the particularly clear pictures of the chromosomes which result from its use, but mainly because of the belief that it specifically stains those nuclear structures which contain nucleic acid. Thus, because the chromosomes are the only structures in dividing cells which become stained by this method, it is assumed that these organs contain the whole of the nucleic acid of the nucleus, an assumption which is sometimes expressed by the statement that nucleic acid does not occur elsewhere in the nucleus than in the chromosomes. As an aid to studies of the morphology of chromosomes, nucleal staining may be unobjectionable, but when it is employed for ascertaining the distribution of nucleic acid within the nucleus it becomes open to serious criticism. For such a use is based on the assumption that the nucleic acid 'stains' *in situ*, an assumption which ignores the well known fact that the violet dye produced when isolated nucleic acid is submitted to Feulgen's reaction (hydrolysis with dilute hydrochloric acid and treatment of the neutralized product with Schiff's reagent) is soluble in water. Unless nucleic acid in the nucleus behaves differently from isolated nucleic acid, it follows that those nuclear structures which become stained by Feulgen's process do so because they are composed of non-diffusible material which adsorbs or otherwise combines with the diffusible, violet dye, and that

consequently nucleal staining can provide no certain information regarding the distribution of nucleic acid in the nucleus of the living cell. Evidence of an indirect nature supporting these conclusions has been adduced elsewhere (Stedman & Stedman, 1943, 1947). Direct proof is now provided that the hydrolysis of isolated cell nuclei under conditions used in nucleal staining gives rise to diffusible products which react with Schiff's reagent to form a soluble violet dye.

METHODS AND RESULTS

Isolation of cell nuclei. The method employed, which was evolved from that of Stoneburg (1939), has been used continuously in this laboratory since 1943 for the isolation of nuclei from the thymus glands, spleens, livers and tumours of various species. It has not hitherto been described in detail, although the principles on which it is based have been mentioned in publications of a general nature (Stedman, 1944; Stedman & Stedman, 1947). It differs from Stoneburg's method chiefly in two respects: the operations are carried out at a lower acidity, using acetic in place of citric acid, and the use of pepsin has been dispensed with. The detailed procedure is as follows. Ox liver (0.5–1.5 kg.) from a freshly killed animal is minced in a machine, first coarsely and then finely. The resulting pulp is poured into several volumes of 4% acetic acid. The exact proportion of acid to pulp is immaterial as regards the liberation of nuclei, but subsequent operations are facilitated if a ratio of not less than 3:1 is used. The mixture is, without delay, stirred vigorously with a mechanical stirrer for about 15 min., this operation being repeated four times at short intervals. It is then left for 2 hr. to allow froth to drain, a period which may be extended to 12 hr. if the timing of the experiment necessitates leaving it overnight. Much tissue fibre and other non-nuclear material, as well as viscous lumps of impure nuclear material, presumably originating from nuclei ruptured during the mincing process, are then

removed by straining the fluid, first through fine wire gauze, stirring gently if necessary to prevent the gauze from becoming choked, and subsequently through four layers of fine muslin. The filtrate is centrifuged for 20 min. at 2000 rev./min.; this causes the bulk of the free nuclei to sediment, together with a considerable amount of cell debris, particularly of cytoplasmic granules, and some whole cells which have resisted lysis. The turbid supernatant fluid is rejected and the deposit thoroughly mixed with 1% acetic acid and again centrifuged, a process which is repeated as many times (usually ten or more) as is necessary completely to remove the non-nuclear material from the product. The fractionation is controlled throughout by microscopic examination. The procedure is modified to suit particular preparations by, for example, varying the time and rate of centrifugation during successive fractionations, or removing non-nuclear material by any mechanical means which may appear convenient. Thus, it is sometimes necessary at a late stage of the fractionation to centrifuge the product down hard and to remove a small layer of impurity, consisting of whole cells or of a mixture of these with the larger cytoplasmic granules and recognizable in a liver preparation by

Fig. 1. Wet smear of isolated cell nuclei from ox liver. Stained methylene blue. $\times 700$ approx.

its buff or brown colour, by gently washing the surface of the deposit with 1% acetic acid. Alternatively, it may be possible to remove the surface layer, although less satisfactorily, with a spatula. If the whole process has been successfully carried out the product will consist essentially of nuclei of liver cells contaminated only with a few nuclei from cells of a different type. A preparation such as that used in the present investigation is illustrated in Fig. 1. If the nuclei are to be stored or used for the preparation of non-lipoid components, they are dried by treatment during 24 hr. with at least two changes of 4 vol. in each case of cold ethanol, which usually removes a small amount of pigment, followed by suspension in ether and filtration.

