Protein Determination by Nuclear Magnetic Resonance

B.A. COLES, Physical Chemistry Dept., Oxford University, South Parks Road, Oxford OX1 3QZ, England

ABSTRACT

Protein may be determined using a pulsed nuclear magnetic resonance (NMR) spectrometer in conjunction with a relaxation reagent. These reagents exhibit characteristic nuclear relaxation rates, controlled by a paramagnetic ingredient such as copper, and these rates are altered if substances are added which can bind the copper. The method is fast and simple. Sample and reagent are mixed, brought to a standard temperature and transferred to the spectrometer for measurement. Sample throughput can be high since the time for the spectrometer reading is on the order of 30 sec. Compositions and methods of use are given for 2 copper-based reagents together with the results on a number of materials. The alkaline copper reagent gives a very similar response to different types of protein and it appears suitable for meat, fish and for other protein materials low in carbohydrates. It shows a response to carbohydrate which depends strongly on type. The acid copper reagent is intended for measurements of vegetable or seed protein and similar applications where carbohydrate is present. This reagent does not respond to carbohydrates tested; the response to protein depends on the type of protein.

INTRODUCTION

A new instrumental technique for the determination of protein uses a pulsed nuclear magnetic resonance (NMR) spectrometer in conjunction with a "relaxation" reagent. This type of reagent is a solution which contains a paramagnetic material, such as copper, which controls the magnetic relaxation rates of the hydrogen nuclei present in the water. These relaxation rates are readily measured by pulsed NMR techniques. Addition to the reagent of a substance such as protein which can complex or bind the copper will cause measurable and quantitative changes in relaxation rate, as shown in Figure 1.

A protein determination will therefore involve steps (a) mixing a known weight or volume of sample with a standard quantity of reagent, (b) bringing the mixture to a standard temperature, then (c) transferring it to the spectrometer for measurement of the relaxation rate. The difference between this rate and a reference or blank rate from a preparation containing reagent but no sample is a measure of the amount of protein present in the sample. This difference may be converted to quantity by reference to a previously determined calibration factor or chart.

The measurement of relaxation rates by pulsed NMR is a well-established technique, in which a sample is placed in a magnetic field and subjected to a sequence of pulses of radiofrequency from a surrounding coil. In response to these pulses the hydrogen nuclei echo weak radiofrequency signals which are detected by a sensitive receiver and processed to give the relaxation rates. The time required for measurement in the spectrometer is on the order of 30 sec to 3 min; this fast and simple procedure allows a high throughput of samples.

The NMR method offers advantages over Kjeldahl in using a single reagent of low hazard and in having no separation steps, and over optical spectroscopic methods in that there is no requirement for the protein to be soluble or for the sample to yield a clear solution. This paper gives details of the composition and use of 2 relaxation reagents, together with preliminary results obtained in surveying a range of materials to investigate the potential applications and limitations of the NMR method and its relation to Kjeldahl.

EXPERIMENTAL

For use with these reagents a spectrometer must achieve an operator-independent accuracy and reproducibility on the order of 0.1%; an initial part of this work was the development of automatic setting-up and control circuits as well as data-processing circuits to facilitate this performance (1). Two prototype instruments incorporating these features have been constructed, one operating at 15 MHz and one at 2.7 MHz. Transverse relaxation rates were measured using the Carr-Purcell Meiboom-Gill technique (2). The echo amplitudes were measured at 4 and 12 m sec (15 MHz) or at 2 and 8 m sec (2.7 MHz). The values obtained were effective rates since the data processing assumes a single value of rate over the relevant time interval. The use of different conditions may give rates and calibration factors slightly different from those reported

