*Onderstepoort J. vet. Res.* 41 (4), 221—224 (1974)

# THE ELIMINATION OF RIBONUCLEIC ACID INTERFERENCE IN THE SPECTROPHOTOMETRIC DETERMINATION OF PROTEIN CONCENTRATION

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ABSTRACT

OELLERMANN, R. A., 1974. The elimination of ribonucleic acid interference in the spectrophotometric determination of protein concentration. *Onderstepoort J. vet. Res.* 41 (4), 221—224(1974).

A simple, sensitive and rapid spectrophotometric method for the determination of protein concentration in the presence of RNA contaminants is described. Only small spectral differences exist between RNAs from different sources and to eliminate their interference, two wavelengths must be selected where absorbance due to RNA is identical. The isoabsorbance wavelengths must lie between 235 nm and 226 nm, the region where with decrease in wavelength the increase in absorbance is linear for most proteins. From data presented wavelengths of 233,0 nm and 226,0 nm were selected as the most suitable. A linear relationship between difference in absorbance (226,0 nm–233,0 nm) and protein concentration was observed for pure protein preparations up to 300 μg/ml and for protein: RNA mixtures. Protein concentrations determined by this method were comparable with those determined by the colorimetric method of Lowry. Differences in isoabsorbance wavelengths selected in this study and those reported by other workers are discussed.

## INTRODUCTION

It is frequently necessary to determine the protein concentrations of impure biological materials. A method commonly used is the colorimetric procedure developed by Lowry, Rosebrough, Farr & Randall (1951). This method is time consuming and cannot be used over wide concentration ranges. Nucleic acids also interfere with the colour development. Recently Groves, Davis & Sells (1968) and Ehresmann, Imbault & Weil (1973) reported spectrophotometric procedures which were claimed to eliminate interference by nucleic acids. In both reports protein concentration was related to the difference in absorbance at two wavelengths where RNAs reputedly had identical absorbance values. Groves *et al.* (1968) used isoabsorbance wavelengths of 224,0 nm and 233,3 nm whereas Ehresmann *et al.* (1973) claimed that values of 228,5 nm and 234,5 nm gave better results.

In the present investigation the purity of RNA extracts was determined and isoabsorbance wavelengths selected which are suitable for measuring protein concentration by the determination of differential absorbance.

## MATERIALS AND METHODS

### *Nucleic acids*

Single stranded liver (L) and spleen (S) RNA was extracted from normal mice employing the hot phenol procedure at pH 5 as described in detail elsewhere (Oellermann, 1974).

Single stranded RNA from a baby hamster kidney (BHK-21) cell line was extracted from denucleated cells according to Scherrer & Darnell (1962).

Yeast transfer RNA (tRNA), *Escherichia coli* K12 MO 16s+23s ribosomal RNA (rRNA) and calf thymus DNA (T-DNA) were obtained from Miles Laboratories (Pty) Ltd, Cape Town.

### *Purified proteins*

Crystallized bovine albumin fraction V, bovine IgG, chromatographically pure ribonuclease (RNase), crystalline deoxyribonuclease (DNase) and crystalline trypsin were obtained from Miles Laboratories (Pty) Ltd, Cape Town.

### *Miscellaneous protein preparations*

Bovine serum was provided by N. T. van der Walt, Department of Biochemistry, and tuberculin PPD without phenol preservative by the Department of Bacteriology, Onderstepoort.

Received 26 August 1974—Editor

### *Buffers*

Acetate buffers of pH 4, 5, 6 and 7 were prepared by mixing 0,1 M sodium acetate and 0,1 M acetic acid in the desired proportions.

Phosphate buffers of pH 6, 7 and 8 were prepared by mixing 0,1 di-sodium hydrogen phosphate and 0,1 m sodium di-hydrogen phosphate.

Tris-HCl buffers were prepared by adjusting the pH of 0,1 M tris (hydroxymethyl) aminomethane with hydrochloric acid to pH 6, 7, 8 and 9.

