533. *Deoxy-sugars. Part XIII. Some Observations on the Feulgen Nucleal Reaction.*

By W. G. Overend.

Some of the problems of the Feulgen nucleal reaction have been studied. The intensities of the colours produced when Schiff's reagent is added to some 2-deoxy-pentose and -hexose derivatives have been compared with those produced by adding the reagent to the normal sugar analogue. The value of the reaction in indicating the location in cells of deoxypentose nucleic acids is discussed.

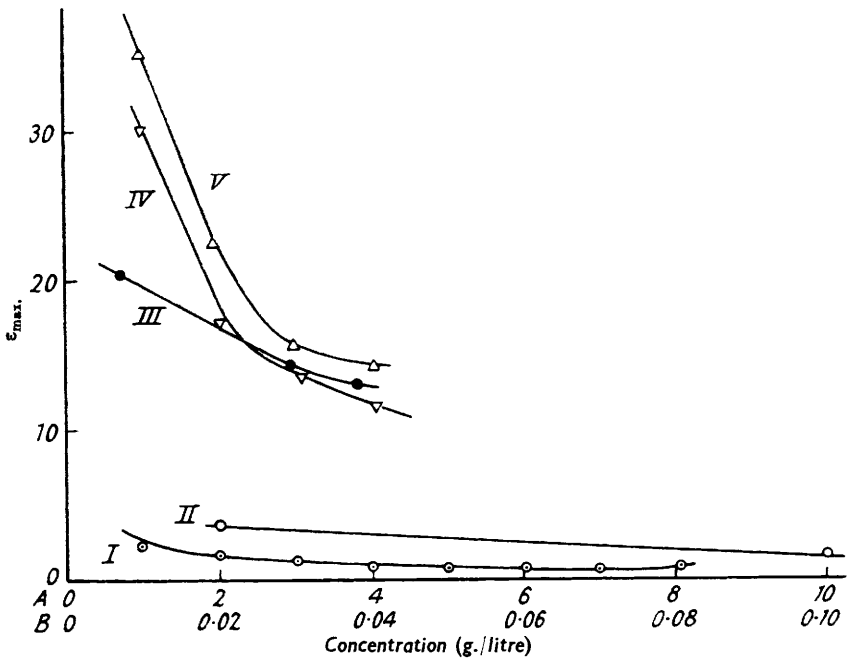
The important rôle which deoxypentose nucleic acids play in the nucleal material of cells (Mirsky *et al.*, *Proc. Nat. Acad. Sci.*, 1942, **28**, 344; *Adv. Enzymol.*, 1943, **3**, 1; Avery, MacLeod, and McCarty, *J. Exp. Med.*, 1944, **79**, 137) necessitates an understanding of the mechanisms of the reactions whereby they are detected. Two of these reactions are those introduced by Dische (*Mikrochemie*, 1930, **8**, 4) and Feulgen (*Z. physiol. Chem.*, 1923, **128**, 154; 1924, **135**, 203). Recently Stacey and his colleagues (*Nature*, 1946, **157**, 740; *J.*, 1949, 1222) reported an explanation of the chemical reactions leading to the formation of the characteristic blue colour shown by deoxyribonucleic acid when subjected to the Dische diphenylamine reaction. They indicated that the reactive moiety of the nucleic acid molecule was its carbohydrate component, 2-deoxy-D-ribose. A detailed examination of this sugar has been described by these authors (*J.*, 1949, 1879, 2836) who have also discussed the specificity of the Dische test (Overend, Shafizadeh, and Stacey, *J.*, 1950, 1027). Measurements on the colours produced in Feulgen tests with nucleic acids have been reported recently by Di Stefano (*Proc. Nat. Acad. Sci.*, 1948, **34**, 75).—The present communication is a continuation of a preliminary report on the chemistry of the Feulgen reaction (Stacey, Li, and Overend, *Nature*, 1949, **163**, 538).

