# A Study of the Conditions and Mechanism of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid

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(*Received* 29 *June* 1955)

Of the colour reactions available for the determination and identification of deoxyribonucleic acid (DNA), the reaction with diphenylamine in a mixture of acetic and sulphuric acids at 100° (Dische, 1930) has been perhaps the most widely used. The present study arose from the observation that a more intense colour was sometimes produced if, instead of being heated at 100° for 10 min., the reaction mixture was allowed to stand overnight at room temperature. As a result of this observation the procedure has been modified, principally by adding acetaldehyde to the reagents and by allowing the solution to stand for about 17 hr. at 30° instead of heating it at 100°. The modified method is 3·5 times as sensitive as Dische's original procedure, and several substances which interfere in the original method do not do so in the modified procedure.

Some observations on the mechanism of the reaction have been made; in particular it was discovered that there is a liberation of inorganic orthophosphate from DNA during the early stages of the reaction. This finding has a bearing on the structure of DNA.

The modified method has already been used in an investigation of nucleic acid metabolism during bacteriophage multiplication (Burton, 1955).

## MATERIALS AND METHODS

*Deoxyribonucleic acid (DNA).* Two samples of high-molecular-weight calf-thymus DNA were used. Sample I was kindly given by Dr F. W. Putnam and had been prepared according to Hammarsten (1924). Sample II was kindly given by Dr P. W. Kent and had been prepared according to Kay, Simmons & Dounce (1952). In addition, a commercial sample of lower molecular weight (British Drug Houses Ltd.) was also used.

DNA was isolated from bacteriophage T2r which had been obtained by confluent lysis as previously described, and purified by three cycles of differential centrifuging (Burton, 1955). A portion (20 ml.) of a preparation containing $10^{11}$ infective bacteriophage/ml. was shaken with 5 ml. of $CHCl_3$ for 1 hr. and centrifuged, and the clear supernatant poured into 2 vol. of ethanol. After 2 hr. at −10°, the fibrous DNA was removed by a glass rod and washed with 80% (v/v) ethanol on a sintered-glass filter. The material was resuspended in 5 ml. of water and dialysed overnight against distilled water. It was re-precipitated with ethanol as before and dissolved in 5 ml. of water.

DNA was isolated from *Escherichia coli* B grown in 6 l. of mannitol medium (Burton, 1955) for 48 hr. with aeration until a thick suspension was obtained (about 1 mg. dry wt./ml.). The bacteria were harvested by centrifuging, washed with 1·5 l. of water, and suspended in 400 ml. of M-NaCl containing 0·7% of sodium dodecyl sulphate at pH 7. The suspension was agitated in the Waring Blendor for 5 min. and centrifuged. The precipitate, which contained most of the bacterial nucleic acid, was suspended in 100 ml. of M-NaCl and shaken vigorously with 100 ml. of $CHCl_3$. The layers were allowed to separate and the aqueous layer was then shaken with a further 100 ml. of $CHCl_3$. After separation, the aqueous layer was centrifuged. The slightly opalescent supernatant was poured into 200 ml. of 96% (v/v) ethanol and allowed to stand overnight at −10°. The fibrous precipitate was collected by a glass rod, washed on a sintered-glass filter with 80% (v/v) ethanol and dissolved in 11 ml. of water. At this stage the material gave an orcinol reaction (Ogur & Rosen, 1950) equivalent to 1·7 $\mu$moles of ribose/ml., and in the diphenylamine reaction it gave a reaction equivalent to 5·8 $\mu$g. atoms of DNA-P/ml. The DNA was purified by adsorption of the ribonucleic acid (RNA) on charcoal. Norit (Harrington Bros. Ltd.) was found to be suitable and the procedure of Zamenhof & Chargaff (1951) was followed. The recovery of DNA in this step was 90%. It was precipitated with 2 vol. of ethanol as above, collected and dissolved in 12 ml. of water. This solution was centrifuged in order to remove most of the contaminating charcoal, and stored in the frozen state at −10°.

All the DNA samples were essentially free of RNA, as indicated by the orcinol reaction. The samples reacted as if they contained 0·053–0·085 mole of ribose/g. atom of DNA-P. These values are somewhat less than those recorded by previous workers (Schneider, 1945; Mayers & Spizizen, 1954). This apparent ribose content is due partly to the unspecificity of the orcinol reaction, since deoxyribose gave a reaction equivalent to 0·094 mole of ribose/mole of deoxyribose.

The calf-thymus DNA samples I and II contained no inorganic phosphate, but both the *Esch. coli* and T2 DNA samples contained 7% of their P as inorganic phosphate.

*Standard DNA solutions.* The highly polymerized calf-thymus DNA preparations were used. A stock solution was prepared by dissolving DNA at about 0·4 mg./ml. in 5 mM-NaOH. From this, working standards were prepared every 3 weeks by mixing a measured volume of the stock standard with an equal volume of N-$HClO_4$ and heating at 70° for 15 min. Both standards were stored in the refrigerator; no deterioration of the stock standard was detected after 6 months.

Deoxyribose, if available, is also a convenient standard. Aqueous solutions (1 mM) were stable for 3 months in the refrigerator, but after 8 months at room temperature they gave only 80–90% of the colour given by fresh solutions.

