The Estimation of Deoxyribonucleic Acid in the Presence of Sialic Acid: Application to Analysis of Human Gastric Washings

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1. Sialic acid has been found to interfere with three colorimetric reactions used for the estimation of DNA: a modified diphenylamine reaction at 100° (Dische, 1930), the nitrophenylhydrazine method (Webb & Levy, 1955) and the diphenylamine reaction at 30° (Burton, 1956). 2. Evidence is presented that sialic acid is present in hydrolysates obtained from gastric wash-out material. 3. A mathematical method for correcting for interference from sialic acid in the diphenylamine reaction at 30° is described. 4. The diphenylamine reaction has been modified to make it suitable for the estimation of DNA in the presence of sialic acid. The modifications are to increase the concentration of diphenylamine to 2% and to perform the reaction at 6–13° for 48 hr. These modifications increase the sensitivity 25% above Burton's (1956) modification of the diphenylamine reaction. 5. The precipitation, extraction and recovery of DNA from gastric wash-out material have been investigated.

When a modification (Slater & Lovell, 1961; Croft, 1963) of the original diphenylamine method (Dische, 1930) for the estimation of DNA was applied to the fluid obtained by washing out the human stomach with 0.9% sodium chloride solution, a purple colour, noticeably different from the pale blue produced by DNA, was observed in some specimens. A more specific diphenylamine method (Burton, 1956) appeared to give the same colour with both gastric wash-out fluid and with pure DNA, but spectrophotometric examination showed that some gastric wash-out specimens gave absorption spectra that were different from that given by DNA.

Lee (1963) found that sialic acids interfered with the estimation of DNA by Dische's diphenylamine method at 100°, and he suggested that sialic acids are a common cause of interference in the estimation of DNA in biological tissues. Burton (1956) found very few substances that interfered with his modification of the diphenylamine reaction, but he did not study sialic acids as possible causes of interference. Our material was derived from humans, in whom the only type of sialic acid so far isolated is *N*-acetylneuraminic acid (Gottschalk, 1960). As sialic acid is known to cause a purple colour when heated with diphenylamine reagent at 100° (Werner

& Odin, 1952; Bohm, 1958; Slater & Lovell, 1961), it seemed probable that it was interfering in the reactions used with our gastric wash-out material.

The present paper describes a study of the effect of sialic acid on the estimation of DNA by three recognized colorimetric methods. The diphenylamine method has been modified to allow the estimation of DNA in the presence of sialic acid. We have also investigated the extraction procedure of Schneider (1945) as applied to gastric wash-out material.

MATERIALS AND METHODS

2% Diphenylamine reagent. A 1 g. sample of diphenylamine (A.R. grade) was dissolved in about 40 ml. of acetic acid (A.R. grade) in a 50 ml. volumetric flask. Then 1.0 ml. of conc. H₂SO₄ was added and the volume made up to 50 ml. with acetic acid. Finally 0.25 ml. of the aqueous acetaldehyde solution described below was added. The reagent should be used on the day of preparation.

Aqueous acetaldehyde solution. A 2 ml. sample of acetaldehyde was diluted to 100 ml. with water and the solution stored at 4°. Pipetting was made easier if the acetaldehyde and pipette were first cooled to about 4°.

Sterile bovine plasma albumin. This solution contained 0.3 g./ml.

DNA solution. Stock standard DNA solution was prepared from highly polymerized calf-thymus DNA (Sigma Chemical Co., St Louis, Mo., U.S.A.). It contained 40 mg. of DNA/100 ml. in 5mM-NaOH and was stored at 4°. The solution used contained 0.836 µg. atom of P/ml.

DNA hydrolysates: working standards. Samples (0.25, 0.5 and 1.0 ml. respectively) of stock standard DNA solution

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in glass-stoppered test tubes were made up with water to 6.8 ml., and 0.2 ml. of 50% (w/v) trichloroacetic acid solution was added to each. The DNA solutions were heated at $90 \pm 1^\circ$ for exactly 15 min., cooled in running tap water and stored at 4° . The hydrolysates were stable for at least 6 months.

Sialic acid. Sialic acid (*N*-acetylneuraminic acid) solution contained 1 mg./ml. in water.

Other reagents. *p*-Nitrophenylhydrazine, trichloroacetic acid and perchloric acid were A.R. grade.

