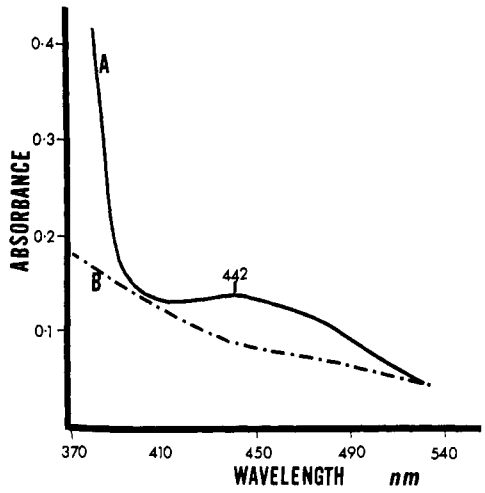

Figure 2. Spectral analysis of egg exudate with and without sodium hydrosulfite (A = egg exudate; B = reduced egg exudate)

Thin-layer chromatographs of fractions 4 and 5 on cellulose layers were developed with 1-butanol–acetic acid–water (4:1:5) and these showed one fluorescent spot each, with R_f values of 0.24 and 0.25, respectively. Pure riboflavin had an R_f value of 0.46 in the same solvent system. These differences in R_f values may be due to the conjugation of riboflavin with other components in fractions 4 and 5.

The exact identity of fraction 4 could not be established by spectral analysis, as the patterns did not agree with the absorption maxima either for riboflavin or flavoprotein.

In conclusion, it appears that the egg exudate is a heterogenous mixture of at least five moieties, as determined by gel filtration. Fractions 1 and 2, on the basis of their biological activity and chemical analysis, would appear to be similar to ovomucoid in the native egg albumen. The exact nature of fractions 3 and 4 could not be fully established, although their analysis would indicate that these are derived from carbohydrate-rich albumen moieties, while fraction 5 appears to be a degradation product of egg flavoproteins.

LITERATURE CITED

Baker, A. C. M., Manwell, C., *Br. Poultry Sci.* **3,** 161 (1962).
Boas, N. F., *J. Biol. Chem.* **204,** 553 (1953).
Bragg, P. D., Hough, L., *Biochem. J.* **78,** 11 (1960).
Deutsch, H. F., Morton, J. I., *Arch. Biochem. Biophys.* **64,** 19 (1956).
Gaitonde, M. K., Dovey, T., *Biochem. J.* **117,** 907 (1970).
Gornall, A. G., Baradawill, C. S., David, M. M., *J. Biol. Chem.* **177,** 751 (1949).
Lineweaver, H., Murray, E. W., *J. Biol. Chem.* **171,** 565 (1947).
Nelson, N., *J. Biol. Chem.* **153,** 375 (1944).
Rhodes, M. B., Bennett, N., Feeney, R. E., *J. Biol. Chem.* **234,** 2054 (1959).
Rhodes, M. B., Bennett, N., Feeney, R. E., *J. Biol. Chem.* **235,** 1686 (1960).
Sale, E. E., Priest, S. G., Jensen, H., *J. Biol. Chem.* **277,** 83 (1957).
Stevens, F. C., Feeney, R. E., *Biochemistry* **2,** 1346 (1963).
Tillmans, J., Philippi, K., *Biochem. Z.* **215,** 36 (1929).
Warren, L., *J. Biol. Chem.* **234,** 1971 (1959).

Received for review October 10, 1971. Accepted March 16, 1972.