*Other chemicals.* 2-Deoxy-L-ribose was kindly given by Dr P. W. Kent. Acetaldehyde was redistilled and stored at 4° as an aqueous solution containing 16 mg./ml. Butyraldehyde was freshly distilled. Formaldehyde was freshly distilled into a suitable volume of water and the concentration of the resulting solution determined iodometrically. Aldol (β-hydroxybutyraldehyde) was prepared according to Claisen (1899) and distilled *in vacuo* at 60° shortly before use. DL-Glyceraldehyde was a commercial sample.

*Diphenylamine reagent.* This is prepared by dissolving 1·5 g. of steam-distilled diphenylamine in 100 ml. of redistilled acetic acid and adding 1·5 ml. of conc. $H_2SO_4$. The reagent is stored in the dark. On the day it is to be used, 0·10 ml. of aqueous acetaldehyde (16 mg./ml.) is added for each 20 ml. of reagent required. Many batches of A.R. acetic acid were found to be suitable for preparing the reagent without prior distillation, but with some samples of acetic acid the reagent developed a blue colour on standing.

*Estimation of phosphorus.* Inorganic orthophosphate was determined by the method of Berenblum & Chain (1938), with the slight modifications described by Bartley (1953). Total phosphorus was determined by the same method after digestion with $H_2SO_4$ and $HNO_3$ (Fiske & Subbarow, 1925).

*Spectrophotometry.* A Unicam SP. 600 spectrophotometer with 1 cm. cells was used.

## RESULTS

### *Method of colour development finally adopted*

In estimating nucleic acids from biological materials a suitable extract in 0·5N-$HClO_4$ should be obtained by a method similar to that described for bacteria below. The details of preparation may have to be modified for different sources.

The nucleic acid extract is diluted with 0·5N-$HClO_4$ so that the final solution contains between 0·02 and 0·25 μg. atom of DNA-P/ml., and a measured volume (1 or 2 ml. as convenient) is mixed with 2 vol. of diphenylamine reagent containing acetaldehyde. Tubes containing known amounts of standard DNA and a blank containing 0·5N-$HClO_4$ but no DNA are also prepared. The colour is developed by incubating at 30° for 16–20 hr. Provided that all tubes are at the same temperature, constancy of the temperature is not essential, and variations, at least between 25° and 35°, do not appreciably affect the colour development. In the present work a constant-temperature bath at 30° was used. The optical density at 600 mμ. is measured against the blank and compared with the values obtained with the standard DNA.

### *Factors influencing the colour development*

*Aldehydes.* When the reagent prepared with redistilled acetic acid was used, the optical density in the absence of added acetaldehyde was only

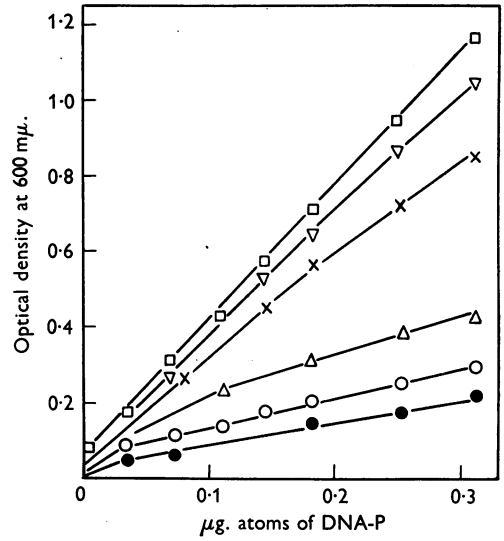


Fig. 1. Effect of acetaldehyde on the optical density obtained with different amounts of DNA. Diphenylamine reagent (2 ml.) added to 1 ml. of calf-thymus DNA (sample I dissolved in 0·5N-$HClO_4$ by heating at 70° for 15 min.). Final concentrations of acetaldehyde (mM): ●, None; ○, 0·12; △, 0·24; ×, 0·61; ▽, 1·21; □, 1·81.

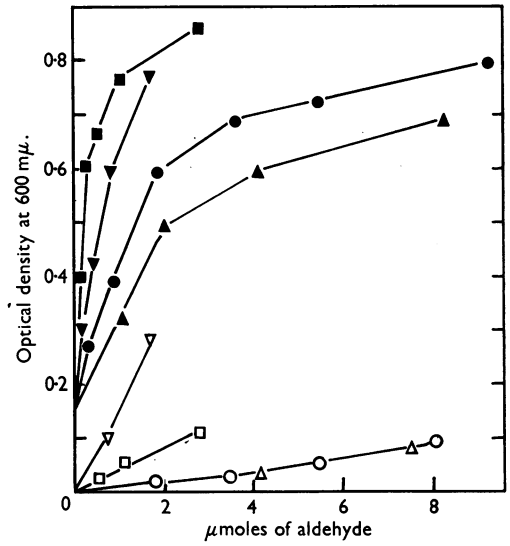


Fig. 2. Comparison of different aldehydes. Diphenylamine reagent (2 ml.) containing the aldehyde added to 1 ml. of calf-thymus DNA (sample II; 0·20 μg. atom of DNA-P) which had been dissolved in 0·5N-$HClO_4$ by heating at 70° for 15 min. Closed symbols with DNA, open symbols no DNA: ● ○, acetaldehyde; ▼ ▽, β-hydroxybutyraldehyde; ▲ △, *n*-butyraldehyde; ■ □, glyceraldehyde.