Gastric wash-out specimens. Gastric wash-out specimens were obtained by the following procedure, devised by D. N. Croft & N. F. Coghill (unpublished work). A plastic Ryle's tube (4.0 mm. bore) was prepared by enlarging the holes near its tip to 1.0 cm. $\times$ 0.3 cm. and attaching along its length a plastic catheter (2.0 mm. bore) ending 9.5 cm. proximal to the hole furthest from the tip. The plastic catheter was about the same length as the Ryle's tube. Tests were performed after the subject had been without food for 12–15 hr. overnight. The prepared Ryle's tube was introduced perorally, with fluoroscopy, so that the tip lay in the stomach over the left edge of the supine subject's vertebral column. The subject was provided with a dental sucker and asked not to swallow, retch or cough during the test. Gastric specimens were obtained by washing the stomach four times with 500 ml. of warm (29 – 35°) 0.9% NaCl containing 0.8 mg. of polyethylene glycol (mol. wt. 4000)/ml. The first wash-out was performed by injecting ten successive 50 ml. volumes of wash fluid down the catheter, withdrawing each as completely as possible through the Ryle's tube with a syringe. The remaining three wash-outs were performed by infusing 500 ml. of wash-out fluid through the catheter and withdrawing it through the Ryle's tube by a continuous suction at a reduced pressure of -15 to -20 cm. H_2O . Each infusion took about 15 min. To prevent destruction of DNA by deoxyribonucleases (Marmur, 1961) the specimens were aspirated directly into 25 ml. of 0.2 M-EDTA, pH 8.

Preliminary treatment of gastric wash-out material. The gastric wash-out specimens were poured into 650 ml. plastic centrifuge bottles, which were immediately placed in ice. About 1 hr. later the specimens were removed and the volumes measured. As some of the specimens might have contained less than 0.01 μ g. atom of DNA P/ml., albumin was added to all the specimens (0.15 ml. of 30% bovine serum albumin solution/100 ml. of gastric wash-out specimen) to aid precipitation of the DNA, as recommended by Burton (1956). After the specimens had been well shaken to mix the albumin solution, 10 ml. of 50% trichloroacetic acid was added for each 100 ml. of volume and the acidified specimens were kept at -16° to -20° for 1–7 days. Storage at low temperature of the acidified wash-out material is essential for the complete separation of the solid phase on subsequent centrifugation.

Extraction and hydrolysis of DNA from acidified gastric wash-out material. The gastric wash-outs were thawed in a shaking water bath at 37° and centrifuged in an MSE Magnum refrigerated centrifuge for 30 min. at 1800 rev./min. ($735 g_{av.}$) at 4° . In each case all but about 20 ml. of the supernatant was discarded and the deposit was loosened and transferred to a 50 ml. glass-stoppered test tube. The plastic container was washed out two or three times with small volumes of cold 5% (w/v) trichloroacetic acid so that the final volume in the test tube was about 50 ml. This

was centrifuged at 2500 rev./min. ($1420 g_{av.}$) for 30 min. at 4° and the supernatant discarded.

To the deposit in the centrifuge tube 4 ml. of 5% trichloroacetic acid was added with stirring. The tube was then heated at $90 \pm 1^\circ$ for 15 min., stirred with a glass rod once during this time, cooled to room temperature, centrifuged at 2500 rev./min. ($1420 g_{av.}$) for 30 min. and the supernatant poured into a 25 ml. graduated test tube. A second hydrolysis was performed on the deposit by adding 3 ml. of 5% trichloroacetic acid and heating for 15 min. at $90 \pm 1^\circ$. The tube was again centrifuged for 30 min. at 2500 rev./min. ($1420 g_{av.}$) and the supernatant added to the first hydrolysate in the graduated test tube. The volume of the combined hydrolysates was recorded. The hydrolysate was stored at -16° to -20° . Before the colour reaction was carried out, the hydrolysate was thawed and centrifuged at 4° at 2500 rev./min. ($1420 g_{av.}$) for 30 min. to remove solids that were separated from the hydrolysate by freezing. The supernatant was decanted and stored at 4° . The hydrolysates were stable for at least 6 months.