Unbuffered physiological saline (0,162 M NaCl), and tris-HCl and phosphate buffers of pH 7 containing 1,0 M sodium chloride were used in some experiments.

Reagent grade chemicals were used. The pH was adjusted with a Radiometer model 26 pH-Meter set on 10 × expanded scale.

### *Spectrophotometric assay*

Ultraviolet absorbance measurements and spectral readings were obtained with a Beckman Acta CIII spectrophotometer. Paired quartz cuvettes with a 10 mm path length were used and scans carried out in the region of 320 nm to 220 nm. Protein concentration was determined by the difference in absorbance at 226,0 nm and 233,0 nm using albumin as standard.

Unless stated otherwise, physiological saline was used as solvent for measuring absorbance of nucleic acid and protein preparations.

### *Colorimetric determination of protein*

The colorimetric procedure for the determination of protein described by Lowry *et al.* (1951) was used.

## RESULTS

### *Absorbance spectra of RNAs*

During the extraction of L-RNA, samples taken from the aqueous phase after successive phenol extractions were dephenolized with diethyl ether and precipitated with ethanol. The absorbance spectra were compared with that of L-RNA finally obtained after ethanol precipitation of the redissolved 1,0 M sodium chloride precipitate (Oellermann, 1974). The data are reproduced in Fig. 1. The wavelength of minimum absorbance in the spectral region between 240 nm and 220 nm (λ Min) decreased from 232,3 nm after the first phenol extraction to 229,2 nm after the third phenol extraction. The value of λ Min was 228,8 nm for the final single stranded RNA preparation and the spectrum did not change with successive salt precipitations.

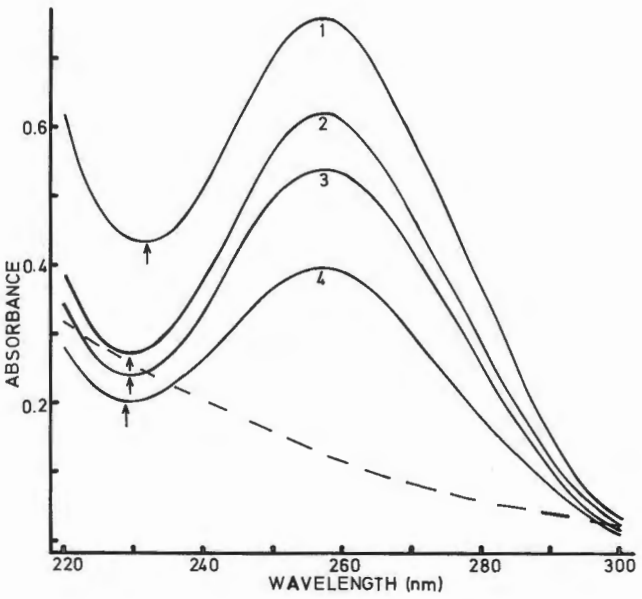


FIG. 1 Absorbance spectrum of mouse liver RNA after (1) first phenol, (2) second phenol, (3) third phenol extraction, and (4) single stranded L-RNA after 1,0 M NaCl precipitation. The broken curve is the spectrum of 1 mg Glycogen/ml

The major contaminant in the RNA preparation with an influence on the absorbance spectrum appears to be protein. Although glycogen could also contribute to the contamination, only high concentrations would significantly change the absorbance spectrum.

The effect of RNA concentration on the absorbance spectra was investigated and the data appear in Fig. 2. Changes in concentration did not result in lateral spectral shifts and $\lambda$ Min remained constant at 228,9 $\pm$ 0,2 nm (mean $\pm$ SD). Similar results were obtained by Ehresmann *et al.* (1973), but at a higher $\lambda$ Min value.