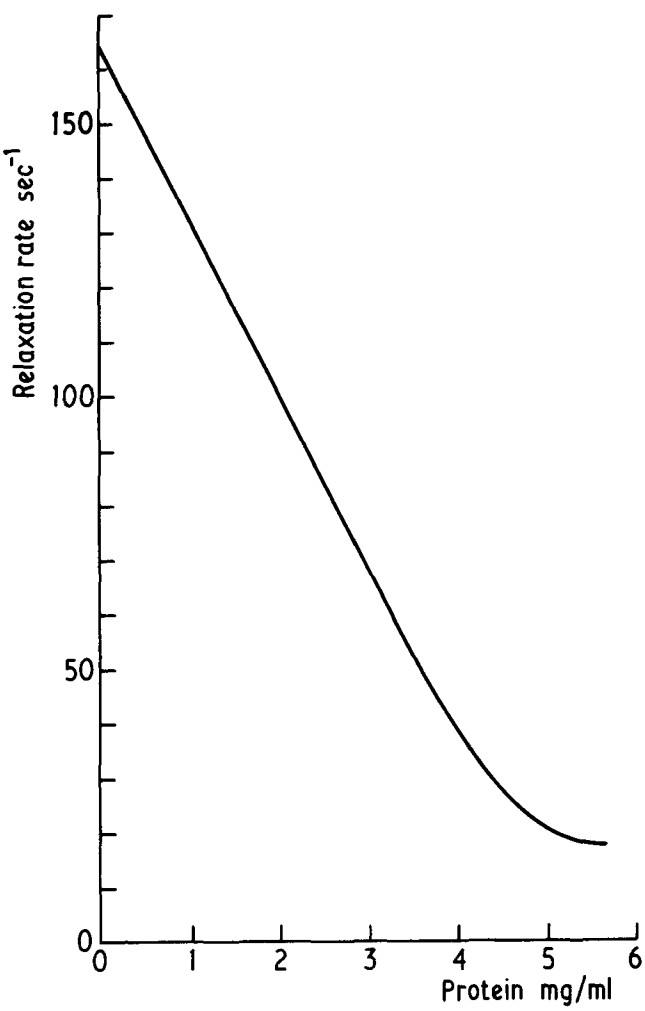

FIG. 1. Response of the alkaline copper reagent to bovine serum albumin, fraction V. (15 MHz, 25 C).

here but this should not affect the use of the method.

The alkaline copper reagent was prepared by adding 1 vol 0.534 M aqueous copper sulfate to 3 vol 6.5 M aqueous sodium hydroxide. This formulation was used for materials in solution or suspension in water, 1 vol sample being added to 4 vol reagent. The blank was therefore prepared by adding 1 vol water to 4 vol reagent, and gave a relaxation rate of 163.2 sec^{-1} at 15 MHz and 25 C. The procedure for dry materials was to use a diluted reagent prepared exactly as for the blank just described, added to the milled or powdered sample.

The acid copper reagent had a composition of 0.108 M copper sulfate and 0.15 M sodium acetate in aqueous solution, adjusted to pH 4.0 by the addition of sulfuric acid, and gave a relaxation rate of 129.2 sec^{-1} at 15 MHz and 25 C. This formulation was used for dry materials, normally in the proportion of 1 ml reagent to 30 mg sample.

Materials for the relaxation reagents were analytical grade. Protein samples were used as received without drying except where stated. Unknown protein contents were determined by the micro-Kjeldahl method substantially in accordance with Baker (3) using sodium sulfate and mercury catalyst tablets and multiplying the total nitrogen obtained by the appropriate conversion factor. Corrections were not made for any nonprotein nitrogen or nucleic acid content since one objective was to relate the NMR method to Kjeldahl.

RESULTS AND DISCUSSION

Alkaline Copper Reagent

This reagent contains copper in the form of the cuprite ion $Cu(OH)_4^{2-}$, which is a highly effective relaxation agent since it is readily accessible to the hydrogen nuclei of the water. Upon the addition of protein, the copper reacts with the peptide linkages (the biuret reaction) to form a complex which is only ca. one-eighth as effective a relaxation agent as the cuprite ion, so the overall relaxation rate falls as shown in Figure 1. For additions of bovine serum albumin up to 3.5 mg/ml of sample-reagent mixture a sufficient excess of cuprite ion remains and a closely linear response of relaxation rate-to-protein quantity is observed which has a gradient of 31.1 sec^{-1}/mg protein/ml. This slope may be treated as a calibration factor for bovine serum albumin (BSA). For albumin additions above 3.5 mg/ml, an increasing curvature sets in as the cuprite ion level is further depleted, until the reagent is completely exhausted; sample quantities should be chosen to avoid using this part of the characteristic.