Diphenylamine colour reaction finally adopted for gastric wash-out specimens. To 0.2 ml. of 60% (w/v) perchloric acid (sp. gr. 1.54) in a glass-stoppered test tube was added 2.0 ml. of hydrolysate. Specimens found to contain more than 0.15 μ g. atom of DNA P/ml. were diluted with 5% trichloroacetic acid to bring them into the range of 0.01–0.15 μ g. atom of DNA P/ml. The blank consisted of 2.0 ml. of 5% trichloroacetic acid. A 2.0 ml. sample of each of the three working-standard DNA hydrolysates was set up with each series of estimations. To each mixture was added 2.0 ml. of 2% diphenylamine reagent and the specimens were placed for 48–54 hr. in a refrigerator, the temperature of which varied between 6° and 13° . After removal from the refrigerator the coloured solutions were kept in ice-water until transferred to the cuvettes. The extinctions were read against a reagent blank at 550 and 600 $m\mu$. The concentration of DNA was calculated from the extinction at 600 $m\mu$; the extinction at 550 $m\mu$ indicated whether significant interference by sialic acid had occurred, as explained below.

An alternative procedure was used in many cases in parallel with the preferred procedure. The reaction mixtures were heated at 30° for 18–24 hr. and the extinctions read against the blank at 550 and 600 $m\mu$. The DNA concentration was calculated by the method described below. The alternative procedure was useful in those cases where sialic acid interference was not great.

RESULTS

Study of Dische's diphenylamine reaction as modified by Slater & Lovell (1961)

In Dische's (1930) diphenylamine method and its modification by Slater & Lovell (1961), the reaction is carried out at 100° . The method of Slater & Lovell (1961) has been adapted for use with gastric material (Croft, 1963). With this adaptation, the absorption spectra of the colours obtained with pure DNA and gastric wash-out hydrolysates differed when less than 0.17 μ g. atom of DNA P/ml. was present. The difference in the absorption spectra was most marked in the range 450–550 $m\mu$, the effect being

to move the maximum extinction to a lower wavelength (Fig. 1). Sialic acid gave an absorption spectrum with peaks at 530 and 700 $m\mu$ (Fig. 2). Heating sialic acid at 90° for 15 min. with 10% (w/v) trichloroacetic acid caused a 50% increase in extinction at 530 and 600 $m\mu$ as compared with a similar hydrolysis with 5% trichloroacetic acid. The colour produced both by some gastric wash-outs and sialic acid increased perceptibly on standing at room temperature. In the gastric wash-out specimens this increase was greater at 550 $m\mu$ than at 600 $m\mu$.

Although it was possible by this method to obtain reproducible DNA estimations when the concentrations of DNA P in the gastric wash-out hydrolysate was above 0.17 $\mu\text{g. atom/ml.}$, poor reproducibility was obtained with less concentrated hydrolysates.

Study of the nitrophenylhydrazine method of Webb & Levy (1955, 1958)

This method was tested with gastric wash-out specimens and with sialic acid. The red colour faded

Fig. 1. Absorption spectra of gastric wash-out and DNA hydrolysates after heating with a 1% diphenylamine reagent (1g. of diphenylamine, 2ml. of conc. H_2SO_4 and 98ml. of acetic acid) at 100° for 10 min. The 1% diphenylamine reagent (2ml.) was added to 2ml. of DNA hydrolysate (0.081 $\mu\text{g. atom of P/ml.}$) or to 2ml. of gastric wash-out hydrolysate, which had not been stored at -20° or centrifuged to remove solid material. $\blacktriangle$, DNA; $\circ$, gastric wash-out.

Fig. 2. Absorption spectra of pure DNA (0.325 $\mu\text{g. atom of P}$) and pure sialic acid (0.33 mg.) hydrolysates after heating with 1% diphenylamine reagent at 100° for 10 min. A 2ml. portion of hydrolysate in 5% trichloroacetic acid was treated with 2ml. of the reagent. Extinctions were read 30 min. after cooling the reaction mixture to room temperature. $\blacktriangle$, DNA; $\square$, sialic acid.

Fig. 3. Effect of temperature on the colour development with DNA. The 2% diphenylamine reagent was added to 0.2ml. of 60% perchloric acid and 2ml. of three DNA hydrolysates each containing 0.080 $\mu\text{g. atom of P}$. The solutions were kept at 35°, 26° and 6-13° respectively and the extinctions at 600 $m\mu$ read at intervals from 4 to 120 hr. $\blacktriangle$, 35°; $\blacksquare$, 26°; $\bullet$, 6-13°.

so rapidly that the method was unsuitable for use with gastric wash-out hydrolysates containing low concentrations of DNA, possibly because of interference by sialic acid.