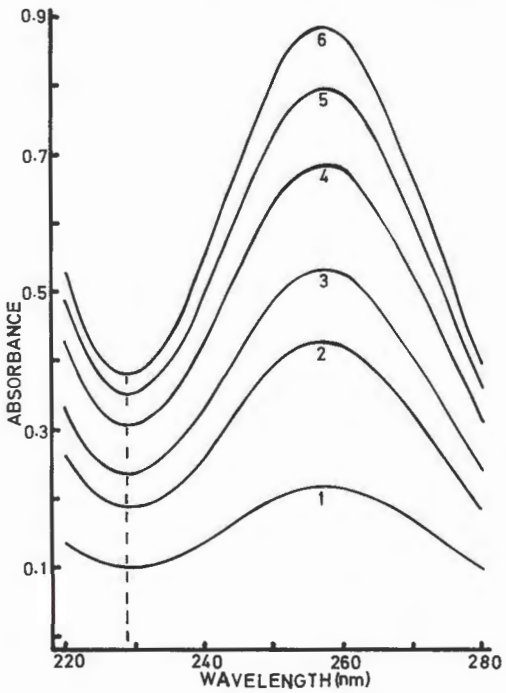


FIG. 2 Absorbance spectra of L-RNA at (1) 8,5 $\mu$g/ml, (2) 17 $\mu$g/ml, (3) 42 $\mu$g/ml, (4) 81 $\mu$g/ml, (5) 63 $\mu$g/ml, and (6) 35 $\mu$g/ml. Curves 1, 2 and 6 recorded at an absorbance span=1. Curves 3 and 5 recorded at an absorbance span=2. Curve 4 recorded at an absorbance span=3

The influence of pH and ionic strength on the absorbance spectra of L-RNA was determined and the results summarized in Table 1. Irrespective of the buffer used, insignificant spectral shifts occurred over the range of pH 5–9, thereby confirming an earlier report (Ehresmann *et al.*, 1973). Increases in ionic strength resulted in very slight spectral shifts towards higher values of $\lambda$ Min, but the shifts were less than those reported by Groves *et al.* (1968).

TABLE 1 Absorbance by L-RNA at different values of pH and ionic strength

| Buffer | pH | $\lambda$ Min (nm) | Wavelength of isoabsorbance relative to | | |
|---|---|---|---|---|---|
| | | | 224,0 nm | 226,0 nm | 228,5 nm |
| 0,1M Acetate..... | 4,0 | 230,2 | 238,0 | 236,0 | 233,0 |
| | 5,0 | 229,3 | 235,1 | 232,9 | 230,2 |
| | 6,0 | 229,0 | 234,6 | 232,5 | 229,5 |
| | 7,0 | 228,8 | 234,7 | 232,3 | 229,0 |
| 0,1M Phosphate..... | 6,0 | 229,0 | 235,0 | 232,6 | 229,5 |
| | 7,0 | 228,7 | 234,5 | 232,3 | 229,1 |
| | 8,0 | 229,0 | 234,6 | 232,3 | 229,4 |
| "+1M NaCl..... | 7,0 | 229,3 | 235,5 | 233,0 | 230,1 |
| 0,1M Tris-HCl..... | 6,0 | 229,2 | 235,5 | 233,0 | 230,0 |
| | 7,0 | 229,0 | 235,3 | 232,6 | 229,6 |
| | 8,0 | 228,8 | 234,4 | 232,4 | 229,3 |
| | 9,0 | 229,0 | 235,3 | 232,7 | 229,6 |
| "+1M NaCl..... | 7,0 | 229,0 | 234,7 | 232,4 | 229,5 |
| 0,162M NaCl..... | — | 228,8 | 234,6 | 232,1 | 229,2 |
| $H_2O$..... | — | 228,5 | 233,8 | 231,8 | 228,5 |

Although DNA has not been mentioned as a contaminant in the L-RNA and S-RNA extracts prepared, its absence cannot be claimed. Different concentrations of T-DNA were therefore added to known concentration of L-RNA and the absorbance data are presented in Table 2. Although the addition of DNA produced a lateral shift in the absorbance spectrum of RNA, only large concentrations resulted in significant changes of $\lambda$ Min with a concomitant change in the isoabsorbance wavelengths observed. Its presence as a contaminant of RNA preparations therefore does not have a major influence on the selection of suitable isoabsorbance wavelengths.

