340. *Deoxy-sugars. Part XX. The Value of the Dische Test for the Quantitative Determination of Deoxypentose Nucleic Acids.*

By W. G. Overend.

The value of the Dische diphenylamine reaction (*Mikrochemie*, 1930, **8**, 4) for the determination of deoxypentosenucleic acids in cellular material has been investigated. It has been shown that amino-acids and purine and pyrimidine bases affect the intensity of the colour developed. Some unsuccessful attempts to modify the reagent are briefly mentioned.

Several workers (cf. Sevag, Smollens, and Lackmann, *J. Biol. Chem.*, 1940, **134**, 523; Seibert, *ibid.*, 1940, **133**, 593) have used the Dische diphenylamine reaction (*Mikrochemie*, 1930, **8**, 4) for the quantitative determination of deoxyribonucleic acid in cellular material. Hoagland *et al.* (*J. Exptl. Med.*, 1940, **72**, 139) estimated the nucleic acid in elementary bodies of vaccinia by using this reaction and stated that the colour produced was stable and that its intensity was proportional to the amount of nucleic acid in the original solution. Recently Hinshelwood and Caldwell (*J.*, 1950, 1415) used the Dische procedure to measure the deoxyribonucleic acid content of *Bact. lactis aerogenes* grown under a variety of conditions.

The test depends on the conversion of the carbohydrate moiety of the nucleic acid into ω-hydroxylævulaldehyde (Deriaz *et al.*, *J.*, 1949, 1222) which then reacts with diphenylamine. Investigations in this Department (*J.*, 1950, 1027) have shown that the test is not specific for 2-deoxy-D-ribose but that it is given by other 2-deoxypentoses. To extend this investigation it was considered worth while to examine the value of the reaction for the quantitative determination of deoxypentose nucleic acid in cellular tissue and bacteria. It is well known that when suitable concentrations (*e.g.*, 0·02—0·2%) of pure deoxypentose nucleic acid are used the intensity of the blue colour developed in the test appears to be proportional to the amount of nucleic acid present. However, Davidson (" The Biochemistry of the Nucleic Acids,"

Effect of added substances on the intensity of colour developed by deoxyribonucleic acid in the Dische test.

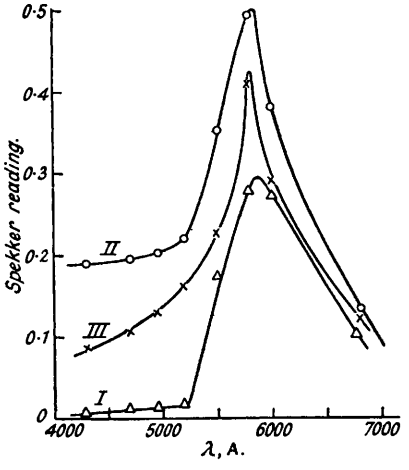
Added substance.	Spekker reading at 5800 A.	Added substance.	Spekker reading at 5800 A.
Amino-acids.			
None	0·280	Tyrosine	0·433
Histidine	0·347	Aspartic acid	0·467
*iso*Leucine	0·363	Arginine	0·470
Serine or lysine	0·373	Phenylalanine	0·480
Alanine	0·400	Leucine	0·510
Cysteine or glycine	0·410	Glutamic acid	0·540
Methionine	0·430	Valine	0·557

Added substance.	$\lambda_{max.}$, A.	Spekker reading.	Added substance.	$\lambda_{max.}$, A.	Spekker reading.
Simple bases.					
None	5950	0·29	Benzylamine	5950	0·51
p-Toluidine	5940	0·47	*iso*Propylamine	5940	0·45
p-Phenylenediamine	5050	0·10	Diethylamine	5950	0·49
Purines and pyrimidines.					
None	5942	0·30	Uracil	5942	0·335
Adenine	5835	0·44	Thymine	5942	0·355
Guanine	5830	0·39	Cytosine	5942	0·380

Methuen Monographs, 1950, p. 60) states that most of the colorimetric sugar reactions are liable to interference by proteins, and hence it is best to remove the protein residue of the tissue before applying the colorimetric tests. Moreover, Webb (personal communication) has shown that the Dische colour is influenced by various concentrations of sodium chloride, but that the effect is not linear.