Study of factors affecting Burton's (1956) modification of the diphenylamine reaction

In Burton's (1956) modification, the diphenylamine reaction takes place at 30° for 18 hr. The diphenylamine reagent contains acetaldehyde, and the DNA extract contains perchloric acid. In our extraction and hydrolysis procedures, 5% trichloroacetic acid was used both as a protein precipitant and hydrolysis agent (Schneider, 1945). The sensitivity of the colorimetric reaction was increased by

raising the diphenylamine concentration from 1.5 to 2%. The addition of 0.2 ml. of 60% perchloric acid to 2 ml. of the 2% diphenylamine reagent and 2 ml. of 5% trichloroacetic acid hydrolysate prevented both the precipitation of the diphenylamine (K. Burton, personal communication) and provided sufficient perchloric acid for optimum colour development (Burton, 1956). With the diphenylamine method at 30°, it suited our working schedule better to allow the colour development to take place for 24 hr. instead of the 16–20 hr. recommended by Burton (1956). There was no marked difference in colour intensity at these two times (Fig. 3).

Effect of temperature on the colour produced by sialic acid. In these experiments sialic acid was heated at 90° with 5% trichloroacetic acid by the procedure described for the working-standard DNA hydrolysates. The resulting mixture is designated sialic acid hydrolysate. The smallest amount of sialic acid detectable at 600 μ when the development of colour took place at 30° was 0.05 mg./ml. of hydrolysate: at 6–13° it was 0.1 mg./ml. of hydrolysate. The absorption spectrum of the coloured solution derived from 2 ml. of sialic acid hydrolysate containing 0.7 mg./ml. (Fig. 4) showed maxima at 550 and 440 μ , the former being the higher. Fig. 5 shows the rate of colour development with sialic acid over 5 days at three temperatures, 35°, 26° and 6–13°. Placing the specimens in the refrigerator at 6–13° substantially decreased the colour developed by sialic acid. The effect of carrying out the diphenylamine reaction on DNA at 35°, 26° and 6–13° is shown in Fig. 3. In each case an almost constant value is reached for the extinctions at 600 μ , the time taken to reach this value being longest at the lowest temperature. The extinction at 600 μ is eventually higher at 6–13° than at 26°.

Fig. 4. Absorption spectra of DNA and sialic acid with Burton's (1956) modification of the diphenylamine reaction. The 2% diphenylamine reagent (2 ml.) and perchloric acid (0.2 ml.) were added to 2 ml. of DNA hydrolysate (0.1 μ -atom of P/ml.) or 2 ml. of sialic acid hydrolysate (0.7 mg./ml.). The solution containing DNA was heated at 32–35° for 21 hr. and was then allowed to stand at room temperature for a further 24 hr. The sialic acid mixture was heated at 35 $\pm$ 1° for 19 hr. $\blacktriangle$, DNA; $\square$, sialic acid.

Fig. 5. Effect of three temperatures on the colour development with sialic acid. The 2% diphenylamine reagent (2 ml.) and 60% perchloric acid (0.2 ml.) were added to 2 ml. of sialic acid hydrolysate (0.4 mg./ml.). The mixtures were kept at 35°, 26° and 6–13° respectively for 118 hr. Δ , 35°; $\square$, 26°; $\circ$, 6–13°.

Fig. 6. Absorption spectra of pure DNA, a gastric wash-out and a mixture of DNA and sialic acid when hydrolysates of these solutions were heated at 30° with 2% diphenylamine reagent. The 2% diphenylamine reagent (2ml.) and 60% perchloric acid (0.2ml.) were added to 2ml. of DNA hydrolysate (0.37 μ g. atom of P) or 2ml. of gastric wash-out hydrolysate or 2ml. of a hydrolysate of DNA (0.37 μ g. atom of P) and sialic acid (1mg.). The colour was developed at 30° for 18hr. and the extinctions were read 4hr. later. Δ , DNA; $\bullet$, gastric wash-out; $\blacksquare$, DNA and sialic acid.

Comparison of colours produced by gastric wash-out hydrolysates, DNA and sialic acid. The absorption spectrum of the coloured solution obtained with a gastric wash-out hydrolysate is shown in Fig. 6. The absorption spectrum from the hydrolysate is similar to, but not identical with, that obtained from DNA and can be closely imitated by a mixture of DNA and sialic acid.