Improved Method for Determination of Sulfur-35 in Plant Material Using Oxygen Flask Combustion and Liquid Scintillation Counting

Noel J. Grundon* and Colin J. Asher

A safe oxygen flask combustion procedure is described for the preparation of ^{35}S-labeled plant material for liquid scintillation counting. Hazards associated with flammable absorbing solutions are eliminated by using deionized water to trap the sample combustion products from the oxygen flask. Sulfur-35 activity in the resulting aqueous solution is then determined in a 1:1:2 mixture of toluene, Triton X-100, and water with an efficiency of 73%. With a 3-l. oxygen flask and a 20-min absorption period, the relationship between sample size and activity recovered was linear up to a sample weight of 1.2 g. Recoveries of ^{35}S added to plant material as sodium sulfate-^{35}S or cysteine hydrochloride-^{35}S were 100.0 ± 2.3% and 99.1 ± 1.2%, respectively. Using five combustion flasks and an automatic liquid scintillation counter, a single operator can process about 50 samples per day.

Oxygen flask combustion offers a convenient means of oxidizing biological materials prior to the estimation of stable or ^{35}S-labeled sulfur (MacDonald, 1961). Since the operation is conducted in a closed system, the volatile losses of sulfur which can occur with wet or dry ashing of samples in open vessels are largely prevented (Beaton *et al.*, 1968).

For the estimation of ^{35}S, it has been usual to trap the combustion products in absorbing solutions similar to those used when estimating ^{14}C (Kalburer and Rutschmann, 1961; Dobbs, 1963). However, the presence of flammable solvents such as methanol, ethanol, and toluene in these solutions greatly increases the fire and explosion hazard of the method. Various procedures have been used to minimize these hazards. Kalburer and Rutschmann (1961) added the trapping solution to the flask before sample ignition but chilled the solution in a Dry Ice–acetone bath before and during sample combustion. Others have avoided the hazard by admitting the trapping solution to the flask only after the combustion had finished (Martin and Harrison, 1962; Dobbs, 1963). However, to introduce a solution at this stage, the internal pressure of the flask must be lowered, either by a lengthy chilling period (Martin and Harrison, 1962) or through the use of elaborate

Department of Agriculture, University of Queensland, St. Lucia, Queensland, Australia, 4067.

ancillary equipment (Dobbs, 1963). Many workers appear to accept the hazard and complete the combustion in strong protective cages to minimize the damage likely to be caused by an explosion (Davidson and Oliverio, 1967). To prevent the flask from shattering if excessive internal pressures are momentarily developed during the combustion, use has been made of plastic discs or rubber stoppers which would burst or eject before the flask would break (Martin and Harrison, 1962; Davidson and Oliverio, 1967). However, operation of these safety devices would necessarily result in loss of the sample combustion products. In this respect, the use of a balloon attached to a sidearm on the flask is to be preferred (Lisk, 1960).

The explosion hazard could be greatly reduced by the use of aqueous trapping solutions. Although such solutions are commonly employed when stable sulfur is being estimated (MacDonald, 1961), the lack of an efficient method for counting weak β-emitting isotopes present in these aqueous solutions has hitherto precluded their use with ^{35}S. However, since the development of emulsion counting techniques (Patterson and Greene, 1965; Fox, 1968), it would appear that this limitation no longer exists.

This paper is primarily concerned with the estimation of ^{35}S activity in labeled plant material and in labeled volatile sulfur compounds of plant origin which have been trapped on activated charcoal (Asher and Grundon, 1970). However, the method may well be useful also in the estimation of ^{35}S in other types of biological material.

METHODS

Combustion Apparatus and Procedure. The 3-l. combustion flask used in these studies is shown in Figure 1. A stopcock in the bottom of the flask facilitates removal of the sample after combustion, and a balloon fitted to the sidearm absorbs sudden pressure changes which might lead otherwise to flask breakage or sample loss.

Before combustion, samples were wrapped in a square of ashless filter paper and placed in the platinum basket, with a small piece of paper being left protruding from the basket to act as a wick. The appropriate amount of trapping solution was added to the flask, the balloon was attached to the sidearm, and the flask was flushed with oxygen. The wick of the sample was then ignited and the combustion head was placed in position and secured by steel springs. When combustion was complete, the flask was allowed to cool for a few minutes and then rotated to wet the walls of the flask with trapping solution. At the end of the absorption period, the trapping solution was drained from the combustion flask by means of the stopcock into a 100-ml volumetric flask. The platinum basket and walls of the flask were then washed with a jet of deionized water, and the washings were collected in the volumetric flask. The volume was then made up to the mark and an aliquot was taken for liquid scintillation counting.