0·15–0·3 of that obtained with the addition of acetaldehyde to the reagent. In the absence of the aldehyde the optical density for a given amount of DNA decreased with increasing DNA concentration, whereas in the presence of sufficient aldehyde the optical density is very nearly proportional to the DNA concentration. The effects of different amounts of acetaldehyde at different DNA concentrations are shown in Fig. 1. Of six aldehydes examined for their ability to replace acetaldehyde, chloral and formaldehyde had no effect. As shown in Fig. 2, DL-glyceraldehyde and DL-β-hydroxybutyraldehyde (aldol) were effective at lower concentrations than acetaldehyde, but aldol gave an appreciable colour in the absence of DNA. Higher concentrations of *n*-butyraldehyde were required. At concentrations which markedly increased the colour development from DNA, acetaldehyde and glyceraldehyde gave little colour in the absence of DNA. No effect of acetaldehyde was detected in the reaction at 100° under the conditions described by Dische (1930).

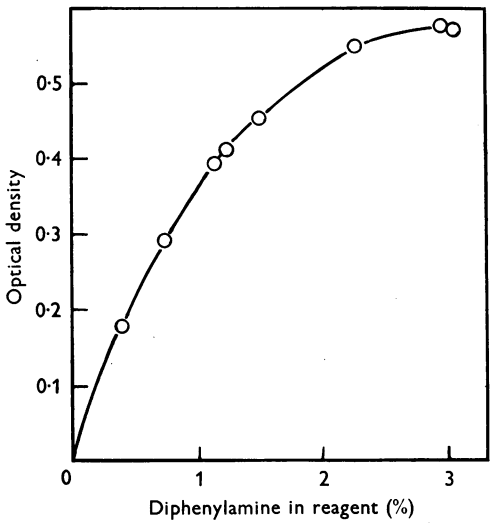


Fig. 3. Effect of diphenylamine concentration. Reagent (4 ml.) added to 2 ml. of calf-thymus DNA (sample II; 0·33 μg. atom of DNA-P) previously dissolved in 0·5N-$HClO_4$ by heating at 70° for 15 min. The concentration of diphenylamine was varied but the amounts of $H_2SO_4$ (1·5%, v/v) and acetaldehyde (0·08 mg./ml.) in the reagent were kept constant.

*Diphenylamine concentration.* The colour intensity increased with increasing diphenylamine concentration in the range used, which was up to a concentration of 3% (w/v) in the reagent (Fig. 3). At higher concentrations diphenylamine sulphate crystallized out of the reagent. A reagent containing 3% (w/v) diphenylamine may be of value if greater sensitivity is required, but otherwise a concentration of 1·5% (w/v) is preferable, since it gives less colour in the absence of DNA.

*Perchloric acid.* The effect of different concentrations of the acid was investigated by using 0·25 μg. atom of DNA-P in 0·20 ml. of 0·5N-$HClO_4$, together with 1·8 ml. of water or aqueous $HClO_4$ and 4·0 ml. of the diphenylamine reagent. The optimum concentrations of $HClO_4$ before the addition of the reagent were found to be between 0·25 and 0·65N, and there was no detectable effect of varying the acid concentration within this range. At 0·05N and at 1N the colour was 92% of the maximum, but at 2N the colour was only 70% of the maximum. The colour given by aqueous solutions of apurinic acid (Tamm, Hodes & Chargaff, 1952) or of deoxyribose in the absence of $HClO_4$ was only about 70% of that obtained with the optimum amount of $HClO_4$.

*Trichloroacetic acid.* This acid is likely to be present in samples taken for estimation, because in Schneider's (1945) procedure nucleic acids may be extracted by 5% trichloroacetic acid at 90°. However, if $HClO_4$ is added so as to give a concentration of 0·5N before the addition of the diphenylamine reagent, trichloroacetic acid does not affect the final optical density, even when present at concentrations as high as 10%.

*Specificity and interference.* Several substances were examined for the amount of colour given with the reagent under the conditions of the DNA estimation. A solution or suspension of each substance at 4 mg./ml. in 0·5N-$HClO_4$ was heated at 70° for 15 min. If, as with materials containing protein, a clear solution was not obtained the solution was centrifuged and the supernatant used. To 0·5 ml. of the solutions thus obtained, 0·5 ml. of 0·5N-$HClO_4$ and 2 ml. of the reagent were added. After developing the colour in the usual way 2 mg. each of the following substances gave no detectable colour: sucrose, glucosamine, rhamnose, soluble starch, alginic acid, lactose, glucose, inositol, ascorbic acid, bovine plasma albumin, glutathione, cysteine hydrochloride, tryptophan, glycine, histidine hydrochloride, potassium gluconate, adenine sulphate, uric acid, adenosine-5′ phosphate, creatine hydrate and chloral hydrate. Under these conditions, the colour given by 4·5 μμg. atoms of DNA-P (i.e. 1·4 μg. of pure DNA) was readily detectable. The following substances, in 2 mg. amounts, gave a detectable colour which was less than that given by 1·4 μg. of DNA: pyruvic acid, tannic acid, fructose, indole, ribose, arabinose, xylose, α-oxoglutaric acid, acid-hydrolysed casein (Difco casamino acids), uracil. Dried human blood, glycogen and a commercial preparation of bile salts (used as an emulsion) gave readings equivalent to 6 μμg. atoms of DNA-P. Commercial

ribonucleic acid (5 mg.) gave a reading equivalent to 35 $\mu\mu$g. atoms of DNA-P (i.e. 11 $\mu$g. of pure DNA). Agar (2 mg.) gave a reading equivalent to 58 $\mu\mu$g. atoms of DNA-P (i.e. 18 $\mu$g. of pure DNA).