Effect of sialic acid on the estimation of DNA

The effect of sialic acid on the colour produced by different amounts of DNA was investigated at 30°

Fig. 7. Effect of sialic acid on the extinction at 600 $m\mu$ of hydrolysates of mixtures of DNA and sialic acid. Experimental details are given in Table 1. $\blacksquare$, 0.3 ml. of DNA; $\square$, 0.2 ml. of DNA; $\bullet$, 0.1 ml. of DNA; $\circ$, 0.05 ml. of DNA; Δ , 0.03 ml. of DNA.

and 6–13°. A mixture of various amounts of sialic acid solution containing 1mg./ml. and various amounts of stock standard DNA solution was made up to 1.8ml. with water, and 0.2ml. of 50% trichloroacetic acid solution was added. After hydrolysis, 2% diphenylamine reagent and perchloric acid were added and the colour was developed at 30° in one experiment and at 6–13° in another. The extinctions were read at 550 and 600 $m\mu$ after 24 and 48 hr. respectively and the results are set out in Tables 1 and 2.

In the colour reaction at 30° the presence of sialic acid clearly depressed the extinction at 600 $m\mu$ due to DNA. At low DNA concentrations sialic acid increased the extinction at 600 $m\mu$, presumably owing to the reaction of sialic acid itself (Fig. 7). At 550 $m\mu$ the extinction of the mixture is higher at all concentrations of sialic acid compared with that of the pure DNA solution.

When the reaction was carried out at 6–13°, sialic acid had similar, but much less marked, effects on the extinction of sialic acid–DNA mixtures.

Table 1. *Extinctions at 550 and 600m μ of mixtures of sialic acid and DNA after hydrolysis and the 2% diphenylamine colour reaction at 30°*

Sialic acid solution containing 1mg./ml. was mixed with DNA solution containing 0.836 μ g. atom of P/ml. The mixture was made up to 1.8ml. with water, and 0.2ml. of 50% trichloroacetic acid solution was added; hydrolysis was carried out for 15min. at 90°. To the hydrolysate were added 0.2ml. of 50% perchloric acid solution and 2ml. of 2% diphenylamine reagent, and the colour was developed at 30° for 24hr.

Composition of mixture		Extinctions of mixtures		E_{600}/E_{550} ratio of mixture	Extinction of mixture minus observed sialic acid extinction		E_{600}/E_{550} ratio (E'_{600}/E'_{550})	Corrected E_{600} for DNA	Observed E_{600} for DNA alone
Sialic acid soln. (ml.)	DNA soln. (ml.)	E_{550}	E_{600}		E'_{550}	E'_{600}			
0.8	0.3	0.458	0.588	1.283	0.246	0.478	1.94	0.668	0.640
0.8	0.2	0.382	0.432	1.120	0.170	0.322	1.89	0.453	0.452
0.8	0.1	0.304	0.268	0.884	0.092	0.158	1.72	0.222	0.222
0.8	0.05	0.241	0.176	0.730	0.029	0.066	2.28	0.100	0.117
0.8	0.03	0.240	0.157	0.654	0.028	0.047	1.68	0.064	0.071
0.8	0	0.212	0.110	0.519	—	—	—	—	—
0.6	0.3	0.432	0.600	1.389	0.271	0.512	1.89	0.658	0.640
0.6	0.2	0.348	0.428	1.230	0.187	0.344	1.84	0.444	0.452
0.6	0.1	0.248	0.246	0.992	0.087	0.162	1.86	0.209	0.222
0.6	0.05	0.206	0.166	0.831	0.045	0.082	1.82	0.107	0.117
0.6	0.03	0.194	0.136	0.855	0.033	0.052	1.58	0.064	0.071
0.6	0	0.161	0.084	0.522	—	—	—	—	—
0.4	0.3	0.400	0.608	1.520	0.282	0.543	1.93	0.660	0.640
0.4	0.2	0.312	0.433	1.388	0.194	0.368	1.90	0.443	0.452
0.4	0.1	0.212	0.237	1.118	0.094	0.171	1.83	0.212	0.222
0.4	0.05	0.160	0.148	0.925	0.040	0.083	1.90	0.107	0.117
0.4	0.03	0.143	0.114	0.797	0.025	0.049	1.96	0.065	0.071
0.4	0	0.110	0.065	0.550	—	—	—	—	—
0.2	0.3	0.369	0.618	1.675	0.304	0.585	1.89	0.633	0.640
0.2	0.2	0.273	0.433	1.587	0.213	0.400	1.88	0.436	0.452
0.2	0.1	0.168	0.233	1.387	0.108	0.200	1.85	0.221	0.222
0.2	0.05	0.114	0.136	1.192	0.054	0.103	1.91	0.116	0.117
0.2	0.03	0.092	0.093	1.011	0.032	0.060	1.88	0.068	0.071
0.2	0	0.060	0.033	0.550	—	—	—	—	—
0	0.3	0.338	0.640	1.893					
0	0.2	0.243	0.452	1.860					
0	0.1	0.122	0.222	1.820					
0	0.05	0.063	0.117	1.857					
0	0.03	0.039	0.071	1.821					

