BBA 3999

ISOLATION AND CHARACTERIZATION OF AN UNUSUAL PROTEIN FROM THE CELL NUCLEUS

LE ROY KUEHL*

Virus Laboratory, University of California, Berkeley, Calif. (U.S.A.)

(Received September 13th, 1962)

SUMMARY

A protein with unusual solubility properties has been isolated from chicken-erythrocyte nuclei. It is insoluble in water and dilute salt solutions at all pH values greater than 5, but is soluble at lower pH's. The protein has been partially purified by chromatography on phosphorylated cellulose.

Some distinguishing features of this protein include high proportions of both the acidic and the basic amino acids, a relatively low molecular weight, and an absence of N-terminal amino groups as determined by the fluorodinitrobenzene method.

INTRODUCTION

During an investigation of the proteins of the chicken erythrocyte nucleus, a protein was found which was unusual in that it was insoluble in water and dilute salt solutions at all pH values greater than 5, but soluble at pH's lower than 4. The present report deals with the purification and characterization of this protein, which is designated the neutral precipitable protein, or, more simply, the N-protein.

A protein fraction with similar solubility properties has previously been isolated from the nuclei of mouse liver, spleen and kidney, and from the nuclei of Ehrlich ascites tumor of mouse by DAVISON[1], who has used the term "neutral precipitate" to designate the material which he investigated.

METHODS

Preparation of P-cellulose

P-cellulose was prepared by treatment of Whatman cellulose powder with phosphorus oxychloride as described by PETERSON AND SOBER[2]. The concentration of phosphate groups in the product was varied by varying the amount of phosphorus oxychloride, the quantities of all other components of the reaction mixture being held constant. Upon completion of the reaction, the pH of the reaction mixture was adjusted to about 6 with hydrochloric acid. The product was then washed as described by PETERSON AND SOBER.

The concentration of phosphate groups in each preparation was determined by

Abbreviations: N-protein, neutral precipitable protein; P-cellulose, phosphorylated cellulose.

* Present address: Max Planck Institut für Biologie, Tübingen (Germany).

titration. A 1-g sample of the cellulose derivative was weighed accurately and suspended in a small amount of water. The resulting slurry was adjusted to pH 4.5 with hydrochloric acid, then titrated to pH 9.5 with a standard solution of sodium hydroxide. A 1-g sample of unmodified cellulose was run as a blank. Duplicate titrations were performed on each preparation. The number of moles of alkali required to bring the pH of the P-cellulose slurry from 4.5 to 9.5 was set equal to the number of moles of phosphate group in the sample.

Protein analyses

Protein was determined by the method of LOWRY *et al.*[3], using histone II (a lysine-rich histone fraction) prepared according to UI[4] as a standard. Histone II gave a color value approx. 10 % higher than that of bovine plasma albumin and approx. 10 % lower than that of histone I (an arginine-rich histone fraction). Moisture corrections were determined by drying separate samples of each protein for 5 h at 103–105° over phosphorus pentoxide *in vacuo*.

Since different proteins give different color values by the LOWRY method, confidence in the accuracy of the analytical values is justified only for the protein against which the method is standardized. To overcome this difficulty, a correction factor was determined for each protein fraction of interest. Triplicate protein determinations were performed on accurately weighed portions of a lyophilized sample of each protein fraction. Appropriate moisture corrections were determined by drying separate portions of the lyophilized material for 48 h at room temperature over phosphorus pentoxide *in vacuo*. From the data thus obtained, a factor for converting the amount of protein as determined by the LOWRY method standardized against histone II to the amount of protein based on dry weight of the lyophilized material was calculated for each fraction. This correction factor, once determined for a particular protein fraction, was subsequently applied to all analytical values for that fraction. Correction factors ranged from 0.94 to 1.09.

Amino acid analyses

Separation and quantitative estimation of the amino acids in acid hydrolyzates of various protein fractions were performed with a Beckman/Spinco amino acid analyzer, Model MS. The prototype of this instrument is described in papers by SPACKMAN, STEIN AND MOORE[5,6]. Factors for converting the areas under the amino acid peaks to μmoles of the corresponding amino acids were determined from calibration runs made with a standard solution containing 1.00 μmole of each amino acid. Two calibration runs were performed, one immediately before, and one immediately after analysis of the protein hydrolyzates.