Many of the substances were also tested for their effect on the reading obtained with a standard amount of DNA. Each tube contained the substance tested, together with 0·165 $\mu$g. atom of DNA-P in 1 ml. of 0·5N-$HClO_4$ and 2 ml. of reagent. The optical density was compared with that of a blank tube containing the substance tested, but no DNA. The effect of 2 mg. of arabinose, fructose, xylose, alginic acid, adenine sulphate, uracil, glycine, histidine, chloral hydrate, α-oxoglutaric acid or manganese pyruvate was less than 2%. The presence of 2 mg. of the following substances reduced the reading: potassium gluconate (4%), acid-hydrolysed casein (10%), tryptophan (12%), ethylenediamine (9%) and diethylenetriamine (23%). Sodium chloride (20 mg.) reduced the colour by 5%. The materials found to have greatest effects were ascorbic acid, cysteine and triethylenetetramine; 0·4 mg. of ascorbic acid reduced the colour by 76%, but 0·02 mg. reduced the colour by less than 2%; 2 mg. of cysteine hydrochloride reduced the colour by 69%; 2 mg. of triethylenetetramine reduced the colour by 54%.

Comparison of these findings with those for the reaction at 100° (Dische, 1930; Cohen, 1944; Ahlström, Euler, Fischer, Halm & Högberg, 1946; Overend, 1951) shows that the modified procedure is both more specific and less susceptible to interference by the presence of other compounds.

It will be seen from Fig. 2 that both glyceraldehyde and aldol (β-hydroxybutyraldehyde) give some colour with the reagent in the absence of DNA and acetaldehyde. The colours given by these two hydroxyaldehydes are increased by the presence of acetaldehyde, but even so are much less than those obtained with equimolar amounts of deoxyribose. In the presence of acetaldehyde, 0·45 $\mu$mole of glyceraldehyde gave the same colour as 0·021 $\mu$g. atom of DNA-P, and 1·6 $\mu$mole of aldol gave the same colour as 0·13 $\mu$g. atom of DNA-P. The colour from aldol is blue and that from glyceraldehyde is yellow or, at higher concentrations, yellow-green.

*Rate of colour development.* It was observed that, in the early stages, the reaction mixture first appeared purple and later became blue. As will be seen from Fig. 4, the maximum of the absorption spectrum was at 560 m$\mu$. after 1 hr. and changed to 600 m$\mu$. after 7 hr. These changes occurred whether the reaction was performed with DNA or with deoxyribose but, as will be seen from Fig. 5, in the first 3 hr. the colour developed more rapidly with the DNA standard solution than with deoxyribose. The absorption spectra of the reaction mixtures were identical for both substances after 10 hr.

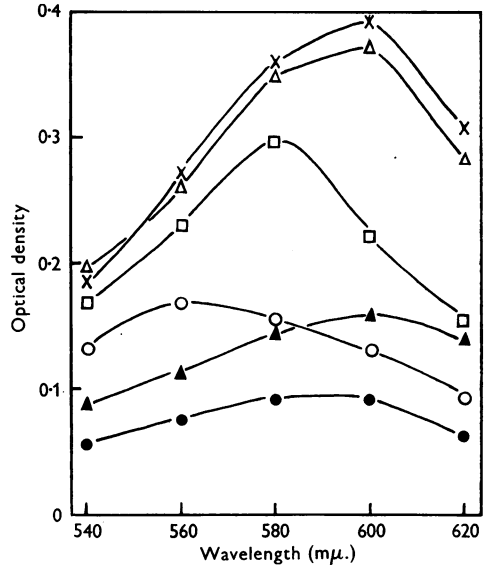


Fig. 4. Absorption spectra after different periods of colour development at 30°. Diphenylamine reagent (2 vol.) added to calf-thymus DNA (commercial material; 0·112 mg. atom of organic P/l. dissolved in 0·5N-$HClO_4$ by heating at 70° for 15 min.). Time of reaction at 30°: (*a*) with acetaldehyde (0·08 mg./ml.) added to the reagent: ○, 1 hr.; □, 2·2 hr.; △, 7 hr.; ×, 24 hr.; (*b*) no acetaldehyde: ●, 10 hr.; ▲, 24 hr.

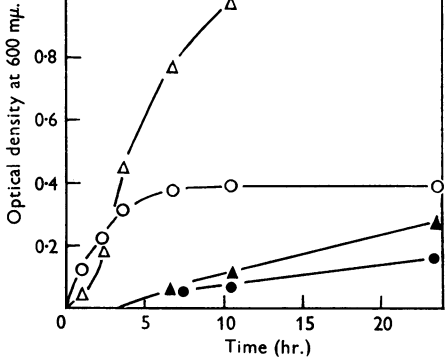


Fig. 5. Comparison of the rates of colour development with DNA and with 2-deoxy-L-ribose. DNA as for Fig. 4: ○, with acetaldehyde; ●, no acetaldehyde. Deoxyribose (0·18 mM in 0·5N-$HClO_4$) mixed with 2 vol. of reagent: △, with acetaldehyde; ▲, no acetaldehyde.