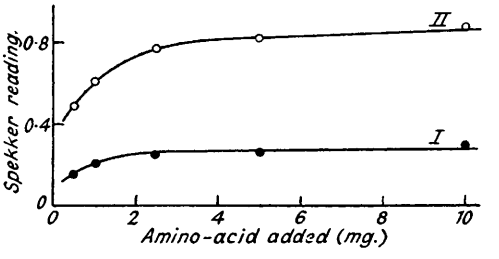
Comparative Dische tests were carried out according to the method described by Deriaz *et al.* (*loc. cit.*), using deoxyribonucleic acid (isolated from soft herring roes by Mirsky and Pollister's method) as control and with other samples of the same nucleic acid to which had been added separately half its weight of protamine and histone. The intensities of the colours developed were measured in the Spekker photoelectric absorptiometer, with Ilford filters 601—608 (λ, 4300—6800 A.). Results are represented in Fig. 1 and show that both proteins enhance the intensity of the colour produced. Since in the acid medium used proteins would be hydrolysed, a series of amino-acids were next tested, with results shown in the Table. In all cases the colour intensity was enhanced. Simple amines had the same effect, although *p*-phenylenediamine interfered with the colour produced and instead of blue a pink colour resulted. Finally, those purine and pyrimidine bases which would be present whenever the

FIG. 1.



I. *Control. Deoxyribonucleic acid* (DRNA) *alone.*
II. DRNA + *protamine.*
III. DRNA + *histone.*

FIG. 2.



I. DRNA *with glycine added.*
II. DRNA *with* DL-*valine added.*

test is used to estimate nucleic acid contents of cellular material were shown to increase the intensity of colour. The effect of the purines was greater than that of the pyrimidines, but neither group of compounds had as great an effect as the amino-acids. Finally, by using glycine and DL-valine the effect of the concentration of the amino-acids on the intensity was examined (see Fig. 2).

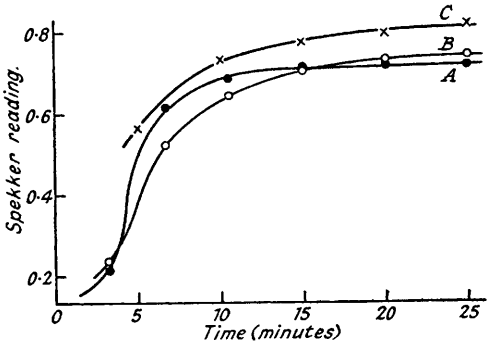
In the procedure adopted by Deriaz *et al.* (*loc. cit.*) the mixture of the nucleic acid and Dische reagent was heated at 100° for 3·25 minutes and we have mainly used this method. However, Woodhouse (*Brit. J. Cancer*, 1949, **3**, 510) concluded that in this period not all the deoxypentose nucleic acid had reacted and stated that 15 minutes' heating was necessary to get a maximum colour. Hinshelwood and Caldwell (*loc. cit.*) and Hoagland *et al.* (*loc. cit.*) found 10 minutes' heating to suffice for maximum colour development. Since our experiments were comparative, under rigidly controlled conditions, the time of heating was immaterial provided that it was sufficiently prolonged to give a colour measurable with accuracy on the Spekker instrument. However, we would state that we agree with Woodhouse, if measurements are to be made of the concentrations of nucleic acid solutions. Fig. 3 illustrates this contention. Moreover it shows also that in experiments with added amino-acid the colour is fully developed in this period.

The results obtained show that great caution must be exercised before using this test for the accurate determination of deoxypentose nucleic acid in cellular material because of the interference of other substances also likely to be present, such as amino-acids, purines, and pyrimidines. The method can, of course, be used to determine the deoxypentose nucleic acid in

pure solutions of unknown concentration. The results reported do not necessarily invalidate earlier work described in which this method of estimation was used. Much of this earlier work was merely comparative and not of great accuracy. Results of Hinshelwood and Caldwell's later work (*loc. cit.*) can only be accurate if errors are constant throughout or cancel each other out. However, their general conclusions are not materially altered since the effects now reported are within the limits of their experimental error.

During this work several unsuccessful attempts were made to prepare modified Dische reagents which would prove more suitable. Replacement in the reagent of the concentrated sulphuric acid by either trichloroacetic or trifluoracetic acid resulted in no colour formation when the test was made in the normal manner. If concentrated hydrochloric acid was used instead of concentrated sulphuric acid the reagent gave a colour when heated with deoxyribonucleic acid, but this was less intense than that obtained with the normal reagent. The addition of phosphate ions to the normal reagent had no effect on the intensity.

FIG. 3.



A. *Control* (DRNA *alone*).
B. DRNA *with glutamic acid added.*
C. DRNA *with* DL-*valine added.*

EXPERIMENTAL.