The 4% acetic acid employed in the above method produces two effects. Its primary function is to

cause the lysis of the cells with liberation of the nuclei. While its efficiency in this respect is very great towards tissue used within 1 hr. of its excision from an animal, it diminishes rapidly with time if the tissue is kept. Nevertheless, unminced tissue can be kept without any appreciable deterioration for at least 4 days provided it is kept frozen hard in solid carbon dioxide. Concentrations of acetic acid of less than 4% are less efficient in producing lysis. Thus, in an experiment on ox liver in which 2% acetic acid was employed, the proportion of whole cells which remained was so great that difficulty was experienced in isolating any nuclei at all, although a parallel experiment on a separate portion of the same tissue using 4% acid gave normal results.

A subsidiary function of the acetic acid is to produce a certain measure of fixation of the nuclei without which they would coalesce during the many operations required for their isolation. This fixation entails, however, the maintenance of an acid medium during their fractionation in the centrifuge, for if water is used for this purpose the nuclei gradually undergo fragmentation. The use of 1% acetic acid maintains a sufficient acidity to prevent this.

Determination of phosphorus. Briggs's (1922) colorimetric method as modified by Fiske & Subbarow (1925), with reagents prepared according to King, Haslewood & Delory (1937), has been used in the following form. About 6 mg. of material dried to constant weight at room temperature in a high vacuum over P_2O_5 , or 1 ml. of solution, is accurately weighed by difference, or pipetted, into a 10 ml. digestion flask. Conc. H_2SO_4 (0.90 ml.) is then added, the mixture digested over a small flame until the organic material is completely oxidized (18–24 hr.), transferred to a 25 ml. flask and made up to volume. To a sample (2 or 4 ml.) in a 10 ml. flask, 1 ml. of ammonium molybdate reagent and 0.2 ml. of reducing reagent are added and the mixture made up to volume. After 15 min. the colour intensity is measured in a Spekker absorptiometer, and the P content read from a standard curve. In order to check the result, a second determination is made in which the intensity of colour is directly compared with that of a phosphate solution containing the exact amount of P found in the first estimation. The H_2SO_4 content of the various solutions used in the absorptiometer is kept constant by the use of molybdate reagents made up in water, 2.5 and 5N- H_2SO_4 , respectively, for 4 and 2 ml. samples and blank (or standard).

Fixation of nuclei. Cell nuclei, prepared from ox liver in the manner described above, were thoroughly mixed with excess (30 ml./6–8 g. of undried nuclei) of a mixture of 100 ml. of ethanol and 30 ml. of glacial acetic acid (ethanol-acetic acid, called by cytologists 'acetic alcohol'). After 2 hr. the fixative was removed in the centrifuge and the fixed nuclei washed twice with water.

Feulgen hydrolysis. This was effected with N-HCl at 60°. As, however, the nuclei would not disperse readily in acid of this temperature and concentration, the following procedure was used. The fixed nuclei, still contained in the centrifuge tube, were intimately mixed with 25 ml. of cold water, and heated to 60° in a water bath maintained at this temperature. With continuous stirring, 25 ml. of 2N-HCl, preheated to

60°, were then added. Stirring was continued for 10 min., when the mixture was removed from the water bath, rapidly cooled and centrifuged. The residue was washed twice with ethanol, suspended in ether and filtered. The residual nuclei and the acid hydrolysis fluid were kept for examination and analysis.

Procedure and results. One typical experiment will be described. A preparation of nuclei from ox liver was divided into three portions, 1, 2 and 3, with wet weights of 6.547, 8.287 and 6.675 g. respectively. Portion 2, which was used as a control, was dried with ethanol and ether and gave a product weighing (air dried) 1.565 g. Its phosphorus content was 2.82%, corresponding with a nucleic acid content of 28.4%. On exhaustive extraction (Stedman & Stedman, 1947) with 0.1N-sulphuric acid it yielded 21.5% of its weight of histone sulphate. Portions 1 and 3, with calculated dry weights of 1.237 and 1.282 g. respectively, were fixed in 'acetic alcohol'. After fixation, portion 1 was dried with ethanol and ether. The product weighed 1.150 g., contained 2.9% phosphorus, corresponding with 29.4% of nucleic acid, and yielded 15.8% of its dry weight of histone sulphate. Fixation thus caused a loss of 7% of the dry weight of the nuclei and of 26.5% of their histone content. Portion 3 was submitted directly to Feulgen hydrolysis. The residual nuclei, after drying with ethanol and ether, weighed 0.836 g. and failed to yield even a trace of histone on extraction with 0.1N-sulphuric acid. The combined processes of fixation and hydrolysis thus produced a loss in dry weight of 34.8%. The material contained 3.15% phosphorus, equivalent to 31.8% of nucleic acid. If no loss of phosphorus had occurred, however, it should have contained the equivalent of 43.8% of nucleic acid. Phosphorus equivalent to 27.4% of the nucleic acid originally present in the nuclei was thus lost during Feulgen hydrolysis. Moreover, under conditions (extraction with 0.05N-sodium hydroxide in the presence of sodium acetate) which enable nucleic acid to be isolated as solid sodium salt from normal nuclei from which histone has been removed, no solid sodium nucleate could be extracted from the hydrolysed nuclei. The treatment did, it is true, yield a small amount of material, but this was precipitated by ethanol as a sticky oil and evidently consisted of a mixture of degradation products of nucleic acid.