*Acid treatment of the DNA.* In the experiments described above the DNA was heated for 15 min. at 70° in 0·5 N-$HClO_4$ before use. This was done so that the behaviour of the DNA would be similar to that of the $HClO_4$ extracts obtained from bacteria (see below). This treatment of the DNA increased the rate of colour development (Fig. 6), but caused a small drop in the final optical density, which amounted to 7·5% after heating for 30 min. and 14% after heating for 60 min. Heating with 5% trichloroacetic acid at 90° for the same times had similar but slightly less effects on the optical densities obtained.

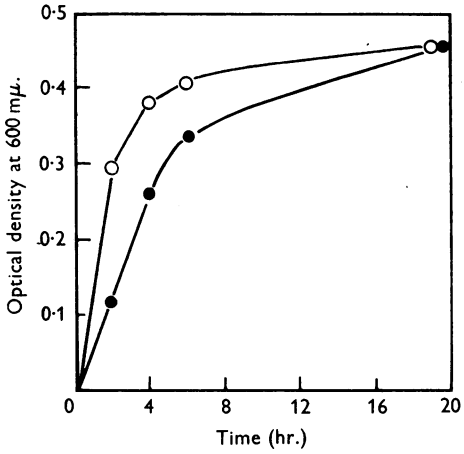


Fig. 6. Pretreatment of DNA with dilute $HClO_4$ at 70° and its effect on the rate of colour development. Reagent (2 ml.) added to 1 ml. of calf-thymus DNA (sample I, 0·135 μg. atom of DNA-P) in 0·5 N-$HClO_4$: ○, DNA heated at 70° for 15 min. in 0·5 N-$HClO_4$ before adding the reagent; ●, DNA not heated with $HClO_4$. (Although the DNA did not dissolve in the $HClO_4$ without heating, it dissolved when the reagent was added.)

*Comparison of deoxyribose and DNA*

The optical densities obtained with the DNA samples from three sources (calf thymus, *Esch. coli* and bacteriophage T2) were compared with those obtained from a standard solution of deoxyribose over a range of concentrations between 0·03 and 0·1 mM deoxyribose or 0·06 and 0·2 mg. atom of DNA-P/l. in the solution before addition of the reagent. For the four DNA samples (calf-thymus samples I and II, *Esch. coli*, and bacteriophage T2) the ratios of moles of apparent deoxyribose to g. atoms of organic phosphorus were respectively 0·475, 0·475, 0·485 and 0·460. For each of the DNA samples these ratios were constant within ± 1·5% over the range of concentrations studied.

*Liberation of inorganic phosphate*

To obtain further information about the reaction between DNA and diphenylamine, the amount of inorganic orthophosphate which appeared in the reaction mixture was measured. It was found that approximately 12% of the DNA-P appeared as inorganic phosphate in 20 min. at 30° and after 150 min. between 24 and 25% of the DNA-P was liberated. This liberation of phosphate required the presence of diphenylamine but was independent of the addition of acetaldehyde. It also occurred when the acetic and sulphuric acid mixture was replaced by formic acid, but not if the mixture of acids was replaced by methanol.

The amounts of inorganic phosphate liberated from calf-thymus DNA in the presence of diphenylamine following different periods of heating in trichloroacetic acid at 90° are shown in Table 1. The table also shows the small amounts of phosphate found under similar conditions in the absence of diphenylamine. It will be seen that the amount of phosphate released in the presence of diphenylamine is not appreciably influenced by the time of the previous treatment with trichloroacetic acid.

Table 1. *Liberation of inorganic phosphate from deoxyribonucleic acid*

Calf-thymus DNA (sample II; 20·4 μg. atoms of P) was suspended in 20 ml. of water and dissolved with the aid of 12 μmoles of NaOH. Samples were heated at 90° with 0·1 vol. of 55% (w/v) trichloroacetic acid as indicated, cooled and mixed with 2 vol. of 1·5% diphenylamine dissolved in (*a*) formic acid, or (*b*) acetic acid containing 1·5% (v/v) of $H_2SO_4$. Control mixtures were also prepared not containing diphenylamine. Inorganic phosphate was determined after incubating at 30° for 17 hr.

| Time of pretreatment at 90° (min.) | Inorganic phosphate liberated (% of total DNA-P) | | | |
|---|---|---|---|---|
| | Formic acid | | Acetic acid + $H_2SO_4$ | |
| | Diphenylamine | No diphenylamine | Diphenylamine | No diphenylamine |
| 0 | 24·5 | — | 26 | — |
| 4 | 26·4 | 0·7 | 27·3 | 1·4 |
| 6·5 | — | 1·0 | 28·2 | 1·5 |
| 15 | 27·0 | 3·4 | 26·5 | 2·1 |
| 40 | 31·0 | 4·5 | 30 | 4·3 |

The method of phosphate estimation used in these experiments (Berenblum & Chain, 1938) was not interfered with by the presence of 1 ml. of the diphenylamine reagent nor by 1 ml. of 1·5% diphenylamine in formic acid. The method is based on the blue colour formed by the reduction of phosphomolybdate which has been extracted from acid solution into *iso*butanol. Although the blue colour from the diphenylamine–DNA reaction is also extracted into the *iso*butanol, it does not interfere in the phosphate estimation since it does not absorb appreciably at 750 mμ., which is the wavelength of the light which was used for measuring the optical density of the phosphomolybdate blue colour.

DNA from *Esch. coli* behaves similarly to that from calf thymus. In the presence of the diphenylamine reagent 25·5% of the organic phosphorus appeared as inorganic phosphate; only 1·1% was liberated by the acetic and sulphuric acid mixture alone.