Liquid Scintillation Counting. All measurements were carried out on a Beckman LS 100 liquid scintillation counter (Beckman Industries, Fullerton, Calif.) located in an air-conditioned room. Voltage, amplifier, and discriminator settings (adjustable plug-in discriminator) were adjusted for maximum operational efficiency in accordance with the manufacturer's recommendations.

Samples were counted in 21-ml low-potassium glass vials. In each case, the sample was stored in darkness inside the counter for at least 2 hr before counting to allow for temperature equilibration, and for any photo-induced fluorescence to decay to background levels. Temperature in the sample compartment was found to remain constant at approximately 25°C.

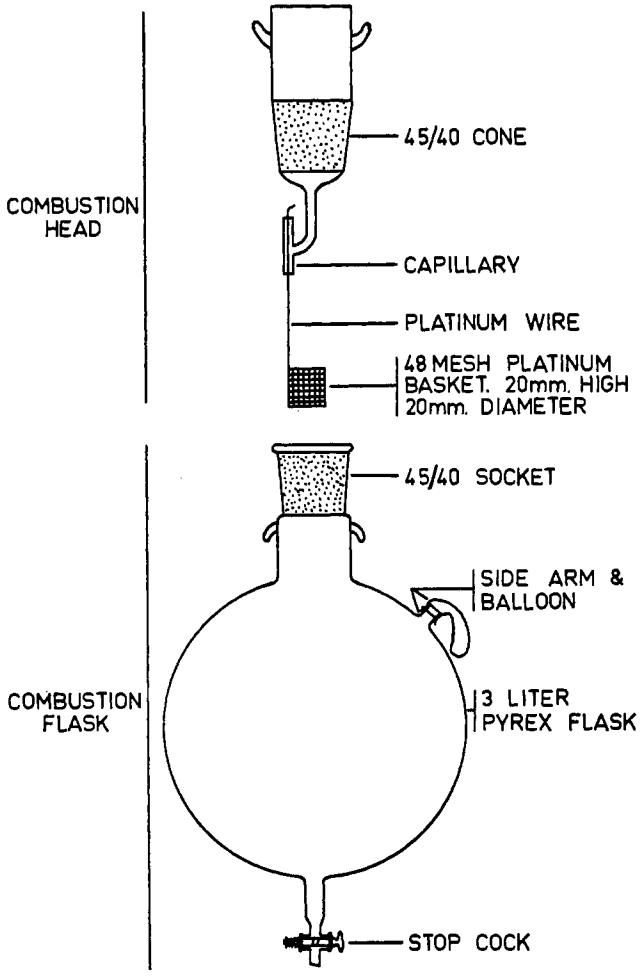

Figure 1. Three-liter oxygen combustion flask used for oxidation of ^{35}S-labeled samples

Composition of the counting mixture was optimized in terms of the ratio toluene–Triton X-100 (Rohm & Haas, Philadelphia, Pa.)–water, after considering emulsion stability and merit values for labeled aqueous sulfate solutions, using 0.5% w/v 2,5-diphenyloxazole (PPO) as the fluor. (Merit value is defined as the product of counting efficiency and water content of the mixture, when both are expressed as percentages.) Effect of fluor concentration was then examined. Technical grade Triton X-100 and toluene were used throughout, as it has been shown that counting efficiencies are not improved by purification of Triton X-100 (Greene *et al.*, 1968) or toluene (Tanielian *et al.*, 1964). Spectrograde PPO was used, as this was the only grade available. Standard aqueous solutions of $Na_2{}^{35}SO_4$ were obtained from the Radiochemical Centre, Amersham, U.K.