Divergent views exist concerning the deductions to be drawn from results obtained by using the Feulgen test (cf. Danielli, *Symp. Soc. Exp. Biol.*, 1947, **1**, 101; *Quart. J. Microscop. Sci.*, 1949, **90**, 67; Di Stefano, *loc. cit.*; Stedman and Stedman, *Symp. Soc. Exp. Biol.*, 1947, **1**, 232). In part these misunderstandings are due to incomplete evidence about the reactions involved. The general view is that the acid hydrolysis breaks the purine–sugar glycosidic linkage in deoxyribonucleic acid, and that the deoxypentose is then able to exist to some extent in its straight-chain aldehydo-form, and it is in this form that it reacts with Schiff's reagent (*Annalen*, 1866, **140**, 102), forming a reddish compound. This general view contains many assumptions. For example it is assumed that in this test a deoxypentose (*i.e.*, 2-deoxy-D-ribose) would react to some extent at least in its aldehydo-form with Schiff's reagent, and that under the test conditions only the purine bases are cleaved from the nucleic acid. Moreover it is assumed that the colour developed is not due to the deoxypentose partly decomposing in the acid conditions to yield ω-hydroxylævulaldehyde which then reacts with Schiff's reagent to give a soluble dye, subsequently adsorbed on the proteins present in the cell. Stacey *et al.* (*loc. cit.*) showed that this aldehyde was responsible for the development of colour in the Dische diphenylamine test. The decrease in colour intensity in the Feulgen test which results from over-hydrolysis was believed to be caused by some secondary reaction.

The validity of some of these assumptions has been investigated. The intensities of the colours produced by normal hexoses and pentoses and their 2-deoxy-analogues, when Schiff's reagent was added to them, were measured quantitatively. Originally we intended to report our results in graphic form by plotting the wave-length (λ, A.) against the molecular extinction coefficient ($\varepsilon_{max.}$) (Stacey *et al.*, *loc. cit.*), but unfortunately the colour intensities produced were not directly proportional to the concentrations of the solutions. Fig. 1 illustrates the results

obtained when the values of $\varepsilon_{max.}$ are plotted against concentration for a series of normal and 2-deoxy-sugars and their derivatives. Consequently we have carried out all our measurements on solutions of the same concentration (*c*, 2·4) and have plotted Spekker readings directly against wave-length. In this way only comparative values of the colour intensities at a certain concentration are obtained, but the results are sufficiently marked to illustrate the subsequent argument. Strict control of the temperature and air contamination is important since both affect the intensities of the colours obtained. It was impossible to reproduce exactly results of Spekker readings with different batches of reagent. The differences between the values obtained for various sugars are however always constant. The comparative measurements shown in Figs. 2 and 3 were carried out with one batch of reagent and those in Figs. 4, 5, and 6 with another.

FIG. 1.



Scale A.
I. D-*Galactose.*
II. D-*Glucose.*
III. 2-*Deoxy*-D-*ribose.*

Scale B.
IV. 2-*Deoxy*-D-*galactose.*
V. 3 : 5 : 6-*Trimethyl* 2-*deoxy*-D-*galactofuranose.*

Fig. 2 shows the results obtained with D-glucose, D-galactose, and D-ribose, and their 2-deoxy-analogues. In all cases the deoxy-sugar shows a much increased intensity of colour compared with its normal analogue. This effect is most pronounced in the hexose series, and it will be observed that 2-deoxy-D-galactose gives a more intense colour than 2-deoxy-D-glucose. In this connection it is noteworthy that other derivatives of D-galactose are known (*e.g.*, 3 : 6-anhydro-D-galactose) which exist predominantly in the aldehydo-form (Haworth, Jackson, and Smith, *J.*, 1940, 620). The sugar component of deoxyribonucleic acid is fixed in its furanose form. This has been proved by Levene and Tipson (*J. Biol. Chem.*, 1935, **109**, 623; *Science*, 1935, **81**, 98) for the thymidine component of the nucleic acid, and more recently for the other deoxyribonucleosides (Todd, private communication). Consequently we investigated next the effect, on the intensity of the colour developed, of preventing the sugar existing in its pyranose form. This was achieved by suitable protection of the hydroxyl group at $C_{(5)}$. Protection by methylation has no effect since D-glucose and its 2 : 3 : 4 : 6-tetramethyl derivative give practically identical Spekker readings (Fig. 3). If the sugars can only exist in furanose or aldehydo-forms, the intensity of colour developed when Schiff's reagent is added, is much enhanced. For example D-glucofuranose 5 : 6-monocarbonate (Haworth and Porter, *J.*, 1929, 2796) and 2-deoxy-D-glucofuranose 5 : 6-monocarbonate (Hughes, Overend, and Stacey, *J.*, 1949, 2846) both show intenser colours than do D-glucose and 2-deoxy-D-glucose when treated according to the procedure described. The most intense colour obtained in the later experiments