After 17 hr. at 30° in 70% (v/v) formic acid, both fructose 1:6-diphosphate and ribonucleic acid yielded about 10% of their phosphorus as inorganic phosphate. This was not affected by the presence of 1% of diphenylamine. Under similar conditions no appreciable amount of phosphate was liberated from 3-phosphoglyceraldehyde or from glucose 6-phosphate, whether diphenylamine was present or not. It was also found that 1% aniline in 70% (v/v) formic acid can cause the liberation of phosphate from DNA, but at a slower rate than with diphenylamine.

*Extraction of DNA from* Escherichia coli

The diphenylamine reaction, as described in this paper, has been used to estimate DNA in *Esch. coli* (Burton, 1955). On preliminary examination, the extraction procedure which was used in this work appeared to extract 95% of the DNA. The extraction has been reinvestigated more thoroughly and now appears to extract only 90–93%, but such a difference does not affect the conclusions obtained in the previous work. By increasing the time of extraction with 0·5N-$HClO_4$ from 10 to 15 min. it is possible to increase the efficiency of extraction to 95–96%.

The procedure is based on those of Schneider (1945) and of Ogur & Rosen (1950). Each sample of the culture should contain at least 0·15 μg. atom of DNA-P with not less than 0·01 μg. atom of DNA-P/ml. of culture. Each sample is acidified with 2·5N- or 12N-$HClO_4$ to a final concentration of 0·25N and centrifuged after chilling for 30 min. The precipitate is broken up with a glass rod and stirred with 0·5 ml. 0·5N-$HClO_4$. A further 3·5 ml. of 0·5N-$HClO_4$ is added and the suspension is heated at 70° for 15 min. with occasional stirring. After centrifuging, the supernatant is decanted into a 10 ml. graduated tube. The precipitate is

Table 2. *Extraction of deoxyribonucleic acid from bacteria*

$HClO_4$ (final concentration 0·25N) was added to measured volumes of *Esch. coli* B cultures grown in a mannitol–ammonium salts medium (Burton, 1955). After cooling at 0° for 30 min. they were centrifuged and the precipitate was extracted with acid. The first two extractions (4 and 3 ml. respectively) were as indicated. The third extraction was with 1·5 ml. of 0·5N-$HClO_4$ for 15 min. at 70°. DNA was estimated on the extracts and the residue.

| First 2 extractions | | | DNA (μμg. atom of P) | | | | | DNA in first two extracts |
|---|---|---|---|---|---|---|---|---|
| Conditions | Time (min.) | Vol. of culture (ml.) | 1st extract | 2nd extract | 3rd extract | Residue | Total DNA/ml. of culture | (% of total DNA) |
| Expt. I | | | | | | | | |
| 0·5N-$HClO_4$ at 70° | 10 | 10 | 418 | 185 | 43 | 22 | 66·8 | 90 |
| | 20 | 10 | 580 | 56 | 11 | 14 | 66·1 | 96 |
| | 30 | 10 | 565 | 54 | 11 | 14 | 64·4 | 96 |
| N-$HClO_4$ at 70° | 10 | 10 | 282 | 217 | 59 | 19 | 57·7 | 86 |
| | 20 | 10 | 299 | 185 | 56 | 19 | 56·1 | 86 |
| 5% Trichloroacetic acid at 90° | 5 | 10 | 425 | 146 | 34 | 25 | 63·0 | 91 |
| | 10 | 10 | 533 | 68 | 17 | 18 | 63·6 | 95 |
| Expt. II | | | | | | | | |
| 0·5N-$HClO_4$ at 70° | 10 | 10 | 584 | 608 | 95 | 32 | 132 | 91 |
| | | 6 | 372 | 354 | 52 | 22 | 133 | 91 |
| | | 4 | 275 | 214 | 36 | 17 | 135 | 90 |
| | | 3 | 216 | 162 | 21 | 13 | 137 | 92 |
| | 15 | 10 | 925 | 327 | 39 | 18 | 131 | 95 |
| | | 6 | 587 | 192 | 27 | 12 | 136 | 95 |
| | | 4 | 401 | 126 | 16 | 12 | 138 | 95 |
| | | 3 | 305 | 92 | 10 | 8 | 139 | 95 |

re-extracted in the same way with a further 3 ml. of 0·5N-$HClO_4$ the two extracts are mixed and the volume is measured. A portion (1 or 2 ml., as convenient) of the extract is used for the diphenylamine reaction.

Table 2 shows the effects of some different conditions of extraction. After the extractions, as indicated, the DNA remaining in the residue was determined by suspending in a total volume of 10 ml. of 0·5N-$HClO_4$ and adding 2·0 ml. of the reagent. The material which did not dissolve remained white, although the supernatant developed the blue colour. This insoluble material was centrifuged off after the colour development and before measuring the optical density of the supernatant. It will be seen that extraction with 0·5N-$HClO_4$ was more efficient than extraction with N-$HClO_4$. Extraction with 5% trichloroacetic acid at 90° was virtually as effective as the extraction with 0·5N-$HClO_4$. The efficiency of extraction is not dependent on the amount of bacteria. The results of Table 2 also show that the amount of culture needed may be reduced by using a single extraction with 0·5N-$HClO_4$ for 20–25 min. at 70°, since under these conditions a more concentrated extract is obtained. When the DNA content of the culture is calculated from the total volume of precipitate plus extract, the value obtained is 94–95% of the total.