RESULTS AND DISCUSSION

Counting of Standard Aqueous Sulfate Solutions. Stability and Physical Appearance of Mixtures. The physical appearance and counting efficiency of the toluene–Triton X-100–water emulsion system have been shown to be sensitive to temperature, nature and concentration of solutes in solution, and method of emulsion formation (Patterson and Greene, 1965; Benson, 1966; Turner, 1967; van der Laarse, 1967; Williams, 1968; Fox, 1968; Greene *et al.*, 1968). Re-

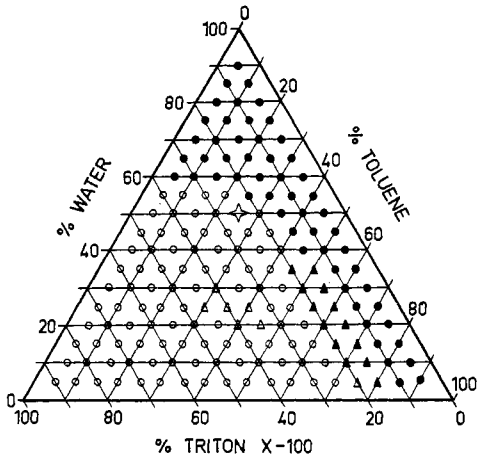

Figure 2. Stability and physical appearance of counting mixtures at 25°C. Open symbols represent clear or translucent mixtures, solid symbols represent opaque. Triangles denote unstable mixtures. Mixture selected for routine counting indicated thus (◇)

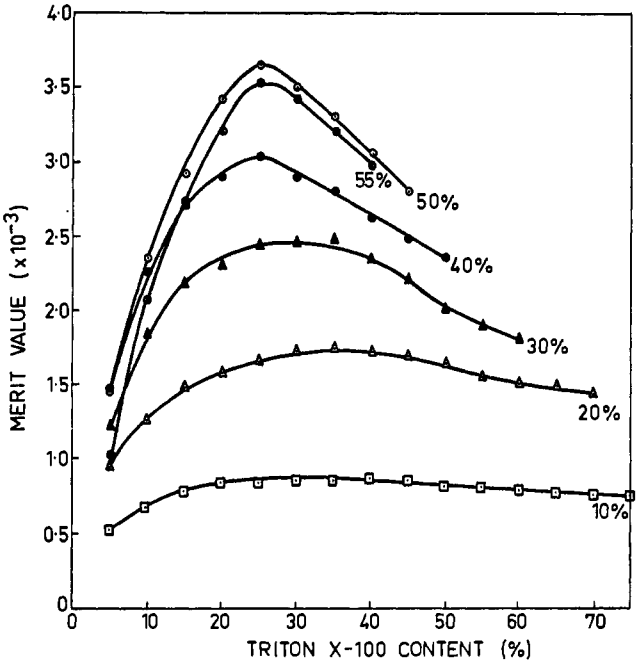

Figure 3. Effect of composition of counting mixture on merit value for mixtures counting 10 to 55% water

sults of van der Laarse (1967) and Fox (1968) clearly demonstrate the need to examine a wide range of mixtures before choosing the optimum mixture for routine use under the prevailing experimental conditions.

The manner in which the physical appearance of toluene–Triton X-100–water emulsions varied with changing composition of the mixture is shown in Figure 2. Mixtures ranged from glass-clear through translucence to opaque white. For simplicity and ease of comparison with results reported by other workers, the mixtures were classified into just two classes —those which were either clear or translucent and those which were opaque. Stable mixtures were defined as those showing no separation of phases after standing for 7 days.