Correction for effects of sialic acid in the reaction at 30°. The results in Table 1 show: (i) that the extinctions at 550 and 600m μ of a mixture of sialic acid and DNA are less than the sum of the extinctions of each component, measured separately; (ii) DNA alone gives an E_{600}/E_{550} ratio 1.85; (iii) sialic acid alone gives an E_{600}/E_{550} ratio 0.52; (iv) the corrected extinction ratios (E'_{600}/E'_{550}) of the values obtained by subtracting the observed sialic acid extinction from the extinction of the mixture are almost the same as for DNA alone (mean value 1.88). Thus the extinction due to DNA is depressed by sialic acid by the same factor at 550m μ as at 600m μ ; the sialic acid extinction and the depressed DNA extinction

are then additive. If so, values of E_{600} for sialic acid and the depressed E_{600} for DNA can be calculated from E_{550} and E_{600} for the mixture by the equations:

$$\text{Depressed } E_{600} \text{ for DNA} = 1.39 E_{600} - 0.72 E_{550}$$

$$E_{600} \text{ for sialic acid} = 0.72 E_{550} - 0.39 E_{600}$$

The plot of the depressed E_{600} for DNA against the concentration of DNA at each sialic acid concentration in the mixtures gave a series of straight lines. Fig. 8 shows a linear relationship between the slopes of these lines and the E_{600} due to sialic acid alone. From the equation of this line it was shown that the true DNA E_{600} value can be obtained from

Table 2. *Extraction of DNA from nine gastric wash-outs*

Bovine albumin and trichloroacetic acid were added to gastric wash-outs (respective concentrations 0.45 mg./ml. and 50 mg./ml.), which were then kept at -16° to -20° for 24–96 hr. After thawing, the centrifuged precipitate was extracted with trichloroacetic acid in four different ways. Expt. 1: The precipitate was heated with 10 ml. of 5% trichloroacetic acid for 15 min. at 90° ; the residue was re-extracted similarly with 5 ml. of 5% trichloroacetic acid, except for specimen DE17/10, when 10 ml. was used for the second extraction; a correction formula was used in calculating DNA concentration. Expt. 2: The precipitate was heated with 10 ml. of 5% trichloroacetic acid for 30 min. at 90° ; the residues were heated with 5 ml. of 5% trichloroacetic acid for 15 min.; no correction formula was necessary. Expt. 3: 10% trichloroacetic acid was used for the first and second extractions and 5% for the third; all extractions were performed at 90° for 15 min.; 10 ml. of the acid was used for the first extraction and 5 ml. for the second and third; no correction formula was necessary. Expt. 4: 5% trichloroacetic acid was heated with the precipitates at 90° for 15 min.; the first extractions were performed with 4 ml. of the acid and all subsequent extractions with 3 ml.; no correction formula was necessary.

	Specimen	Diphenylamine method (temp.)	DNA (μ g. atom of P)				Total DNA estimated in specimen	DNA in first two extracts (% of total DNA)
			First extract	Second extract	Third extract	Fourth extract	Residue	
Expt. 1	B ₁ 16/10	30°	0.187	0.047	0.020	—	0	92
	B ₂ C17/10	30	0.660	0.099	0.023	—	0	97
	DE17/10	30	1.740	0.349	0.058	—	0	97
Expt. 2	X30/10	30	0.909	0.191	0.033	—	—	97
Expt. 3	Y30/10	30	0.727	0.286	0.030	—	—	97
Expt. 4	A20/2	6–13	0.552	0.169	0.050	0.015	0	92
	A21/2	6–13	1.910	0.634	0.187	0.068	0	91
	B ₁ 25/3	6–13	0.907	0.117	0.053	0.025	—	93
	B ₁ 26/3	6–13	5.080	4.060	0.309	0.137	—	95

the depressed E_{600} for DNA by dividing it by the fraction:

$$1 - (1.92 E_{550} - 1.04 E_{600})$$

Combining these factors, we obtain:

$$\text{Corrected } E_{600} \text{ for DNA} = \frac{1.39 E_{600} - 0.72 E_{550}}{1 - (1.92 E_{550} - 1.04 E_{600})}$$

Column 9 of Table 1 gives the corrected E_{600} values for DNA calculated by this formula.