The precipitation of the DNA from the culture, together with the bacterial protein, appears to be complete if the culture contains 0·01–0·03 $\mu$g. atom of DNA-P/ml., since no DNA was detectable in the supernatants and also since after threefold dilution of a culture which contained 0·03 $\mu$g. atom of DNA-P/ml. the same amount of DNA was found in the precipitate as without dilution. However, after tenfold dilution of the same culture only 80% of the DNA was recovered in the precipitate. This loss was mainly the physical loss of small particles of the precipitate into the supernatant; it was prevented by adding bovine serum albumin (0·3–0·5 mg./ml. of culture) which gives a greater bulk to the precipitate but does not affect the extraction of the DNA from the precipitate. Alternatively, in estimating DNA in dilute cultures, the bacterial cells may be centrifuged before treatment with $HClO_4$.

## DISCUSSION

It has already been pointed out in the previous sections that the modified procedure is both more sensitive and specific and less susceptible to interference than is the original diphenylamine reaction at 100°. The sensitivity is similar to that of the most sensitive colour reactions of DNA which have been studied. These reactions are (*a*) with indole and hydrochloric acid (Ceriotti, 1952), (*b*) with cysteine and sulphuric acid (Dische, 1930; Stumpf, 1947; Brody, 1953), (*c*) the Feulgen reaction (e.g. Dondero, Adler & Zelle, 1954) and (*d*) a reaction with *p*-nitrophenylhydrazine (Webb & Levy, 1955).

The indole and Feulgen reactions are less specific than the cysteine, the nitrophenylhydrazine or the modified diphenylamine reactions. These last three reactions do not give any appreciable colour with ribonucleic acid or non-deoxy sugars. However, the specificity and the possible interference by other compounds do not appear to have been studied as thoroughly for the reactions (*b*) and (*d*) as for the modified diphenylamine reaction. Of these three reactions the diphenylamine reaction is the most convenient to use as the cysteine–sulphuric acid reaction requires solutions of sulphuric acid so concentrated that, because of their viscosity, they are not easily pipetted with accuracy, while the *p*-nitrophenylhydrazine method involves an extraction with *n*-butyl acetate. The diphenylamine reaction may therefore be more readily adaptable for ultramicroanalysis.

### *Mechanism of the reaction*

No colour was detected with 0·5 $\mu$mole of thymidylic acid, which is more easily hydrolysed than cytidylic acid (Manson & Lampen, 1951; Dische, 1955). In contrast to the stability of the links between pyrimidines and deoxyribose, those between purines and deoxyribose are very labile, so it appears that diphenylamine reacts with the sugar residues originally combined with purines in the DNA. The ratios of apparent deoxyribose to total phosphorus are in agreement with this mechanism and with the fact that DNA's of different origin all appear to have a purine:pyrimidine ratio very close to unity (Chargaff, 1955).

It follows from this that the inorganic phosphate liberated from DNA in the early stages of the diphenylamine reaction very probably arises from the phosphate bridges between two adjacent purine nucleotides in the polynucleotide chains. If this is so, the liberation of 25% of the total phosphorus in the DNA would be in agreement with the view that there is a random distribution of purine and pyrimidine residues along the polynucleotide chains. As discussed by Brown & Todd (1955) and by Chargaff (1955), there is at present scanty evidence about the sequence of the nucleotides in DNA, but this reaction may prove to be a useful tool when the other products of the reaction and the action of the acid alone have been studied.

The mechanism of this phosphate elimination is unknown; no similar amine-catalysed reaction has been described. However, deoxyribose 5-phosphate (Racker, 1952) and the phosphates of $\beta$-aldehydo alcohols (Meyerhof & Lohmann, 1934; Brown & Todd, 1955) are labile to acid, and possibly the

condensation of the aldehyde group with a suitable amine increases this lability.

The fact that the development of the colour is slower with deoxyribose than with acid-treated DNA indicates that free deoxyribose is not an intermediate in the diphenylamine reaction. The effect of diphenylamine on the phosphate liberation shows that the amine combines with the DNA before the phosphate is released from the sugar. Neither of these findings agrees with the views of Deriaz, Stacey, Teece & Wiggins (1949) and Overend, Shafizadeh & Stacey (1950), who consider that, in the reaction at 100°, ω-hydroxylaevulaldehyde is formed by the action of the acid on the DNA or deoxyribose and subsequently condenses with the diphenylamine. It is, of course, conceivable that condensation products (e.g. Schiff's bases) between the amine and deoxyribose or ω-hydroxylaevulaldehyde are the actual intermediates. However, for the deoxyribose derivative to be an intermediate in the reaction of DNA and diphenylamine, it would have to be formed more slowly from deoxyribose itself than from the deoxyribose residues in DNA which has been treated with 0·5N-perchloric acid for 15 min. at 70°.

The action of acetaldehyde and some other aldehydes in potentiating the colour development is obscure, but it might conceivably involve a reaction analogous to that of Doebner (1894), in which two aldehyde molecules and an aromatic amine react, with ring closure. Deoxyribose can take part in this type of reaction (Pesez, 1950), but chloral and formaldehyde do not (Velluz, Pesez & Amiard, 1948). These two aldehydes have no effect in the diphenylamine reaction.

The nature of the coloured compounds formed in the diphenylamine reaction is unknown. The changes of the absorption spectrum in the reaction at 30° show that at least two compounds are formed; these may be identical with two of the six compounds detected by Deriaz *et al.* (1949) in the reaction at 100°.