A comparison of the results of this study, conducted at 25°C, with those of Fox (1968) at 21°C or van der Laarse (1967) at 3°C shows that the number of opaque mixtures decreases wih increasing temperature. Since opaque mixtures

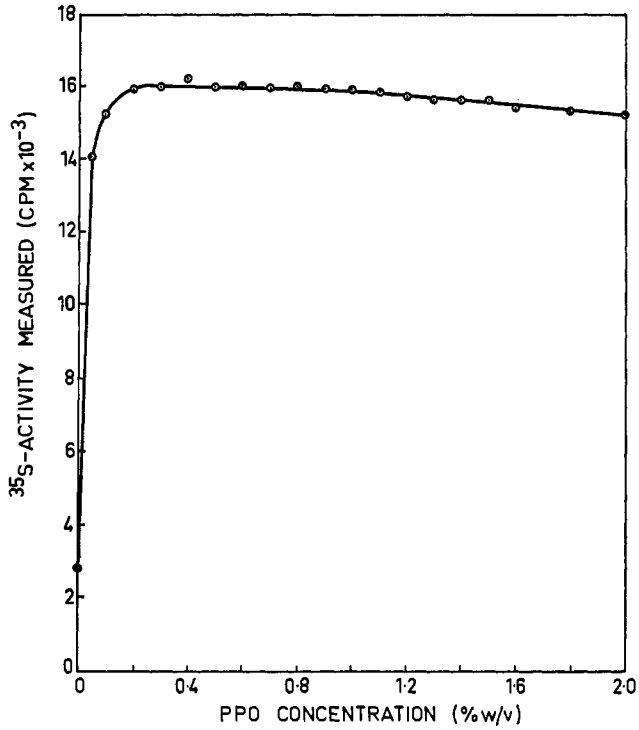

Figure 4. Effect of PPO concentration on ^{35}S-activity measured using a mixture containing toluene, Triton X-100, and water in the ratio of 1:1:2

have lower counting efficiencies than clear or translucent mixtures, it would appear that up to 25°C at least higher counting temperatures are advantageous in that they increase the number of mixtures with potentially useful high counting efficiencies.

Although some of the unstable mixtures were found to have quite high initial counting efficiencies, the progressive loss of counting efficiency during phase separation made these mixtures inconvenient to use on a routine basis. Consequently it was decided to study further only the stable mixtures.

MAXIMIZATION OF COUNTING SENSITIVITY. Since water is a powerful quenching agent in liquid scintillation counting, the counting efficiency generally decreases as the water content of the counting mixture increases (Patterson and Greene, 1965; van der Laarse, 1967; Fox, 1968). However, in the case of aqueous samples, the absolute activity of the counting mixture is directly proportional to the water content. Hence, to maximize the sensitivity of the emulsion-counting method, it is necessary to effect a compromise between increases in absolute activity achieved by increasing the amount of aqueous sample added to the mixture, and the corresponding decreases in counting efficiency. The simplest method of doing this is to select the mixture with the highest merit value (Bruno and Christian, 1961; van der Laarse, 1967; Fox, 1968). In the present study, merit values were found to increase with increasing water content up to 50% water (Figure 3). Beyond 50 to 55% water, mixtures became opaque (Figure 2) and both counting efficiency and merit values decreased sharply.

On the basis of these results, the mixture containing 25% toluene, 25% Triton X-100, and 50% water (*i.e.*, 1:1:2) was selected for routine counting. With this mixture, it was found that ^{35}S could be determined with a counting efficiency of 73%.

EFFECT OF PPO CONCENTRATION. Benson (1966) found that in a 10:5:4 mixture of toluene, Triton X-100, and water counted at −2°C, counting efficiency was very sensitive to