Calculation of true DNA extinction for the reaction carried out at 6–13°. From Fig. 8, showing the depressing effects of sialic acid on the observed extinctions at 600 m μ , it is clear that in most cases no correction is required. It is not possible to apply the method used for correcting the extinctions at 30° , as there is no constant extinction ratio for sialic acid. Therefore it is best to take the observed extinction at 600 m μ as being due to DNA.

Efficiency of the procedure used for the extraction of DNA from gastric wash-out material

Table 2 shows that two hydrolyses of gastric material extract over 90% of the extractable DNA. After one hydrolysis, extraction varied from 53 to

Fig. 8. Effect of sialic acid on the corrected extinctions at 600 m μ for DNA. Corrected extinctions of DNA at 600 m μ were plotted against the corresponding amount of DNA in hydrolysates of DNA-sialic acid mixtures. The slopes of the resulting straight lines are plotted against the calculated sialic acid extinction of these mixtures. Data are taken from Table 1.

84%. No DNA was detected in the residues (Expts. 1 and 4) by the method that Burton (1956) used for bacterial residues. The extinction results suggested

that the gastric residues contained mainly sialic acid, which could have prevented the detection of small amounts of DNA. Table 2 also shows that no more DNA is extracted by increasing the hydrolysis time or the concentration of trichloroacetic acid. The extraction procedures of Expts. 2 and 3 increased the colour caused by sialic acid in the diphenylamine reaction. It therefore appears that two hydrolyses at 90° for 15 min. with 5% trichloroacetic acid is satisfactory for gastric material.

Losses of DNA during the processing of gastric wash-out specimens and hydrolysates. Two recovery experiments were performed. After two gastric wash-out specimens (450 ml. and 530 ml.) containing polyethylene glycol and EDTA had been cooled in ice they were homogenized for 3 min. and each was divided into two. To one of the halves of each specimen DNA (0.836 $\mu\text{g. atom}$ of DNA P) was added. To each half of the 450 ml. specimen 0.35 ml. of 30% bovine albumin and 23 ml. of 50% trichloroacetic acid were added, and to each half of the 530 ml. specimen 0.4 ml. of 30% bovine albumin and 27 ml. of 50% trichloroacetic acid were added. The specimens were stored at -16° to -20° for 24 hr. and then thawed, centrifuged at 1800 rev./min. (735 g_{av}) and the deposits hydrolysed twice with 4 ml. and 3 ml. of 5% trichloroacetic acid respectively for 15 min. at 90°. The hydrolysates were stored at -16° to -20° and then thawed and centrifuged. The DNA was estimated on the hydrolysates by the 2% diphenylamine reaction at 6-13°. In the 450 ml. specimen 0.252 $\mu\text{g. atom}$ of DNA P was recovered in one half and 1.048 $\mu\text{g. atoms}$ of DNA P in the half to which DNA had been added (79.6% recovery). In the 530 ml. specimen 0.244 $\mu\text{g. atom}$ of DNA P was found in one half and 1.028 $\mu\text{g. atoms}$ of DNA P in the half to which the DNA had been added (78.4% recovery).

No DNA was detectable by the 2% diphenylamine method at 6-13° in the supernatant of four gastric wash-out specimens that had been centrifuged after the addition of albumin and trichloroacetic acid. However, the concentration of DNA in these specimens would have varied from 0.002 to 0.005 $\mu\text{g. atom}$ of DNA P/ml., a value below the sensitivity of the method, if all of it had remained in the supernatant.