Protein samples for analysis were hydrolyzed 39 h at 110° *in vacuo* in redistilled 5.7 M hydrochloric acid. A protein:hydrochloric acid ratio of approx. 1:500 was employed. After hydrolysis the samples were evaporated to dryness over sodium hydroxide. To remove last traces of free hydrochloric acid the residues were suspended in water and evaporated to dryness several times. No corrections for destruction of amino acids during hydrolysis were made.

Tryptophan, which is destroyed during acid hydrolysis, was estimated separately by two different methods. Colorimetrically tryptophan was determined by WINKLER'S modification of the Hopkins–Cole procedure[7]. A solution of the pure DL-amino acid

served as the standard. Tryptophan was alternatively estimated as described by BEAVEN AND HOLIDAY[8]. By this method the tryptophan content of a protein is determined from its ultraviolet absorption spectrum in 0.1 M sodium hydroxide solution. Some of the protein fractions in the present study were not soluble in 0.1 M alkali. In these instances the protein was suspended in 1 M sodium hydroxide and incubated at 50° for 30–120 min to dissolve the protein. Tryptophan was determined from the ultraviolet absorption spectrum of the resulting solution. The method of BEAVEN AND HOLIDAY simultaneously yields a tyrosine value for the protein under consideration. Tryptophan values obtained by the ultraviolet absorption method were in satisfactory agreement with those obtained by the colorimetric procedure.

End group determinations

Amino end group analyses were performed by the FDNB method as described by LEVY[9]. It proved more convenient to suspend the protein sample for analysis in about 100 times its weight of water than to use the proportions recommended by LEVY. To maintain a FDNB:protein ratio similar to that recommended by LEVY, the concentration of FDNB in the ethanolic solution was reduced to 1 % (v/v). DNP-proteins were hydrolyzed in redistilled 5.7 M hydrochloric acid for 16 h at 105° *in vacuo*. The protein:hydrochloric acid ratio was approx. 1:200. For detection of N-terminal glycine and proline an additional sample of the DNP-protein was hydrolyzed for 4 h at 105° with concentrated hydrochloric acid.

Preparation of nuclei

Chicken-erythrocyte nuclei were prepared by a modification of the method of DOUNCE AND LAN[10]. Blood was usually collected by heart puncture in a syringe wetted with a 1 % heparin solution. In some instances larger quantities of blood were obtained by severing the carotid arteries and bleeding a number of chickens into a bucket containing, for each liter of blood to be collected, 50 mg of heparin dissolved in 25 ml of 0.15 M sodium chloride. The erythrocytes were sedimented and the supernatant solution, along with the white cells, which form a layer on top of the packed erythrocytes, were discarded. The erythrocytes were washed three times with 0.15 M sodium chloride solution and suspended in 0.15 M sodium chloride to a volume equal to the original volume of blood. To each 100 ml of this suspension was added 100 mg of digitonin suspended in 5 ml of 0.11 M sodium phosphate buffer (pH 6.9). Laking of the erythrocytes was complete in 10 min. The nuclei were sedimented and washed with 0.15 M sodium chloride solution until the washings were colorless, then suspended in 0.15 M sodium chloride to a volume 2.5 times that of the original volume of blood. The isolation procedure was performed at 5°.

Preparation of crude N-protein

To 100 ml of a suspension of nuclei, prepared as described above, was added 100 ml of 3 M sodium chloride solution. The resulting thick gel was blended a few minutes in a Waring blendor, giving rise to a viscous, slightly turbid solution. To this solution was added with stirring 140 ml of 95 % ethanol prechilled to −80°. In this manner the DNA was nearly quantitatively precipitated as a fibrous mass, which was wound around a stirring rod and removed. A lipid-rich protein fraction, representing about 30 % of the protein in the isolated nuclei, accompanied the DNA.