was with a sugar which could only exist in its aldehydo-form, namely diethylidene *aldehydo*-L-xylose, and this seems to indicate conclusively that this development of colour is associated with the aldehydo-form. Moreover, unlike other sugars, when the Schiff's reagent was added to this aldehydo-sugar, a quite strong colour developed immediately. In Fig. 4 the results of measurements with various methylated derivatives of 2-deoxy-D-ribose are recorded; of these derivatives 3 : 5-dimethyl 2-deoxy-D-ribofuranose gives the most intense colour with Schiff's reagent. As expected, 3 : 4-dimethyl 2-deoxy-D-ribopyranose gives a slightly less intense colour than 2-deoxy-D-ribose. Fig. 5 shows the results of similar measurements with various derivatives of 2-deoxy-D-galactose. These measurements show clearly that 2-deoxy-sugars give a much more intense colour with Schiff's reagent than do their normal analogues under comparable conditions, an effect enhanced if they are prevented from existing in the pyranose form.

FIG. 2.

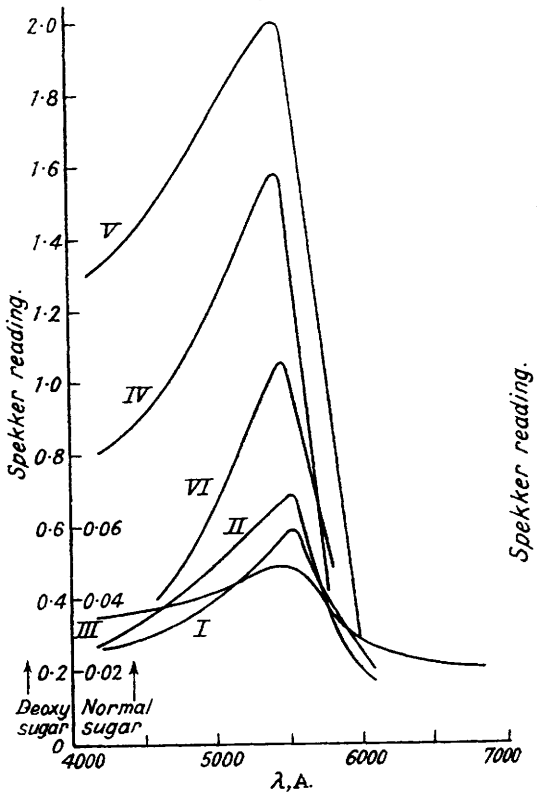


FIG. 3.

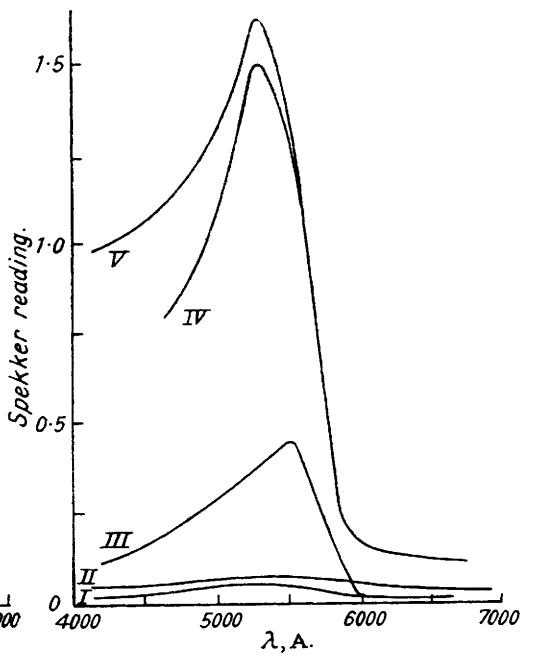


FIG. 2.—*I. Anhydrous* D-*glucose. II.* D-*Galactose. III.* D-*Ribose. IV.* 2-*Deoxy*-D-*glucose. V.* 2-*Deoxy*-D-*galactose. VI.* 2-*Deoxy*-D-*Ribose.*