None of the deposits obtained from centrifuging three gastric wash-out hydrolysates after storage at -16° to -20° contained detectable amounts of DNA. The deposits were suspended in 5% trichloroacetic acid (5 ml.) to which were added perchloric acid (2 ml.) and 2% diphenylamine reagent (2 ml.), and the colour was developed at 6-13°. After 48 hr. the coloured solutions were centrifuged and the extinctions at 550 and 600 $m\mu$ were read. The extinction ratio suggested that the colour was caused by sialic acid. The hydrolysate deposit of

another specimen was resuspended in 1 ml. of its own hydrolysate to which was added 4 ml. of 5% trichloroacetic acid, 2.0 ml. of perchloric acid and 2 ml. of 2% diphenylamine reagent, and the colour was developed at 6-13°. This specimen did not contain more DNA than 1 ml. of its own hydrolysate put through the same procedure.

DISCUSSION

Initial treatment of gastric wash-out material and the extraction of DNA. The initial treatment of the gastric wash-outs was designed to precipitate all the DNA. As the DNA content of many of the gastric wash-out specimens was less than 0.003 $\mu\text{g. atom}$ of DNA P/ml., albumin was added to aid precipitation of DNA by trichloroacetic acid (Burton, 1956). In the two recovery experiments in which the final DNA concentration was about 0.004 $\mu\text{g. atom}$ of DNA P/ml., the average recovery was 79%. This value is to be compared with the 91-95% efficiency of extraction of DNA from the gastric wash-out deposit. It is possible that part of the added DNA was not precipitated and remained in the supernatant despite the addition of albumin. In the gastric wash-out specimens much of the DNA is contained in cellular material that would come down during storage at -16° to -20° and centrifugation whether or not albumin was added, so that the loss may be less than suggested by the recovery experiments.

Storage at -16° to -20° is a useful modification of the method. It leads to precipitation of mucus and cellular material in the gastric wash-out specimen and, after the hydrolysis of the precipitate, storage at -16° to -20° removed, without detectable loss of DNA, solid material that contained sialic acid.

Modifications to the diphenylamine colorimetric reaction. Burton (1956) has shown that the modified diphenylamine reaction at 30° is about 3.5 times as sensitive and more specific than the original diphenylamine reaction at 100°. He found that the sensitivity of the method was similar to that of four other colour reactions for DNA. We have further increased the sensitivity of the method by 25% by increasing the concentration of diphenylamine from 1.5 to 2.0% and by allowing the colour to develop at 6-13° for 48 hr.

Sialic acid interfered with the diphenylamine reaction at 100°, the nitrophenylhydrazine method and to a less extent the diphenylamine reaction at 30°. This interference was considerably diminished in the diphenylamine reaction by performing it at 6-13° and, in most cases, could be ignored. Sialic acid interference in the DNA-sialic acid mixture experiments was significant only when the extinction at 600 $m\mu$ due to DNA was below 0.045. In

none of the hydrolysates from 115 gastric wash-outs examined was sialic acid considered to have interfered significantly.

Duplicate estimations 4 days to 2 months apart by the 2% diphenylamine method at 6–13° on 12 of these hydrolysates, which contained 0.016–0.264 $\mu\text{g. atom}$ of DNA P/ml., differed by less than 3% of their means.

Use of a mathematical correction. Dische (1955), using the diphenylamine reaction at 100°, found that the extinction at 595m μ minus the extinction at 650m μ gave better agreement between duplicate samples than when a single reading at 595m μ was used. We found in gastric wash-out specimens that this correction was not satisfactory for the diphenylamine reaction at 100° or 30°. A correction that used readings at 550, 600 and 650m μ (Allen, 1950) was also unsatisfactory for some gastric wash-outs analysed by the diphenylamine reaction at 100° and 30°. With mixtures of DNA and sialic acid analysed by the 2% diphenylamine reaction at 30°, the observed extinction at 600m μ gives the concentration of DNA in the specimen to within 10%, if this extinction is between 0.200 and 0.650 and the extinction ratio is more than 60% of that of the standard (Table 1). In other cases a correction formula must be used. The coefficients of the correction formula are based on an extinction ratio (E_{600}/E_{550}) 1.85 for DNA; if this ratio differs by more than ± 0.07 they may have to be modified accordingly.

It is possible that the correction formula over-corrects for the effect of sialic acid. In 13 cases where the DNA in the gastric material was estimated both at 6–13° and 30°, the values at 30° were higher by an average of 10% than the values at

6–13°. As the interference due to sialic acid is much less at the lower temperatures, the results obtained at 6–13° are more likely to estimate the true DNA content.

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