Other proteins give a response similar to that shown in Figure 1, with the slope and therefore the calibration factor to be used in determinations varying slightly according to type, as given in Table I. These factors are grouped closely within the range defined by bovine serum albumin and casein, and the narrowness of this range demonstrates the good correlation with protein content determined by Kjeldahl.

Values inside this range are also obtained for Promine D, suggesting that carbohydrate interference (if present) is small for soya isolate. Values for yeast were also inside the range, demonstrating an advantage of the NMR method in that the protein is not required to be soluble. Sample material must, however, be finely dispersed in the reagent and this may require the use of a high speed mixer in some cases. The single-cell protein gave an exceptionally low value, which may reflect a high nucleic acid level contributing a nonprotein nitrogen content, since no correction was made for nonprotein nitrogen in the Kjeldahl determination.

TABLE I

Protein Calibration Factors with the Alkaline Copper Reagent

Material	Calibration factor (sec^{-1}/mg/ml, 15 MHz, 25 C)
Bovine serum albumin, fraction V	31·1
Gelatin, swine skin	28·1
Casein	27·2
Meat: Veal	29·0
Beef	28·3
Pork	28·6
Turkey	27·6
Fish: Cod	28·6
Single-cell protein[a]	24·9
Soya: Promine D[b]	28·9
Yeast[c]	27·5

[a]Not corrected for nonprotein nitrogen. See Experimental.
[b]May have carbohydrate interference. See Experimental.
[c]Protein insoluble in this reagent.

The biuret reaction is relatively slow, and ca. 20 min (at 25 C, depending on the sample material) is required for the relaxation rate to reach a steady value. This period may be reduced by measuring the samples at a shorter, fixed time after mixing and using an adjusted calibration factor, by standardizing and measuring at a higher temperature or by applying a brief heat treatment (e.g., 10 min at 65 C) to the sample-reagent mixture.

The alkaline copper reagent also shows a response to carbohydrate, and while this response may be used for the determination of carbohydrate in samples (4), the presence of carbohydrate in a protein sample may interfere. Similarly, interference may arise from other materials able to bind copper under alkaline conditions. The degree of interference and maximal permissible levels may be estimated from a comparison of the reagent's response to protein (Table I) with that from other materials expressed in the same units of sec^{-1}/mg/ml. Values observed for carbohydrate range from -20 to +41, apart from sorbitol at +139. Examples of other compounds are: ammonium sulfate, -3.5; sodium citrate, -1.2; sodium potassium tartrate, +0.28; glycine, -1.9; histidine hydrochloride, +11.1.

Alkaline Copper Reagent—Accuracy and Linearity

Errors from sampling inhomogeneous material and in determining weights or volumes would affect NMR just as other methods and need not be discussed here. Factors specific to the NMR method are those which determine the accuracy of measurement of the relaxation rates, and the fact that it is a difference between 2 rates (blank and sample) which is required.

The prototype instruments are able to measure relaxation rates to ca. 0.1% with a reading time of ca. 30 sec. Precision may be improved by the normal procedures such as using longer integration times or averaging a number of readings. The error on the difference between blank and sample rates would be equivalent to ca. 0.15% of the blank rate. Good practice dictates that the protein load should be chosen so as to make this difference as large as possible without entering the reagent exhaustion region (e.g., for albumin, ca. 3 mg/ml would be used). The accuracy on the determination is then on the order of 0.24%. This assumes adequate temperature control since the relaxation rates have temperature coefficients in the region of 1.5% per degree C.

Errors could arise in comparing samples of different

protein content if the relationship between relaxation rate and protein quantity is not accurately linear, and therefore, the apparent linearity of Figure 1 from 0 to 3 mg/ml has been investigated. The procedure used was a 3-point test for curvature, repeated for different sections of the characteristic. Two sample-reagent mixtures were prepared from the same albumin solution, one at full concentration and one at half concentration by using either 1 or 2 aliquots of solution as appropriate. Together with a blank prepared at the same time, this yielded points at albumin concentrations of zero, C/2 and C. Different albumin solutions were used to vary C over a 3:1 range. This procedure avoids the need for highly accurate independent determinations of the protein content of weak solutions.