The acid hydrolysis fluid (total volume, 50 ml.) contained 5.53 mg. of phosphorus, equivalent to 55.8 mg. of nucleic acid, compared with phosphorus equivalent to 98.4 mg. lost by the nuclei. The material represented by the difference between these two figures was doubtless removed during the washing of the nuclei after hydrolysis. The hydrolysis fluid gave a strong Molisch reaction. When 1 ml. was neutralized in the cold with alkali and treated with 5 ml. of Feulgen's reagent, a violet colour was pro-

duced of the same intensity as that given by 6 mg. of nucleic acid after hydrolysis. The total hydrolysis fluid thus contained chromogenic material equivalent to 300 mg. of nucleic acid. On the other hand, Dische's reagent gave no colour with the hydrolysis fluid in the cold, but when 1 ml. was heated with the reagent on a water bath for 3 min. a blue colour developed which was slightly less intense than that given by 1 mg. of nucleic acid treated directly with the reagent under the same conditions. On the basis of Dische's reaction, the hydrolysis fluid thus contained chromogenic material equivalent to less than 50 mg. of nucleic acid. The large discrepancy between the values obtained with these colour reactions may be due, partly, to the unsuitability of the Feulgen reaction for quantitative work. It also suggests, however, that the chromogenic materials responsible for the two reactions are not identical.

DISCUSSION

The above experiments have shown that, when applied to isolated nuclei from liver cells, the hydrolytic process used in staining histological specimens by Feulgen's method causes a profound change in their chemical composition. The nuclei lose no less than 35% of the organic material which they contain, and this loss includes the whole of the histone and much of the nucleic acid originally present. Unfortunately, the data obtained do not permit an accurate calculation of the proportion of the latter which is lost. This is due to the extensive degradation which this substance undergoes, as demonstrated both by the diffusion of fragments into the hydrolysis fluid and by our inability to extract solid sodium nucleate from the residual nuclei. Our analyses do, it is true, show that phosphorus corresponding with 27% of the nucleic acid originally present is lost from the nucleus. But this is not a measure of the total amount of nucleic acid actually lost, for fragments of the nucleic acid, such as thymic acid, retaining the whole of its phosphorus may be, and doubtless are, to some extent retained by the nucleus. A better estimate of the loss due to nucleic acid is given by the difference between the total loss of weight and that due to histone. This involves the assumption that no appreciable amount of material other than histone and hydrolysis products of nucleic acid is lost from the nucleus, an assumption which we have reason to believe is substantially correct. The method, however, suffers slightly in precision from the fact that the amount of sulphuric acid combined with the histone is not known. No great error will, however, be made if, for the sake of calculating a numerical figure, it is assumed that a yield of 21.5% of histone sulphate was due to a loss of 20% of histone base from the nucleus, in other words, that the histone sulphate

contained about 7 % of sulphuric acid. The loss due to nucleic acid is then $35 - 20 = 15\%$. Since the nuclei originally contained 28 % of nucleic acid, it follows that over 50 % of the nucleic acid was, in some form or other, lost from the nucleus during hydrolysis. This figure would, moreover, almost certainly have been greater had the nuclei been washed with water after hydrolysis instead of being dried directly with ethanol and ether.

It is thus clear that the hydrolysis with hydrochloric acid of the nucleic acid present in cell nuclei resembles that carried out with pure nucleic acid in a test tube in so far as both lead to the formation of one or more soluble and diffusible substances which react with Schiff's reagent to yield a soluble, violet dye. They thus confirm our view of the mechanism of the staining reaction. The objection has been raised to this view that any soluble substances formed in the reaction would diffuse out of the nucleus before the addition of Schiff's reagent. Thus, Baker & Sanders (1946) state that 'if the reaction product can diffuse through the tissues away from the place where it was first formed, it can also diffuse into the fluid in the jar and thus be lost'. As our experiments show, this is indeed the case. But the rate of diffusion of simple substances in the nucleus is much smaller than in water.* Because of this there is a tendency during the initial stages of hydrolysis for the products to accumulate in the nucleus, and it is no doubt for this reason that the intensity of staining depends, other conditions being equal, upon the time of hydrolysis. This intensity, in fact, at first increases with time, reaches a maximum and then diminishes. It is thus clear that, during the period between the commencement of hydrolysis and the optimum time, hydrolysis products of nucleic acid are being formed in the nucleus at a greater rate than they are diffusing out; thereafter, owing to the exhaustion of the supply of nucleic acid, the reverse holds. In this connexion it is interesting to note that, according to Di Stefano (1948), the optimum time of hydrolysis for sections fixed in 'acetic alcohol' is 12 min., while in our experiments the time of hydrolysis was restricted to 10 min. This gives added weight to our interpretation, for it is evident that in a period of hydrolysis shorter than the optimum approximately 50 % of the weight of nucleic acid originally present in the nucleus has diffused in fragments into the hydrolysis fluid.