Dische Tests.—(a) *Control.* Herring roe deoxypentosenucleic acid (1·6 mg.) (isolated by Mirsky and Pollister's method) was dissolved in water (3 c.c.), and Dische reagent (cf. Deriaz *et al.*, *loc. cit.*) (6 c.c.) was added. The mixture was heated on a boiling water-bath for 3·25 minutes, then cooled for 5 minutes, and the intensity of the blue colour developed was measured in the Spekker photoelectric absorptiometer with Ilford filters 601—608 (λ, 4300—6800 A.). The experiments were conducted in duplicate.

λ, A.	4300	4700	4900	5200	5500	5800	6000	6800
Average Spekker reading ...	0·005	0·014	0·019	0·070	0·178	0·280	0·275	0·100

(b) *With added proteins.* The experiments were repeated in precise detail except that in one series of tubes protamine (0·8 mg.; B.D.H.) was added, and to another series histone (0·8 mg.). Results are shown in Fig. 1.

(c) *With added amino-acids.* In another series of experiments amino-acids (0·8 mg.) were added. In other respects the conditions were identical with those described. Experiments, conducted in duplicate, are reported in the Table (p. 1484).

(d) *With amines added.* Tubes were prepared each containing deoxyribonucleic acid (1·6 mg.) in water (3 c.c.) and Dische reagent (6 c.c.). To duplicate sets of tubes simple amines (0·8 g.) were added. Results are in the Table.

(e) *With purines and pyrimidines added.* A similar procedure was adopted using 0·8 mg. of adenine, guanine, uracil, cytosine, and thymine. Results are shown in the Table.

Effect of the Amino-acid Concentration on the Intensity of the Dische Colour.—Tubes were prepared each containing herring roe deoxyribonucleic acid (1·6 mg.) in water (1 c.c.) and Dische reagent (4 c.c.). To duplicate sets of tubes glycine solution (1 c.c.) of varying concentration was added. The tubes were heated in a boiling water-bath for 3·25 minutes, then cooled for 5 minutes, and the intensity of colour measured on the Spekker Photoelectric Absorptiometer using Ilford filter no. 606 (cf. Fig. 2).

Glycine added (mg.)	10·0	5·0	2·5	1·0	0·5
Average Spekker reading	0·312	0·279	0·255	0·223	0·184

The experiment was repeated using DL-valine instead of glycine.

DL-Valine added (mg.)	10·0	5·0	2·5	1·0	0·5	0·2
Average Spekker reading	0·881	0·830	0·784	0·610	0·490	0·433

Rate of Development of Dische Colour.—(a) *Control.* Herring roe deoxyribonucleic acid (8·2 mg.) was dissolved in water (13 c.c.), and Dische reagent (26 c.c.) was added. The solution was heated at 100° and at noted intervals of time aliquots (5 c.c.) were withdrawn and the intensity of colour was

measured on the Spekker photoelectric absorptiometer using Ilford filter no. 606. Results are shown in Fig. 3.

(b) *With glutamic acid added.* A similar procedure was adopted with a solution containing deoxyribonucleic acid as in (a) (8·2 mg.), and glutamic acid (4·0 mg.) in Dische reagent (26 c.c.) and water (13 c.c.). See Fig. 3 for results.

(c) *With* DL-*valine added.* The procedure was repeated on a solution prepared by dissolving deoxyribonucleic acid (6·66 mg.) and DL-valine (9·6 mg.) in water (12 c.c.) and Dische reagent (21·2 c.c.). Results are represented in Fig. 3.

Attempts to Prepare a Modified Dische Reagent.—(*a*) Twice recrystallised diphenylamine (0·4 g.) was added to AnalaR acetic acid (40 c.c.) and trichloroacetic acid (1·1 c.c.). This reagent (4 c.c.) was added to a solution of deoxyribonucleic acid (1·6 mg.) in water (2 c.c.), and the solution was heated at 100° for 3·25 minutes and then cooled. No colour developed. A similar result was obtained if trifluoracetic acid was used instead of trichloroacetic acid.

(*b*) Twice recrystallised diphenylamine (0·4 g.) was added to AnalaR acetic acid (40 c.c.) and concentrated hydrochloric acid (1·1 c.c.). Deoxyribonucleic acid (1·6 mg.) was added to water (3 c.c.) containing this reagent (6 c.c.). After being heated at 100° for exactly 3·25 minutes the solution was cooled and the colour intensity measured in the usual manner.

λ, A.	4300	4700	4900	5200	5500	5800	6000	6800
Spekker reading	0·043	0·052	0·086	0·111	0·132	0·172	0·155	0·075

(Cf. control experiments on p. 1486 using the normal reagent.)

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