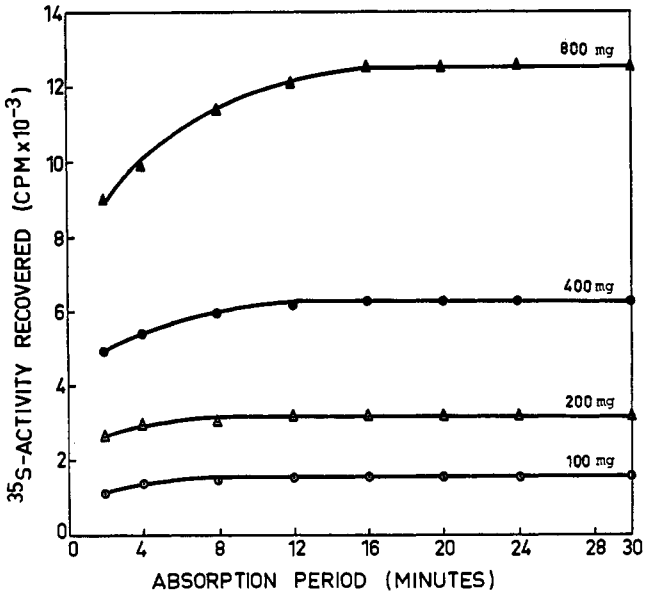

Figure 5. Effect of sample weight and absorption period on ^{35}S-activity recovered from the oxygen flask

Table I. Effect of Hydrogen Peroxide Concentration and Volume of Trapping Solution on ^{35}S-Activity Recovered from Combustion of 500 mg of Labeled Plant Material (Values Are Means of Three Replicates, Expressed as cpm $\times 10^{-5}$)

Volume of trapping solution, ml	100-Volume hydrogen peroxide added (% v/v) 0.0	0.5	2.0	Mean
1	17.68	17.72	17.65	17.68
10	17.65	17.67	17.71	17.68
40	17.65	17.71	17.72	17.69
Mean	17.66	17.70	17.69	

Table II. Recovery of ^{35}S from the Oxygen Flask after Combustion of Plant Material or Activated Charcoal to Which Labeled Standards Had Been Added (Values are Means of Ten Replicates)

Sodium sulfate	100.0 ± 2.3[a]	98.0 ± 1.8
L-Cysteine hydrochloride	99.1 + 1.2	100.1 ± 1.5
1-Butanethiol	1.1 ± 0.2	97.5 ± 1.9

[a] Percent recovery of sulfur $= \frac{(\text{cpm recovered} \times 100)}{(\text{dpm in standard})} \times \frac{100}{73}$.

PPO concentration, the maximum efficiency being obtained at 0.55% w/v PPO, with much lower efficiencies occurring above or below this concentration. By contrast, in the present study, the counting efficiency of the 1:1:2 mixture at 25°C was only slightly affected by PPO concentration over a range of 0.2 to 2.0% w/v (Figure 4). These results suggest that 0.3% w/v PPO is sufficient for use with the 1:1:2 counting mixture, and that minor variations in PPO concentration will have little effect on counting efficiency.

Trapping of Combustion Products. Effect of Peroxide. Dilute hydrogen peroxide solutions have been employed to ensure complete oxidation of the sample sulfur to sulfate (MacDonald, 1961). The peroxide concentration is reported to be important when sample sulfur is being estimated as sulfate, since oxidation may be incomplete if the concentration is too low, and persulfate may be formed if the concentration is too high (Wagner, 1957). However, since the oxidation state of the sulfur is unlikely to affect the measurements of ^{35}S activity, hydrogen peroxide concentration will be important in the present case only if it affects the efficiency with which ^{35}S-labeled combustion products are trapped.

To test for possible effects of peroxide on trapping efficiency, a series of 500-mg samples of labeled plant material were combusted using different peroxide concentrations and different volumes of trapping solution. The absorption period was 30 min. It was found that the addition of up to 2% v/v of 100-volume "Analar" hydrogen peroxide to deionized water had no detectable effect on trapping efficiency (Table I).

Although as little as 1 ml of deionized water was found to be sufficient to trap the ^{35}S-labeled combustion products of a 500-mg plant sample, a much larger volume of water was needed for thorough rinsing of the sample basket and walls of the flask. In practice it was found convenient to use 5 ml of deionized water to trap the combustion products, and approximately 80 ml of deionized water to rinse the flask and platinum basket.

Effect of Absorption Period. Figure 5 shows the relationship between absorption time and ^{35}S-activity recovered for samples varying in weight from 100 to 800 mg. The absorption time was taken from when the combustion head was inserted in the flask to when any unabsorbed gases were flushed from the combustion flask with a stream of air.