The solution remaining after removal of the DNA and lipoprotein was clear. This solution was poured into an equal volume of 0.1 M sodium citrate solution adjusted to pH 7.0 and the resulting mixture allowed to stand overnight. During this time the N-protein precipitated and settled to the bottom of the container. The overlying clear solution, which contained the histone fraction representing about 60 % of the protein present in the isolated nuclei, was decanted and the underlying suspension centrifuged to sediment the protein precipitate. The sediment was washed with three 7-ml portions of distilled water and dissolved in 7 ml of 0.01 M hydrochloric acid (pH 2.0) or in 7 ml of 0.2 M citric acid (pH 2.0). Insoluble material was removed by centrifuging 60 min at 78000 × *g*. The entire preparative procedure was performed at a temperature of 5°.

N-protein was sometimes precipitated by dialysis against distilled water or 0.05 M phosphate buffer (pH 7.0) rather than by addition of 0.1 M sodium citrate solution. In one instance the 0.1 M sodium citrate was replaced by an equal volume of 0.05 M sodium phosphate buffer (pH 7.0). These modifications were, in general, less satisfactory than the first-described method.

Reprecipitation of N-protein

If a solution of N-protein, prepared as described above, is dialyzed against 0.05 M sodium phosphate buffer (pH 7.0) or, in the case where the protein is dissolved in 0.01 M hydrochloric acid, is simply mixed with an equal volume of the phosphate buffer, about 40 % of the protein fails to precipitate. The tryptophan content of the reprecipitated protein is about 5 times that of the protein remaining soluble, indicating that a separation of two distinctly different protein fractions has occurred. If the reprecipitated protein is dissolved in acid and reprecipitated a second time, about 15 % of the total protein remains soluble. Very little protein remains soluble upon a third reprecipitation. Crude N-protein which has been purified by two or three reprecipitations at neutral pH will be designated reprecipitated N-protein.

Characterization of N-protein

Solubility

N-protein is insoluble not only at neutrality, but also in the alkaline pH region. Even in 0.1 M sodium hydroxide it fails to dissolve. The solubility in the acid pH region as a function of pH and ionic strength is given in Fig. 1. An increase in either pH or ionic strength results in a decrease in solubility of the protein. Interestingly, at higher pH values the effect of ionic strength on solubility becomes less; at pH 5 ionic strength has little or no influence on the solubility of N-protein.

Chromatographic behavior

The similarity between N-protein and a previously described protein fraction, the neutral precipitate, has been indicated in the INTRODUCTION. DAVISON succeeded in separating the latter protein fraction into three subfractions by chromatography on CM-cellulose at low pH (see ref. 1). Attempts to separate N-protein into subfractions by means of a similar chromatographic system were not successful.

Attempts to chromatograph N-protein on Dowex-50, a sulfonic acid resin; on Amberlite IRC-50, a carboxylic acid resin; and on a commercial preparation of P-cellulose were unsuccessful. In salt solutions in which the protein is soluble (pH

0–3.6; I 0.001–0.4) it was, in every case, bound completely and irreversibly to the resin. In the pH range 0–3.6 the carboxyl groups of Amberlite IRC-50 are largely in the unionized form[11]. In this case, at least, multiple ion–ion interactions between the charged groups of the protein and those of the resin do not seem to account for the irreversible binding. Indeed, it was observed that urea, which presumably has little effect on ion–ion interactions, could, in high concentrations, release part of the N-protein from Amberlite IRC-50.

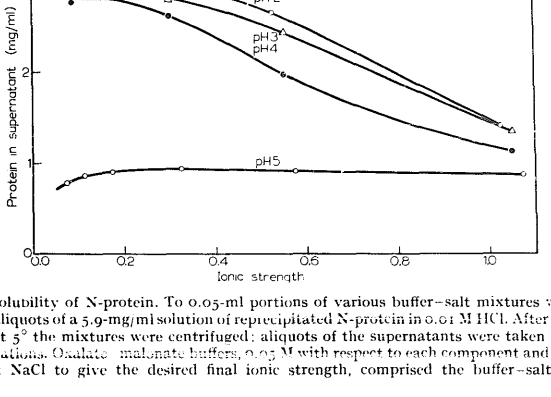

Fig. 1. Solubility of N-protein. To 0.05-ml portions of various buffer–salt mixtures were added 0.05-ml aliquots of a 5.9-mg/ml solution of reprecipitated N-protein in 0.01 M HCl. After incubating 75 min at 5° the mixtures were centrifuged; aliquots of the supernatants were taken for protein determinations. Oxalate–malonate buffers, 0.05 M with respect to each component and containing sufficient NaCl to give the desired final ionic strength, comprised the buffer–salt mixtures.