TABLE 2 Influence of added T-DNA on the absorbance measurements by L-RNA

| Nucleic acid | $\lambda$ Min (nm) | Wavelength of isoabsorbance relative to 226,0 nm |
|---|---|---|
| 20 $\mu$g L-RNA/ml..... | 229,0 | 232,8 |
| "+2 $\mu$g T-DNA/ml..... | 229,0 | 233,2 |
| "+6 $\mu$g T-DNA/ml..... | 229,4 | 233,3 |
| "+10 $\mu$g T-DNA/ml..... | 230,0 | 234,0 |
| "+14 $\mu$g T-DNA/ml..... | 230,0 | 234,2 |
| 30 $\mu$g T-DNA/ml..... | 230,4 | 235,2 |

Different RNAs could possibly give different absorbance spectra. The results obtained for RNA from different sources are therefore presented in Table 3. Contrary to the results obtained by Groves *et al.* (1968) only slight differences were observed between the absorbance spectra of RNA from different sources, thereby corroborating observations for tRNA in particular (Ehresmann *et al.*, 1973). The mean isoabsorbance wavelength for all sources of

RNA relative to 226,0 nm was 233,0 nm. By comparison Groves *et al.* (1968) reported values of 224,0 nm and 233,3 nm and Ehresmann *et al.* (1973) 228,5 nm and 234,5 nm (cf. Table 1).

TABLE 3 Absorbance by different sources of RNA

| Source of RNA | λ Min (nm) | Wavelength of isoabsorbance relative to 226,0 nm |
|---|---|---|
| L-RNA | 228,8[a] | 232,1 |
| S-RNA | 229,6 | 233,2 |
| BHK21-RNA | 229,3 | 232,7 |
| tRNA | 229,7 | 233,5 |
| rRNA | 229,9 | 233,7 |

[a] Values presented are the average of triplicate determinations for each of 10 preparations of L-RNA and S-RNA, 2 preparations of BHK21-RNA and 1 preparation of tRNA and rRNA.

*Absorbance spectra of purified proteins*

Albumin has most frequently been used as a standard in quantitative protein determinations. The absorbance spectra of different concentrations of albumin were therefore compared with a number of purified proteins (Fig. 3). Different proteins at the same concentration display differences in absorbance and albumin can therefore only be regarded as a suitable standard for those proteins with absorbance spectra closely related to it. It is also evident that the increase in absorbance of different proteins is essentially linear only in the region between 235 nm and 226 nm, a factor to be considered in the selection of isoabsorbance wavelengths.

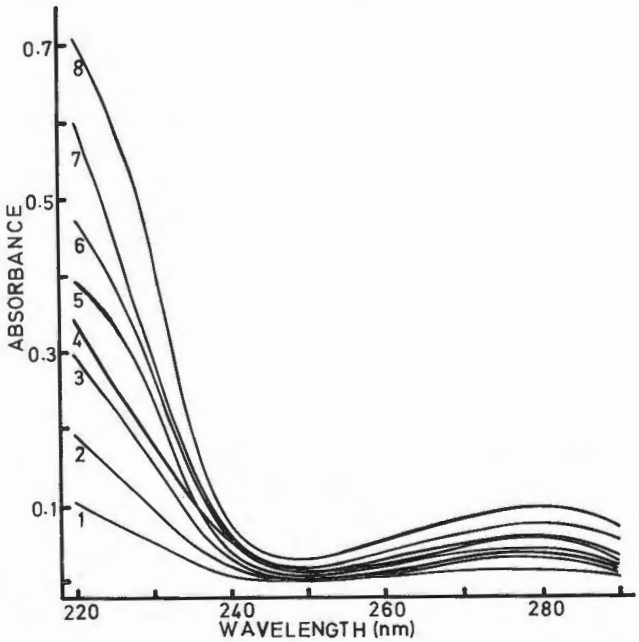


FIG. 3 Absorbance spectra of purified proteins: (1) 10 μg Albumin/ml, (2) 20 μg Albumin/ml, (3) 30 μg Albumin/ml, (4) 40 μg RNase/ml, (5) 40 μg DNase/ml, (6) 40 μg bovine IgG/ml, (7) 60 μg Albumin/ml, and (8) 60 μg Trypsin/ml.