FIG. 3.—*I.* D-*Glucose. II.* 2 : 3 : 4 : 6-*Tetramethyl*-D-*glucopyranose. III.* D-*Glucofuranose* 5 : 6-*monocarbonate. IV.* 2-*Deoxy*-D-*glucose. V.* 2-*Deoxy*-D-*glucofuranose* 5 : 6-*monocarbonate.*

Finally measurements were carried out on guanosine, a deoxyribonucleotide mixture isolated from the enzymic degradation of deoxyribonucleic acid, probably containing a preponderance of pyrimidine types (Overend and Webb, forthcoming publication), and a purine deoxyribonucleotide; results are shown in Fig. 6. The most intense colour was obtained from the purine deoxyribonucleotide, and this was much more intense than that from guanosine, of which the molecular concentration was much greater.

A consequence of these measurements is that even if the sugar-base glycoside linkages in ribonucleic acid are cleaved during the Feulgen test, the freed reactive group at $C_{(1)}$ of the sugar and Schiff's reagent do not react to give a colour as intense as that which results from similar treatment of deoxyribonucleic acid. Another factor which influences the intensity of the colour developed is the presence of phosphate groups; this is indicated in preliminary experiments and is being further developed.

When the sodium salt of a fibrous sample of deoxyribonucleic acid, isolated from soft herring roe by essentially Mirsky and Pollister's method (*J. Gen. Physiol.*, 1946, **30**, 101), was treated with Schiff's reagent after being kept at pH 2·0 at 100° for 2·75 minutes, it immediately became coloured, and the intensity of the colour became visibly progressively greater. No colour

FIG. 4.

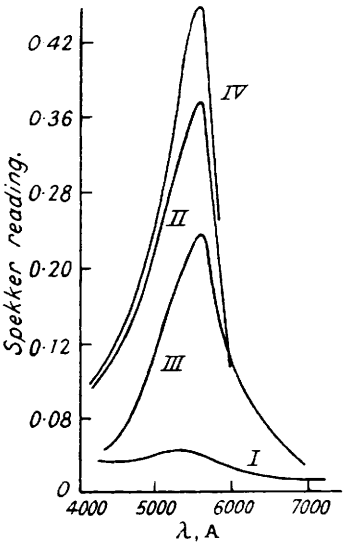


FIG. 5.

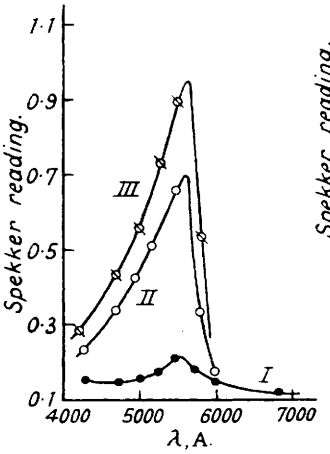


FIG. 6.

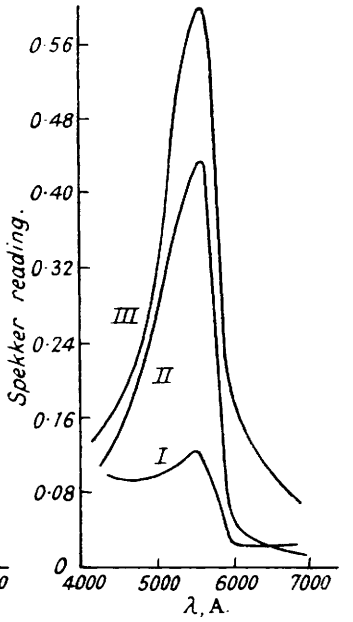


FIG. 7.

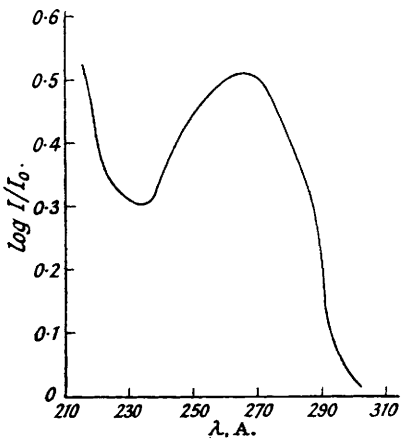


FIG. 4.—D-*Ribose*. *II*. 2-*Deoxy*-D-*ribose*. *III*. 3 : 4-*Dimethyl* 2-*deoxy*-D-*ribopyranose*. *IV*. 3 : 5-*Dimethyl* 2-*deoxy*-D-*ribofuranose*.