With a view to eliminating the irreversible binding of the protein by the resin, chromatography was attempted on P-cellulose preparations with lower contents of phosphate group than the usual commercial products. On preparations with about 0.025 as high a concentration of phosphate groups as the commercial resins, it proved possible to separate reprecipitated N-protein into three main fractions, designated in order of their appearance from the column as Fractions 1, 2, and 3. The exact conditions required for chromatography of the protein were determined by gradient elution using solutions of salt and urea. Subsequently the procedure was modified to make use of the more convenient technique of stepwise elution.

Solutions containing divalent metal ions were more effective in eluting the protein from the modified cellulose than sodium chloride solutions of equivalent ionic strength. Quantitative elution of the protein could not be accomplished with salt solutions alone. To elute the final protein fraction it was necessary to use solutions containing both salt and urea.

A typical elution pattern is presented in Fig. 2. In this pattern 6 % of the total applied protein (as determined by absorbancy) has emerged from the column unretarded; 20 % is found in peak 1, 31 % in peak 2 and 28 % in peak 3. In this instance recovery of the applied protein was 85 %; recoveries greater than 95 % were, however, occasionally obtained. From 1 to 3 % of the total applied protein always emerged

unretarded from the column. When the column was overloaded the proportion of this protein increased. The pattern shown in Fig. 2 was obtained from a slightly overloaded column.

The chromatographic behavior of the protein was extremely sensitive to the concentration of phosphate groups on the cellulose. This is illustrated in Table I, in which are tabulated the concentrations of Ba^{2+} and urea necessary to elute the various fractions from columns of P-cellulose containing various amounts of phosphate group per gram of resin. Corresponding fractions from different columns had equivalent tyrosine and tryptophan contents and were, therefore, presumed to be essentially identical.

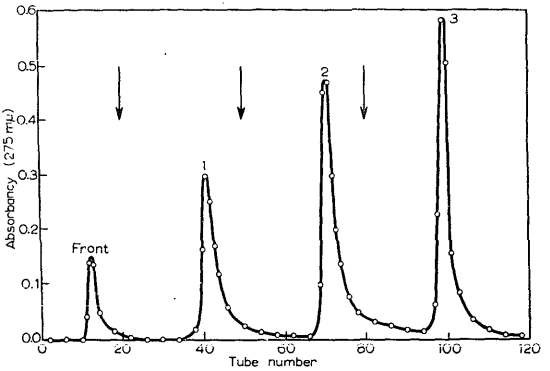

Fig. 2. Chromatography of reprecipitated N-protein. A 4.0-ml aliquot of a 0.01 M HCl solution containing 4.2 mg/ml of reprecipitated N-protein was applied to a 1.1 × 28 cm column of P-cellulose previously equilibrated with 0.01 M HCl. The P-cellulose employed contained 0.015 mmoles of phosphate/g resin. Elution was begun with 0.01 M HCl; at the position indicated by the first arrow the effluent was changed to 0.008 M $BaCl_2$–0.01 M HCl; at the second arrow, to 0.020 M $BaCl_2$–0.01 M HCl; and at the third arrow, to 3.5 M urea–0.025 M $BaCl_2$–0.01 M HCl. The flow rate was maintained at about 16 ml/h; 2.0-ml fractions were collected. The amount of protein in each fraction was estimated from the absorbancy at 275 mμ.

TABLE I

INFLUENCE OF PHOSPHATE GROUP CONCENTRATION ON CHROMATOGRAPHIC BEHAVIOR OF N-PROTEIN ON P-CELLULOSE

Reprecipitated N-protein was chromatographed on 1.1 × 28 cm columns of P-cellulose of varying phosphate group concentrations. Elution was accomplished with 0.01 M HCl solutions containing concentration gradients of $BaCl_2$ and urea.