*Spectrophotometric determination of protein concentration*

The difference in absorbance (△ Abs) of different concentrations of albumin at the selected isoabsorbance wavelengths (226,0 nm and 233,0 nm) was plotted against protein concentration in Fig. 4.

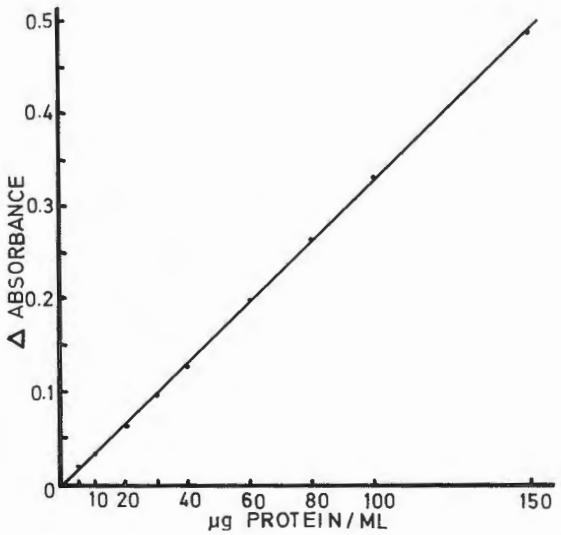


FIG. 4 Relationship between △ Abs (226,0 nm–233,0 nm) and albumin concentration

A straight line passing through zero △ Abs at zero protein concentration was obtained. Although not shown in Fig. 4, linearity in the relationship △ Abs/protein concentration was observed for albumin concentrations up to 300 μg/ml. A △ Abs of 1 corresponded to a concentration of 298,5 μg protein/ml.

The △ Abs (226,0 nm–233,0 nm) was also measured for different albumin concentrations in the presence of a series of L-RNA concentrations. The results are presented in Table 4. From the observed △ Abs, the protein concentrations calculated from Fig. 4 agreed closely with the theoretical values of 40 and 60 μg albumin in the presence of 0–36 μg L-RNA. Ehresmann *et al.* (1973) report such close agreement between the theoretical and observed values only for concentrations up to 10 μg RNA per 50 μg protein.

To test the validity of the spectrophotometric determination further, the present method using △ Abs (226,0 nm–233,0 nm) was compared with the colorimetric procedure (Lowry *et al.*, 1951). The results appear in Table 5. Very close agreement was observed for both bovine serum and tuberculin assayed at different protein concentrations, thereby confirming an earlier report (Ehresmann *et al.*, 1973).

## Discussion

The spectrophotometric determination of proteins in biological materials must take into consideration the contribution to absorbance measurements by major contaminants like RNAs, DNAs and, possibly, polysaccharides. Two spectrophotometric procedures were developed to eliminate interference by nucleic acids (Groves *et al.*, 1968; Ehresmann *et al.*, 1973). Both procedures were based on the measurement of differences in absorbance at two wavelengths, in the 220 nm to 240 nm region, at which nucleic acid absorbance measurements were identical. These values were 224,0 nm and 233,3 nm (Groves *et al.*, 1968) and 228,5 nm and 234,5 nm (Ehresmann *et al.*, 1973). The difference in absorbance between the selected wavelengths was attributed to protein. Both groups of workers claimed to have used pure RNA preparations in the determination of isoabsorbance wavelengths for RNA. In the present study it has been shown that from all possible contaminants in RNA extracts protein results in the most marked

