An Improved Diphenylamine Method for the Estimation of Deoxyribonucleic Acid

Many plant tissues contain very low levels of DNA and in such circumstances it is necessary to utilize a method of estimation of extreme sensitivity coupled with a low reagent blank. In this respect it is our experience that the Burton diphenylamine method[1] is the most satisfactory. This procedure has been simplified, the blank considerably reduced and the sensitivity increased.

The standard Burton method was investigated using calf-thymus DNA in 10 per cent perchloric acid as standard. The reagent consists of a 2 per cent diphenylamine and 1·5 per cent sulphuric acid solution in glacial acetic acid containing 0·08 mg/ml. of acetaldehyde (all 'AnalaR' grade). To 1 ml. test solution was added 2 ml. reagent, the mixture was incubated at 30° C overnight and the resulting blue colour read at 595 mμ on a spectrophotometer. It was noted that the reagent rapidly turned green when the acetaldehyde was added, so it was omitted from the reagent and added to the final mixture as 0·1 ml. of 1·6 mg/ml. It can be seen that the blank is reduced to a third (Table 1).

Table 1. Effect of adding Acetaldehyde last and of leaving out Sulphuric Acid on the Diphenylamine Reaction

	Optical density at 595 mμ	
	Blank/water	20 μg DNA/ml./blank
Standard Burton method	0·120	0·168
Acetaldehyde added last	0·044	0·177
As above and H_2SO_4 omitted	0·028	0·184

It was further shown that the addition of sulphuric acid to the reagent was unnecessary since the test solution is already in 10 per cent perchloric acid and on leaving it out the blank was still further improved (Table 1).

Turbidity is almost invariably developed on incubation of the reaction mixture, due to precipitation of impurities in the DNA extracts of plant tissues. This source of error was eliminated by measuring the 595–700 mμ optical density difference. This difference reading results in a zero blank and a straight line calibration. In order to obtain consistently good blanks it was found to be essential that the diphenylamine be of recent manufacture, and the reagent is best used on the day of preparation.

Finally, an increase in sensitivity of more than 70 per cent was obtained by increasing the concentration of diphenylamine from 2 to 4 per cent and the test to reagent volume from 1 : 2 to 2 : 2 (Table 2).

Table 2. DNA Calibration with Varying Diphenylamine (DPA) Concentrations and Reagent to Test Volumes

	1 ml. DNA + 2 ml. 2% DPA			2 ml. DNA + 2 ml. 4% DPA		
	595 mμ	700 mμ	595–700 mμ	595 mμ	700 mμ	595–700 mμ
Blank/water	0·024	0·028	−0·004	0·018	0·023	−0·005
5 μg/ml. DNA/blank	0·044	0·005	0·039	0·086	0·011	0·075
10 μg/ml. DNA/blank	0·092	0·009	0·083	0·158	0·010	0·148
20 μg/ml. DNA/blank	0·179	0·008	0·171	0·306	0·012	0·294
40 μg/ml. DNA/blank	0·356	0·021	0·335	0·606	0·026	0·580

The final procedure is therefore to add 2 ml. of 4 per cent diphenylamine in glacial acetic acid to 2 ml. of test solution of DNA in 10 per cent perchloric acid followed by 0·1 ml. of aqueous 1·6 mg/ml. acetaldehyde; after incubation at 30° C overnight the optical density difference at 595–700 mμ is read against the 595–700 blank.

The method has been successfully used to estimate DNA in hypocotyls of *Lupinus albus*[2], in pea internodes, in pollen and in a variety of micro-organisms including yeast and *Protomyces inundatus*.

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Possible Synthesis of Polyribonucleotides of Known Base-triplet Sequences

For the exact assignment of the genetic code for amino-acids, polyribonucleotides of known base triplet (or doublet) sequences are necessary.

Recently, Jacob *et al.*[1] have described the synthesis of deoxyoligonucleotides of known base sequence and their use as templates in the RNA-polymerase reaction. This communication indicates the possibilities of biosynthesis of polyribonucleotides with known base triplet sequences which may be useful in the assignment of code for a few amino-acids.

The DNA polymerase in the absence of a DNA primer catalyses the synthesis of a copolymer dAT from dATP and dTTP after a lag period[2]. These authors have shown that the adenine and thymine bases occur in an alternating sequence in this copolymer.

With dAT copolymer as the priming DNA, RNA polymerase catalyses the synthesis of the polyribonucleotide poly-AU in which adenine and uracil bases are shown to occur in an alternating sequence[3]. This polyribonucleotide poly-AU has a very stable secondary structure[4]. On inspection of the arrangement of bases in the poly-AU:

AUAUAUAUAUAU—U

the base triplets AUA and UAU appear in an alternating sequence. If this polyribonucleotide is treated with nitrous acid, a certain proportion of adenine bases will be deaminated to hypoxanthine[5,6] and there will be regions in the polyribonucleotide with HUH and UHU base triplets in an alternating sequence (H = hypoxanthine). As hypoxanthine behaves like guanosine in its property of stimulating the incorporation of amino-acids into protein in a cell-free system[7], the deaminated portions in the poly-AU will have, as it were, GUG and UGU base triplets in an alternating sequence. As the GUG is the code for glycine, and UGU for leucine and valine[7,8], if nitrous acid-treated poly-AU is taken as a messenger RNA in a cell-free system of protein synthesis, a polypeptide with glycine and leucine or glycine and valine in an alternating sequence will be formed if the code assigned to those amino-acids is correct.

If the dAT copolymer is treated with nitrous acid[5,6], deamination of adenine to form hypoxanthine will result (not necessarily complete), and the copolymer will have regions of poly-dHT. In RNA-polymerase reaction with poly-dHT as the priming DNA and CTP and ATP as the substrate, poly-CA will be formed in which cytosine and adenine bases will be expected to occur in an alternating sequence. Thus the polyribonucleotide:

CACACACACAC—A (also poly-CH)

has CAC and ACA base triplets in an alternating sequence. As CAC is shown to be the code for proline and threonine, and ACA the code for threonine and aspartic acid[7,8], with alternating poly-CA as the messenger RNA a polypeptide in which threonine and aspartic acid or threonine and proline or proline and aspartic acid in an alternating sequence will be formed if the code assigned to those amino-acids is correct.

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