The results of a number of 3-point tests are consistent with the section of the characteristic between 0 and 2.2 mg/ml having a very slight curvature; the midpoint is at 1.1 mg/ml displaced above the straight line by 0.6 sec^{-1} toward higher relaxation rates. However, since the standard deviation (SD) on individual points in this procedure was 0.2-0.3 sec^{-1}, the displacement represents only ca. 2 SD. Within the limits of present experimental precision it may therefore be tentatively concluded that the region between 2 and 3 mg/ml may be treated as linear and that albumin samples yielding 1 mg/ml or less should employ a slightly lower calibration factor of 29.5 sec^{-1}.

TABLE II

Protein Calibration Factors with the Acid Copper Reagent

Material		Calibration factor (sec^{-1}/mg/ml, 15 MHz, 25 C)
Soya: Beans		1·13
HS 70		0·98
Concentrate:	Unico 75	1·11
	Promosoy 100	1·01
	Newpro	1·00
Textured:	Unibit	1·29
	Retail X	1·05
	Retail Y	0·95
Isolate:	Promine D	0·63
	PP500E	0·63
	PP A	0·62
	PP N	0·68
Bovine serum albumin, fraction V		0·61
Beef, freeze-dried		0·40
Pork, freeze-dried		0·38
Sodium caseinate		0·72
Rapeseed, defatted meal		1·32
Sunflower seeds: Volgar		1·48
Pole Star		1·48
Armavirec		1·62
Experimental A		1·29
Experimental B		1·38

Acid Copper Reagent

For applications such as seeds or vegetable protein requiring the measurement of protein in the presence of carbohydrate, the reagent using copper under acidic conditions may prove suitable. It is used in a similar manner to the alkaline copper reagent, and Table II shows the results obtained on a range of soya and seed materials; some nonvegetable proteins are included for comparison. The response is lower and this is compensated by using a greater proportion of sample-to-reagent, typically up to 30 mg/ml. The characteristic is slightly curved, so constant sample size or a calibration chart should be employed for maximal accuracy. Carbohydrate sensitivity has been tested with sucrose, raffinose, stachyose, lactose, fructose, mannitol, sorbitol, potato starch and cellulose and no significant response was found at levels of 5 mg/ml.

At pH 4, copper would be expected to bind to protein at sites associated with free carboxylic groups, and the response with this reagent should therefore principally reflect aspartic and glutamic acid content. The lower sensitivity and the wider range of calibration factors, compared with the alkaline reagent measuring peptide linkages, are therefore expected. Structural changes caused by processing may not greatly affect the NMR method, as evidenced by the similarity of values for soya beans, meal, concentrate, and textured vegetable protein (TVP).

Considerations of curvature and a restriction on the maximal proportion of sample-to-reagent to avoid errors caused by pH displacement, mean that the difference between blank and sample relaxation rates are only about half of those which may be used with the alkaline reagent. Since the error on this difference is the same in each case, the accuracy using the acid copper reagent is reduced and is on the order of 0.5%.

ACKNOWLEDGMENTS

The author is indebted to Mr. I.J. Richmond (Newport Instruments Ltd.); Dr. G.S.D. Weir and Mr. S.I. West (Leatherhead Food R.A.); Dr. R.D. Sketcher (Unimills B.V., Holland); Dr. D. Waddington, Dr. P.J. Lillford and Mr. C.R.T. Brown (Unilever [U.K.] Central Resources Ltd.) for valuable discussions and generosity in providing samples, and to Newport Instruments Ltd. for the loan of a 2.7 MHz spectrometer.

REFERENCES

1. Coles, B.A., J. Magn. Reson. 23:71 (1976).
2. Meiboom, S. and Gill, D., Rev. Sci. Instrum. *29*:688 (1958).
3. Baker, P.R.W., Talanta 8:57 (1961).
4. Coles, B.A., in preparation.

[Received September 13, 1979]