FIG. 5.—*I*. 2 : 3 : 4 : 6-*Tetramethyl* D-*galactose*. *II*. 3 : 4 : 6-*Trimethyl* 2-*deoxy*-D-*galactopyranose*. *III*. 3 : 5 : 6-*Trimethyl* 2-*deoxy*-D-*galactofuranose*.

FIG. 6.—*I*. *Guanosine*. *II*. *Pyrimidine deoxyribonucleotide*. *III*. *Purine deoxyribonucleotide*.

could be washed out of the nucleic acid which after being dried was obtained as fine fibres with a violet colour. It seems that in the Feulgen test a reaction occurs between the available reactive group at $C_{(1)}$ of the deoxypentose and the added Schiff's reagent. Wieland and Scheuing (*Ber.*, 1921, **54**, 2527) postulate that in the reaction of a simple aldehyde with Schiff's reagent a complex is formed between two molecules of the aldehyde and the fuchsin–sulphurous acid reagent. That a change of this nature probably occurs is demonstrated by the fact that addition of a simple aldehyde to Schiff's reagent does not merely restore the colour to fuchsin,

but gives a different colour (often violet). It seems that a similar mechanism probably holds for the Feulgen reaction.

A preliminary examination was made of the bases liberated in the Feulgen test. Material passed into solution which showed a strong, broad ultra-violet absorption band with a maximum at 2670 A. ($\log I/I_0 = 0{\cdot}52$; *c*, 0·82 mg. %) (see Fig. 7). This can be compared with the measurements reported for adenine and guanine, *i.e.* absorption at 2610 A. ($\log I/I_0 = 1{\cdot}8$; *c*, 2·2 mg. %) and 2740 A. ($\log I/I_0 = 1{\cdot}0$; *c*, 2·2 mg. %), respectively. An equimolecular mixture of these purine bases would show in the conditions used an absorption band in the region 2675 A. ($\log. I/I_0 = 0{\cdot}52$; *c*, 0·82 mg. %). The slight difference in position of the observed and the calculated values may be due to several causes. That hydrolysis of some of the pyrimidine–sugar linkages occurs to a small extent was demonstrated by precipitating some silver salts of pyrimidines from the absorbing solution (cf. Schmidt and Levene, *J. Biol. Chem.*, 1938, **126**, 423).

It is well known that if there is overhydrolysis in the Feulgen test the intensity of the colour is much reduced. Lately renewed interest has been shown in the reasons for this and several explanations have been offered (cf. Bauer, *Z. Zellforsch.*, 1932, **15**, 225; Hillary, *Bot. Gaz.*, 1939, **101**, 276; Di Stefano, *loc. cit.*). The work of Stacey and his co-workers on the properties and reactions of deoxy-pentoses and -hexoses, and in particular of 2-deoxy-D- and -L-ribose, indicates that overhydrolysis of the nucleic acid not only brings it into a more diffusible form but also changes the deoxypentose to some extent into ω-hydroxylævulaldehyde. This aldehyde could be responsible for the results described by various workers. Stacey (*loc. cit.*) tentatively suggested that it was also responsible for the development of colour in the normal Feulgen test, but this was criticized by Danielli (*Symp. Soc. Exptl. Biol.*, 1947, **1**, 101). There is little doubt however that if the time of hydrolysis is extended this compound would be formed, and if washing was not exhaustive it would give a coloured complex when Schiff's reagent was added. This coloured complex could be retained by absorption on to cell proteins (cf. Stedman and Stedman, *loc. cit.*).

Simple preliminary experiments (cf. Experimental) indicated that, in the Feulgen test, any simple aldehyde formed during the hydrolysis procedure could be removed by washing. Hence only a large complex molecule could give the colour with Schiff's reagent, and it is believed that it is the nucleic acid molecule which reacts with the reagent.