## SUMMARY

1. The colour reaction of Dische (1930) between diphenylamine and deoxyribonucleic acid (DNA) has been studied and modified. The principal modifications are to add acetaldehyde and to perform the reaction for several hours at 30° instead of for 3–10 min. at 100°. The modified reaction is more sensitive and specific and less susceptible to interference by other compounds than is the reaction at 100°.

2. The colour reactions of DNA preparations from calf thymus, *Escherichia coli* and bacteriophage T2 have been compared with that of 2-deoxyribose. For the three sources the ratios of moles of apparent deoxyribose to gram atoms of DNA phosphorus were almost identical and between 0·46 and 0·485.

3. In the early stages of the diphenylamine reaction there is a liberation of inorganic orthophosphate from DNA. The amount of phosphorus thus liberated from calf-thymus DNA and from *Esch. coli* DNA is approximately 25% of the total DNA phosphorus and probably arises from the phosphate bridges between adjacent purine nucleotides in the polynucleotide chain. This value is consistent with the view that the purine and pyrimidine nucleotides are randomly distributed along the chain.

4. The conditions for the quantitative extraction of DNA from bacteria have been studied.

5. Evidence on the mechanism of the DNA–diphenylamine colour reaction is discussed and taken to indicate that neither free deoxyribose nor free ω-hydroxylaevulaldehyde are intermediates.

I wish to thank Professor H. A. Krebs, F.R.S., for his interest and encouragement, Dr P. W. Kent for much helpful advice and discussion, and Miss E. Johnson for her technical assistance.

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# The Reduction of Vinylacetate by *Clostridium kluyveri* and its Dependence on Catalytic Amounts of High-energy Acetate

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(*Received* 14 *July* 1955)

Growing cultures of *Clostridium kluyveri* ferment ethanol plus acetate, yielding mainly butyrate, hexanoate and hydrogen (Bornstein & Barker, 1948). Stadtman & Barker (1949*a–e*) and Kennedy & Barker (1951*a*) have investigated the mechanism of butyrate synthesis by this organism, using dried cells and cell-free extracts which catalyse reactions 1 and 2:

$$CH_3.CO_2H + CH_3.CO_2PO_3H_2 + 2H_2 \rightarrow CH_3.CH_2.CH_2.CO_2H + H_3PO_4 + H_2O, \quad (1)$$

$$CH_3.CH_2.CH_2.CO_2H + H_3PO_4 + O_2 \rightarrow CH_3.CO_2H + CH_3.CO_2PO_3H_2 + H_2O. \quad (2)$$

During an examination of possible intermediates, the reduction of vinylacetate (3:4-butenoate) according to reaction 3 was observed (Stadtman & Barker, 1949*e*).

$$CH_2{:}CH.CH_2.CO_2H + H_2 \rightarrow CH_3.CH_2.CH_2.CO_2H. \quad (3)$$

Several other $C_4$ compounds at the same oxidation level as vinylacetate were examined, including crotonate (*trans*-2:3-butenoate), *iso*crotonate (*cis*-2:3-butenoate) and 3-hydroxybutyrate; all were excluded as intermediates in reactions 1 and 2. Crotonate and *iso*crotonate were neither oxidized nor reduced by the preparations. No direct test was carried out with 3-hydroxybutyrate, but the preparations reduced acetoacetate, giving a quantitative yield of 3-hydroxybutyrate, indicating that the latter is not reduced. Vinylacetate was the only compound of those tested which was oxidized and reduced at rates compatible with the role of intermediate. In the presence of a nitrogen gas phase, vinylacetate was shown to undergo two other reactions—an anaerobic oxidation with the evolution of $H_2$ (reaction 4) and a dismutation (reaction 5).

$$CH_2{:}CH.CH_2.CO_2H + H_3PO_4 + H_2O \rightarrow CH_3.CO_2H + CH_3.CO_2PO_3H_2 + H_2, \quad (4)$$

$$2CH_2{:}CH.CH_2.CO_2H + H_3PO_4 + H_2O \rightarrow CH_3.CH_2.CH_2.CO_2H + CH_3.CO_2H + CH_3.CO_2PO_3H_2. \quad (5)$$

However, isotope experiments indicated that free vinylacetate was not a true intermediate in reactions 1 or 2. In the presence of unlabelled vinylacetate, [*carboxy*-$^{14}$C]butyrate gave rise to acetate with a higher specific activity than the vinylacetate recovered at the end of the experiment. Again, in the presence of unlabelled vinylacetate, [*carboxy*-$^{14}$C]acetate yielded labelled butyrate, but no isotope appeared in the residual vinylacetate.

Kennedy & Barker (1951*a*) later observed that dried cells could oxidize butyrate and vinylacetate in the absence of added phosphate, though at a reduced rate, and under these conditions acetoacetate was the main product. These observations led to the hypothesis that the $C_4$ compounds are oxidized and reduced in the form of complexes with a coenzyme, possibly coenzyme A (CoA) (cf. Fig. 2). This concept is supported by the observations reported in the present paper, which indicate that vinylacetate must be activated before it is reduced.

## MATERIALS AND METHODS

*Organism and suspensions of dried cells.* Strain K1 of *Cl. kluyveri*, which was isolated by Barker & Taha (1942) and used in the studies on fatty acid synthesis by Stadtman & Barker, was used throughout.

The dried cell preparation used (Lot YE9) was made by the method of Stadtman & Barker (1949*a*), with the following modifications: (1) a slight change was made in the

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