TABLE 4 Determination of protein concentration in the presence of single stranded RNA by △ Abs (226,0 nm–233,0 nm)

| Albumin by mass (μg/ml) | Measurement | μg L-RNA/ml | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 9 | 18 | 36 |
| 40 | $A_{226,0}$ | 0,268 | 0,279 | 0,300 | 0,364 | 0,444 | 0,624 |
| | $A_{233,0}$ | 0,132 | 0,144 | 0,168 | 0,231 | 0,310 | 0,487 |
| | △ Abs | 0,136 | 0,135 | 0,132 | 0,133 | 0,134 | 0,137 |
| | μg Protein/ml[a] | 40,6 | 40,3 | 39,4 | 39,7 | 40,0 | 40,9 |
| 60 | $A_{226,0}$ | 0,404 | 0,426 | 0,448 | 0,483 | 0,574 | 0,750 |
| | $A_{233,0}$ | 0,202 | 0,225 | 0,250 | 0,287 | 0,375 | 0,553 |
| | △ Abs | 0,202 | 0,201 | 0,198 | 0,196 | 0,199 | 0,197 |
| | μg Protein/ml[a] | 60,3 | 60,0 | 59,1 | 58,6 | 59,4 | 58,8 |

[a] Calculated from Fig. 4

TABLE 5 Comparison between the Lowry determination of protein and the present △ Abs (226,0 nm–233,0 nm) method

| Protein source | Method | Test number | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| | | (μg Protein/ml) | | | | | |
| Bovine serum | Lowry | 24,5 | 27,2 | 48,7 | 58,2 | 73,3 | 78,0 |
| | △ Abs | 25,67 | 28,66 | 50,45 | 61,49 | 76,71 | 73,64 |
| Tuberculin | Lowry | 15,2 | 33,4 | 35,0 | 45,0 | 52,0 | 56,7 |
| | △ Abs | 16,72 | 30,75 | 31,75 | 43,88 | 48,37 | 59,40 |

spectral shift resulting in higher λ Min values compared with that of pure RNA. These results also show that the RNA used was of such purity that any contaminants which might still have been present would not significantly affect the absorbance spectrum recorded (cf. Fig. 1)

A wide latitude in conditions of pH and ionic strength of RNA preparations is permissible without markedly changing the absorbance spectra. It must be remembered, however, that buffer systems like acetate absorb strongly in the 240 nm to 220 nm region. Slight differences in buffer concentrations between the sample and reference cuvettes would be reflected by lateral spectral shifts. Whether or not this could partially explain the difference between the isoabsorbance wavelengths selected in this study and the values reported previously (Ehresmann *et al.*, 1973) is not clear.

In the present study a reading of △ Abs=1 corresponds to 298,5 μg protein, compared to 317 μg protein in the previous study (Ehresmann *et al.*, 1973). The difference is due to the fact that the wavelength span for measuring △ Abs in the present study was 7 nm, as compared to 6 nm (Ehresmann *et al.*, 1973).

From Table 4 it is clear that a very close agreement exists between the theoretical amount of protein present and the calculated concentration based on △ Abs (226,0 nm–233,0 nm), irrespective of the amount of RNA present. Ehresmann *et al.* (1973), however, only reported good agreement if the amount of RNA in the presence of 50 μg protein did not exceed 10 μg. This suggests that their RNA could possibly have been contaminated, thereby contributing to the discrepancy in isoabsorbance wavelengths.

Differences in absorbance spectra exist between different proteins, some of which do not show a linear increase in absorbance with decrease in wavelength, particularly for the spectrum below 226 nm. The selection of suitable isoabsorbance wavelengths is therefore limited by this fact. The experimental evidence presented also supports the reliability of the measurement of △ Abs (226,0 nm–233,0 nm) for the quantitative determination of proteins. The applicability of this procedure to other biological material is supported by the excellent agreement obtained in the comparison with the colorimetric determination of protein (Lowry *et al.*, 1951).

ACKNOWLEDGEMENT

I am most grateful to Messrs P. Carter and M. J. Marx for their expert technical assistance.

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