From these observations it is possible to obtain some idea of what occurs in the Feulgen reaction. The initial acid hydrolysis results in preferential cleavage of the purine bases from the nucleic acid, although some slight hydrolysis of the pyrimidine bases may also occur. Overhydrolysis results in further degradation of the nucleic acid, and the deoxypentose is converted into ω-hydroxylævulaldehyde and then lævulic acid. Although this aldehyde will give a colour with Schiff's reagent it can be removed from tissues, etc., by washing and so is not responsible for the colour obtained when the Feulgen test is performed correctly.

This does not preclude it from being the compound responsible for the colours obtained by Hillary (*loc. cit.*), as originally suggested by Stacey. In the normal procedure when the purine–sugar linkages are cleaved, the sugar can react to some extent in its aldehydo-form and so combine with Schiff's reagent to give a coloured complex. This effect is probably intensified since in nucleic acids the sugar is present in the furanose form, and it has now been demonstrated that the intensity of colour developed is increased when the sugar cannot exist in the pyranose form. Moreover it has been shown that under similar conditions normal sugars give much less intense colours with Schiff's reagent than do their 2-deoxy-analogues. This fact indicates that the test can be used with reasonable certainty to differentiate between ribo- and deoxyribonucleic acids. Although this has been done in the past, no quantitative data have been put forward to support the results obtained. Since the coloured complex molecule formed is relatively large, the sites exhibiting the Feulgen colour are unlikely to be found in the cell far removed from the locations of the deoxyribonucleic acid. It is realised that the ideas proposed represent somewhat ideal conditions in that they apply only to nucleic acids. It is conceivable that other materials occur in cells which could give a colour with Schiff's reagent. Moreover the Feulgen reaction is carried out in cells in the presence of proteins and it is likely that these influence to some extent the formation of colour (cf. Hamazaki, *Nissin Igaku, Tokyo*, 1939, **28**, 46). However as applied to nucleic acids, the mechanism described could account for the development of colour when the Feulgen reaction is correctly performed.

Experimental.

Investigation of the Colours produced by Adding Schiff's Reagent to Certain Sugars.—The intensities of the colours produced by normal hexoses and pentoses and their 2-deoxy-analogues when Schiff's reagent was added to them, were measured quantitatively. The Schiff's reagent used for all the measurements

was the supersensitive aldehyde reagent introduced by Tobie (*Ind. Eng. Chem. Anal.*, 1942, **14**, 405) for the demonstration of free aldehyde groups in certain aldoses. Trial experiments indicated that it gave a negative reaction with ketoses and glycosides. The reagent was stored in a well-stoppered, dark bottle in a cool place. The normal pentoses and hexoses used were commercial samples, purified by repeated recrystallisation, and the deoxy-pentoses and -hexoses and their derivatives were prepared according to the methods of Stacey *et al.* (*J.*, 1949, 1358, 1879, 2836, 2841, 2846). The colours developed were measured in a Spekker photoelectric absorptiometer with Ilford filters 601—608 (λ = 4300—6800 A.) All the measurements except where otherwise indicated were obtained by dissolving the appropriate sugar (0·012 g.) in distilled water (4 ml.) followed by the addition of the standard Schiff's reagent (1 ml.). After the solution had been warmed at 35° for 1 minute the tube was well plugged, cooled rapidly to 17°, and kept at this temperature for 1·25 hours. The intensity of the colour developed was then measured. Results are shown in Figs. 1—5.

Investigation of the Colours produced by adding Schiff's Reagent to a Nucleoside and Some Nucleotides.—The compounds used were guanosine [prepared according to Bredereck, Richter, and Martini (*Ber.*, 1941, **74**, 694)], a deoxyribonucleotide mixture isolated from the enzymic degradation of thymonucleic acid and probably containing a preponderance of the pyrimidine types (Overend and Webb, forthcoming publication), and a purine deoxyribonucleotide kindly supplied by Dr. Chong Fu-Li (late of this Department). The experiments were conducted according to the Widström modification (*Biochem. Z.*, 1928, **199**, 298) of the Feulgen reaction. The materials (0·012 g. of each) were heated separately at 100° for exactly 2·75 minutes in a citrate buffer (pH 2·0; 2 ml.) (cf. Deriaz *et al.*, *J.*, 1949, 1222). After the solution had cooled, distilled water (2 ml.) and standard Schiff's reagent (1 ml.) were added, and the procedure described above followed. Results are shown in Fig. 6.