Fraction	*Concentrations of $BaCl_2$ and urea in eluant*			
	0.010 mmoles P/g resin	*0.013 mmoles P/g resin*	*0.015 mmoles P/g resin*	*0.065 mmoles P/g resin*
1	No well	0.005 M $BaCl_2$	0.008 M $BaCl_2$	Protein bound
2	resolved peaks	0.009 M $BaCl_2$	0.020 M $BaCl_2$	irreversibly
3	obtained	0.010 M $BaCl_2$ 2.5 M urea	0.025 M $BaCl_2$ 3.5 M urea	to resin

In Figs. 3 and 4 are presented patterns obtained upon rechromatography of Fractions 2 and 3. In each of these patterns Fraction 1 is essentially absent, but Fraction 2 and Fraction 3 both appear. These results suggest that Fractions 2 and 3 are two different forms of the same protein—perhaps different aggregation states—in equilibrium with one another. Alternatively, a single protein may have been separated into two peaks through a chromatographic artifact. That Fraction 2 and Fraction 3 represent essentially a single protein species is further indicated by the following: (a) The amino acid compositions of the two fractions, which will be presented in a subsequent section, are very similar. (b) The two fractions are alike in solubility

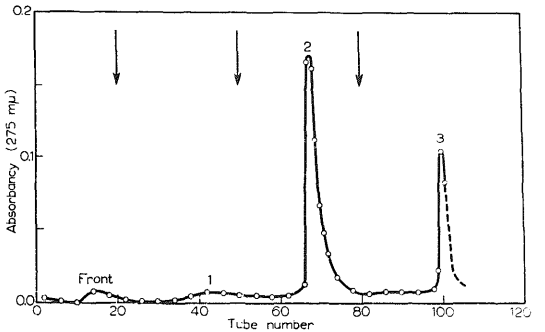

Fig. 3. Rechromatography of Fraction 2. The chromatographic procedure was as described in the text to Fig. 2. A 1.25-ml aliquot of a 0.01 M HCl solution containing 1.7 mg/ml of Fraction 2 was applied to the column.

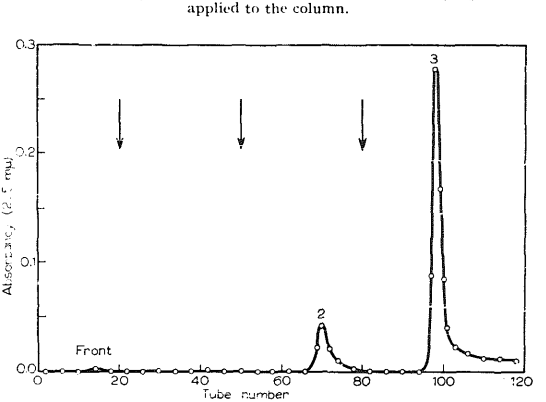

Fig. 4. Rechromatography of Fraction 3. The chromatographic procedure was as described in the text to Fig. 2. A 1.22-ml aliquot of a 0.01 M HCl solution containing 1.5 mg/ml of Fraction 3 was applied to the column.

behavior. Both are soluble at low pH's and insoluble at neutral and alkaline pH values.

On the basis of its solubility, the protein of Fraction 2 and Fraction 3 may be identified as N-protein.

Fraction 1 represents a contaminating protein, distinctly different from the N-protein of Fractions 2 and 3. This conclusion is based on the following evidence: (a) Rechromatography of Fraction 1 gives a pattern in which only small amounts of material appear in the positions of Fractions 2 and 3. (b) As will be shown in a later section, the amino acid composition of Fraction 1 is quite different from that of Fractions 2 and 3. (c) Fraction 1 is soluble not only in the acid, but also in the neutral and alkaline pH regions.

The amount of protein recovered in chromatographic Fractions 2 and 3 usually represented about 6 % of that present in the isolated nuclei. Since some N-protein is undoubtedly lost during the isolation procedure, the amount originally present in the isolated nuclei must be greater than 6 %.