The results show that the length of time necessary for maximum recovery increases with increasing sample size. However, for samples up to 800 mg, a 16-min absorption period appeared adequate. This absorption period compares favorably with the 15- to 20-min absorption periods reported for the flammable trapping solutions by other workers (Kalburer and Rutschmann, 1961; Dobbs, 1963). On the basis of these results, a 20-min absorption period was adopted for all subsequent work.

Effect of Sample Size. When samples of ^{35}S-labeled plant material ranging in weight from 0.02 to 1.4 g were combusted under the standard conditions described above, a precise linear relationship was obtained between sample weight and activity recovered up to a weight of 1.2 g, indicating a constant recovery percentage over this range.

Recovery of Added ^{35}S-Activity. The accuracy of the method was tested by adding known amounts of ^{35}S-activity to samples of oven-dried plant material or activated charcoal before combustion, and measuring the activity recovered. Absolute activities were calculated using a value of 73% for the counting efficiency of ^{35}S-labeled sulfate in the 1:1:2 scintillation mixture. Three sources of ^{35}S-activity were used: sodium sulfate, L-cysteine hydrochloride, and 1-butanethiol, the latter being a volatile sulfur compound boiling at 97–98°C.

Excellent recoveries of ^{35}S-activity were obtained when labeled sodium sulfate or cysteine hydrochloride was added to either plant material or activated charcoal (Table II).

A good recovery was obtained also when labeled butanethiol was added to activated charcoal. This observation is important since activated charcoal provides a convenient trapping material for volatile sulfur compounds of plant origin (Asher and Grundon, 1970). However, the recovery of ^{35}S-activity from butanethiol added to plant material was very low indeed. This low recovery is attributed to escape of uncombusted gaseous butanethiol from the sample, and subsequent retention in the combustion flask due to its low solubility in water.

Whether or not detectable amounts of volatile sulfur compounds remain in plant material after oven drying is not

known. However, recent work has shown that a wide range of plant species released significant amounts of volatile sulfur compounds (0.1 to 2.0% of total sulfur) during oven drying (Grundon and Asher, 1972). Consequently, recoveries of plant sulfur are likely to be incomplete to this extent at least, regardless of the method used subsequently to estimate the sulfur content of the dry plant material.

ACKNOWLEDGMENT

The authors thank Ernest B. Bremmer of the University of Queensland for fabrication of the oxygen flasks used in the study.

LITERATURE CITED

Asher, C. J., Grundon, N. J., *Proc. Int. Grassland Congr. 11th* Australia (1970).
Beaton, J. D., Burns, G. R., Platou, J., The Sulphur Inst. Tech. Bull. No. 14 (1968).
Benson, R. H., *Anal. Chem.* **38,** 1353 (1966).
Bruno, G. A., Christian, J. E., *Anal. Chem.* **33,** 1216 (1961).
Davidson, J. D., Oliverio, V. T., *Atomlight* **60,** 1 (1967).
Dobbs, H. E., *Anal. Chem.* **35,** 783 (1963).
Fox, B. W., *Int. J. Appl. Radiat. Isotopes* **19,** 717 (1968).
Greene, R. C., Patterson, M. S., Estes, A. H., *Anal. Chem.* **40,** 2035 (1968).
Grundon, N. J., Asher, C. J., unpublished data (1972).
Kalburer, R., Rutschmann, J., *Helv. Chem. Acta* **44,** 1956 (1961).
Lisk, D. J., J. AGR. FOOD CHEM. **8,** 119 (1960).
MacDonald, A. M. G., *Analyst* **86,** 3 (1961).
Martin, L. E., Harrison, C., *Biochem. J.* **82,** 18 (1962).
Patterson, M. S., Greene, R. C., *Anal. Chem.* **37,** 854 (1965).
Tanielian, C., Cochie, C., Deluyarchie, G., Laustrait, G., Maillard, D., *Int. J. Appl. Radiat. Isotopes* **15,** 11 (1964).
Turner, J. C., *Int. J. Appl. Radiat. Isotopes* **18,** 557 (1967).
van der Laarse, J. D., *Int. J. Appl. Radiat. Isotopes* **18,** 485 (1967).
Wagner, H., *Mikrochim. Acta* **19** (1957).
Williams, P. H., *Int. J. Appl. Radiat. Isotopes* **19,** 377 (1968).