Treatment of a Partly Hydrolysed Deoxyribonucleic Acid with Schiff's Reagent.—The sodium salt of a fibrous sample of deoxyribonucleic acid isolated from soft herring roe essentially by Mirsky and Pollister's method (*J. Gen. Physiol.*, 1946, **30**, 101) was treated with a citrate buffer (pH 2·0) and 100° for 2·75 minutes. The resulting solution was cooled to 0°. Polymeric material still in solution was precipitated by addition of ethanol (cf. Gulland, Jordan, and Threlfall, *J.*, 1947, 1129), and the precipitate was collected by centrifugation, washed repeatedly with physiological saline and water, and collected by centrifugation. Control experiments indicated that this washing procedure was sufficient to remove any adsorbed aldehydes of low molecular weight, such as ω-hydroxylævulaldehyde. Schiff's reagent was added to an aqueous suspension of the white polymeric material isolated, and this immediately became violet, the intensity of the colour visibly becoming progressively greater. Although it contained excess of Schiff's reagent the supernatant liquid remained colourless even after 48 hours. It was removed by decantation and the coloured complex formed by the combination of the partly hydrolysed nucleic acid and Schiff's reagent was washed with water. No colour could be washed out of it. After being dried, the solid was obtained as fine violet fibres. The colour was light fast for several weeks.

Investigation of the Bases liberated During Mild Acidic Hydrolysis of Deoxyribonucleic Acid.—Fibrous deoxyribonucleic acid (0·0876 g.) and dilute sulphuric acid (0·5N.; 2 c.c.) were heated at 100° for 2·75 minutes. Addition of ethanol (excess) and physiological saline yielded a precipitate (*A*) which was removed and washed by centrifugation. The washings were added to the mother-liquors, giving a total volume of 7 ml. Ultra-violet absorption measurements on this showed a strong broad band at 2670 A. ($\log I/I_0$ = 0·52; *c*, 0·82 mg. %) (see Fig. 7) [cf. adenine and guanine which absorb at 2610 A. ($\log I/I_0$ = 1·8; *c*, 2·2 mg. %) and 2740 A. ($\log I/I_0$ = 1·0; *c*, 2·2 mg. %), respectively].

The mother liquor (1 ml.) was treated with a saturated solution of silver sulphate (2 ml.). A precipitate of the silver salts of guanine and adenine was formed (Schmidt and Levene, *J. Biol. Chem.*, 1938, **126**, 423). After cooling to 0° for 0·5 hour, the precipitate was separated by centrifugation and washed twice, by stirring at 0° with a saturated solution of silver sulphate followed by centrifugation. The solution was combined with the washings and analysed for pyrimidine nitrogen [Found: N(pyrimidine), 2·0%].

The precipitated polymeric material (*A*) was shown readily to develop a colour when excess of Schiff's reagent was added, and its general behaviour was identical with that of the material obtained in the previous experiment.

Effect of a Simple Aldehyde.—Deoxyribonucleoprotein from soft herring roe, isolated as described above, and the protein stripped from it were separately soaked in N-butyraldehyde for 1·5 hours. The proteins were then washed repeatedly (15 times) by being centrifuged with water containing a trace of ethanol (distilled over solid sodium hydroxide). After this treatment excess of Schiff's reagent was added to the solids. Neither developed colour, nor did the reagent. Next a coloured solution was prepared by adding Schiff's reagent (5 c.c.) to N-butyraldehyde (2 drops). The nucleoprotein and stripped protein were separately soaked in it for 1·5 hours, and then washed by being centrifuged ten times with water. Both had strongly adsorbed the dye which was not removed by being washed with water. The stripped protein was steeped in undeveloped Schiff's reagent for 1·5 hours, and then repeatedly washed by being centrifuged with water. On addition of N-butyraldehyde to the washed solid, suspended in water, and shaking the suspension it became coloured showing that it adsorbed the undeveloped reagent.

The author is indebted to Professor M. Stacey, F.R.S., for suggesting this topic and for his interest in the work. He also thanks the Board of the British Rubber Producers' Research Association for financial assistance which enabled him to undertake this investigation. Thanks are due to Dr. E. J. Bourne for kindly supplying a sample of diethylidene *aldehydo*-D-xylose.

CHEMISTRY DEPARTMENT,
THE UNIVERSITY, BIRMINGHAM.

[*Received, April 25th*, 1950.]