It should, however, be mentioned that our results are in complete contradiction with those recently published by Di Stefano (1948). This author, using

* This follows from the fact that, after removing histone from nuclei by extraction with cold 0.1 N- H_2SO_4 , it is necessary to wash the nuclei with many changes of water over a period of a day or more in order to free them completely from the acid.

cytological methods in which the effect of Feulgen hydrolysis on the composition of cell nuclei was followed by photometric measurements according to the methods of Pollister & Ris (1947), has reached the conclusion that, after fixation with 'acetic alcohol', the only loss, whether of proteins or nucleic acid, which occurs after 12 min. hydrolysis is that of the two purine groups from the nucleic acid. These are completely removed after exactly 12 min. hydrolysis, the nucleus retaining the whole of its original content of phosphorus and histone. Nevertheless, during a further 12 min. hydrolysis, after which the nucleus no longer stains with Schiff's reagent, the phosphorus and histone have disappeared completely. Our results, on the other hand, have shown by direct chemical examination of the nuclei that 10 min. hydrolysis suffices to remove the whole of the histone and 27 % of the phosphorus originally present, and we can therefore only conclude that the indirect cytological methods which Di Stefano uses do not possess the accuracy requisite for work of this nature. This conclusion is reinforced by purely chemical considerations. For example, if 24 min. treatment suffices to remove histone completely, there would certainly be an appreciable amount removed in 12 min. Similar arguments apply to the phosphorus of the nucleic acid. While it is probable that thymic acid is the first product of the action of hydrochloric acid on nucleic acid, it is inconceivable, from a chemical viewpoint, that the subsequent action of this reagent would be delayed until all of the nucleic acid had been transformed into thymic acid.

While the primary object of this investigation was the elucidation of the mechanism of nucleal staining, the data which it has yielded also give some information about the structural functions of histone and nucleic acid in the cell nucleus. Thus, the demonstration that the treatment with hydrochloric acid which the method involves completely removes histone from the nucleus indicates, on the assumption that the structures visible in preparations stained by this method are, in fact, complete chromosomes, that this base cannot be a component of the chromosomes, a conclusion which we have already reached on other grounds (Stedman & Stedman, 1944). It further follows that the nucleic acid in combination with histone, which cannot be an inconsiderable proportion of the total amount present, must also be extra chromosomal in function.

SUMMARY

1. A study has been made of the chemical changes produced in isolated cell nuclei by the hydrolytic procedure used in Feulgen's method of staining cytological preparations.

2. The results show that hydrolysis with N-hydrochloric acid for 10 min. at 60° causes a loss of 35 % of the dry weight of the nuclei.

3. This loss includes the whole of the histone originally present.

4. It also includes more than 50 % of the nucleic acid, which leaves the nuclei in the form of diffusible fragments of this material.

5. The hydrolysis fluid, after separation from the nuclei, gives an intense Feulgen reaction with Schiff's reagent.

6. The implications of these results are discussed.

We acknowledge with thanks grants from the Medical Research Council, the Royal Society and the Earl of Moray Research Fund of this University which have contributed to the prosecution of this work.

REFERENCES

- Baker, J. R. & Sanders, F. K. (1946). *Nature, Lond.*, **158**, 129.
Briggs, A. P. (1922). *J. biol. Chem.* **53**, 13.
Di Stefano, H. S. (1948). *Chromosoma, Berlin*, **4**, 282.
Feulgen, R. (1914). *Hoppe-Seyl. Z.* **92**, 154.
Feulgen, R. & Rossenbeck, H. (1924). *Hoppe-Seyl. Z.* **135**, 203.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
King, E. J., Haslewood, G. A. D. & Delory, G. E. (1937). *Lancet*, **1**, 886.
Pollister, A. W. & Ris, H. (1947). *Cold Spr. Harb. Sym. quant. Biol.* **12**, 147.
Stedman, E. (1944). *Edinb. med. J.* **51**, 353.
Stedman, E. & Stedman, E. (1943). *Nature, Lond.*, **152**, 267.
Stedman, E. & Stedman, E. (1944). *Biochem. J.* **38**, xxvi.
Stedman, E. & Stedman, E. (1947). *Cold Spr. Harb. Sym. quant. Biol.* **12**, 224.
Stoneburg, C. A. (1939). *J. biol. Chem.* **129**, 189.