Received for review January 20, 1972. Accepted March 20, 1972. This study was supported financially by The Sulphur Institute, Washington, D.C., U.S.A., and the Australian Commonwealth Department of Education and Science.

Interactions of β-Lactoglobulin with Polyphosphates

Nicholas Melachouris

β-Lactoglobulin aggregated in the presence of polyphosphates. The extent of aggregation was affected by the type of polyphosphate used. At pH 4 or lower, aggregation of β-lactoglobulin resulted in precipitation of the protein in the form of a β-lactoglobulin–polyphosphate complex. Precipitation of β-lactoglobulin and binding of polyphosphate increased with increasing polyphosphate chain length and polyphosphate concentration or decreasing pH. At high protein concentration, the binding became independent of polyphosphate chain length. Temperatures between 5 and 73.5°C did not affect the precipitation and binding markedly. Increased ionic strength reduced significantly β-lactoglobulin precipitation, although the reduction of polyphosphate binding was slight.

Polyphosphates are used extensively by the food processing industry to improve the quality of various food products. Polyphosphates have also been used to isolate proteins from different sources under acidic conditions. Gordon (1945) has utilized the interactions of polyphosphates and proteins to recover proteins from cheese whey by the addition of polyphosphates at pH 3. Hartman (1966) has reported good precipitation of proteins from acid whey after the addition of long-chain polyphosphates at pH 2.5. Sarcoplasmic fish proteins have been isolated from aqueous solutions by precipitation with polyphosphates (Spinelli and Koury, 1970). Differences in polyphosphate binding by the main components of casein have been observed and they have been used to isolate α_s-casein by a simple procedure (Melnychyn and Wolcott, 1967). The effect of polyphosphate chain length on the binding of polyphosphates by gelatin and egg white proteins has been reported (Lyons and Siebenthal, 1966) to be consistent with the law of mass action and dependent on polyphosphate chain length. The main factors determining precipitation of human plasma proteins with polyphosphates have been shown to be polyphosphate concentration, pH, and ionic strength (Nitschmann *et al.*, 1959). Briggs (1940) has contributed significantly to the elucidation of the mechanism of protein–polyphosphate interaction by his study on the binding of metaphosphate by serum albumin.

The objective of this investigation was to study the interactions of polyphosphates with the main protein component of cheese whey, namely β-lactoglobulin. More specifically, we were interested in the effects of various factors on the precipitation of β-lactoglobulin and the binding of polyphosphates by β-lactoglobulin. Finally, changes of the β-lactoglobulin molecule resulting from the treatment with polyphosphates were investigated.

EXPERIMENTAL PROCEDURE

Materials. Two polyphosphates were used in this investigation, sodium polyphosphate with a number-average chain length $\bar{n}$ = 10.3 (commercial sodium hexametaphosphate) and sodium polyphosphate, $\bar{n}$ = 28.1. Both compounds contained a distribution of linear polyphosphate species of varying chain length. The $\bar{n}$ values were determined titrimetrically according to the procedure of Van Wazer *et al.* (1954).

Five additional phosphates were used in one experiment. All the phosphates used in this investigation were available from this laboratory. Crystallized β-lactoglobulin (Pentex, Miles Laboratories, Elkhart, Ind.) was used throughout this investigation. The molecular weight of β-lactoglobulin was assumed to be 36,000.

Measurement of Polyphosphate Binding. Polyphosphate binding was determined by precipitating the protein–poly-

Eastern Research Center, Stauffer Chemical Company, Dobbs Ferry, New York 10522.