Amino acid composition

The amino acid compositions of the three main fractions obtained upon chromatography of reprecipitated N-protein are presented in Table II. The similarity in composition between Fractions 2 and 3, which collectively represent N-protein, has already been mentioned. It should be noted that, although the amino acid compositions of Fractions 2 and 3 are very similar, the analytical value for nearly every amino

TABLE II

AMINO ACID COMPOSITIONS OF CHROMATOGRAPHIC FRACTIONS

Each value, except those obtained from the ultraviolet absorption spectra of the protein fractions, represents the average of two determinations, each made on a separate 39-h hydrolyzate.

Amino acid	*Mole percent amino acid*		
	Fraction 1	*Fraction 2*	*Fraction 3*
Aspartic acid	5.6	9.3	9.6
Glutamic acid	8.5	13.6	15.4
Glycine	14.0	7.7	6.7
Alanine	8.7	9.0	9.2
Valine	7.4	5.4	5.0
Leucine	7.9	8.7	9.7
Isoleucine	5.0	4.4	4.1
Serine	3.0	5.9	5.8
Threonine	6.6	5.1	4.6
½ Cystine	0.00	0.60	0.90
Methionine	1.11	1.8	1.9
Proline	2.7	4.7	3.5
Tryptophan	0.11*	1.1*	1.4*
Phenylalanine	2.2	3.3	3.0
Tyrosine	3.3	2.8	2.5
	3.0*	2.7*	2.2*
Histidine	1.9	2.1	2.3
Lysine	9.7	7.7	7.5
Arginine	12.1	7.2	7.6
Ammonia	13	20	17

* Values obtained from ultraviolet absorption spectra.

acid in Fraction 2 lies between that for the corresponding amino acid in Fraction 1 and Fraction 3. This is most readily explained by assuming that Fraction 2 is still slightly contaminated with Fraction 1.

As seen in Table II, N-protein contains relatively high proportions of both the basic and the acidic amino acids. As judged from the amount of ammonia in the hydrolyzates, a large proportion of the acidic groups occur as the amides so that, notwithstanding a higher mole percent of acidic amino acids than basic ones, the protein is probably somewhat basic. Certainly it is less basic than the histones, however. N-protein is further distinguished from the histones by its content of tryptophan and cystine (or cysteine), amino acids not found in purified histone preparations.

IKAWA AND SNELL[12] have pointed out that upon evaporation of acid hydrolyzates of proteins over sodium hydroxide, glutamic acid may react with serine to form *O*-(γ-L-glutamyl)-L-serine. This compound emerges from the Beckman/Spinco amino acid analyzer shortly after alanine and before cystine, and in a protein hydrolyzate lacking cystine might be mistaken for this amino acid. That this was not the case in the present work is indicated by the following: (a) Fraction 1, which contains both glutamic acid and serine, gave no cystine peak, although hydrolysis of this fraction was performed in exactly the same manner as for Fractions 2 and 3. (b) The cystine peaks of Fractions 2 and 3 were not displaced toward the alanine peak, but rather emerged in the position expected for cystine.

As an estimation of the losses incurred during hydrolysis of the protein fractions and chromatography of the resulting amino acid mixtures, the total weight of the amino acids recovered from each column was determined, corrected for the amount of water gained during hydrolysis of the protein, and compared with the amount of protein originally taken for hydrolysis. The average recovery calculated in this manner for Fraction 1 was 88%, for Fraction 2, 93% and for Fraction 3, 94%.

Ultracentrifugal behavior

In Fig. 5 is presented a pattern obtained upon centrifugation of a solution of reprecipitated N-protein in 0.2 M citric acid (pH 2.0). The protein has separated into two peaks. For the light peak, representing the major portion of the protein, the sedimentation coefficient, $s_{20,w}$, has a value of 0.8 S. The molecular weight of this component is estimated to be between 3000 and 10000. The heavy peak has a sedimentation coefficient of 1.9 S. The molecular weight of this component, which may be an aggregate of the lighter component, probably is between 10000 and 30000.

End group determinations

Freshly prepared, crude N protein has, as determined by the FDNB method, about 12 μmoles of N-terminal alanine/g of protein (1 N-terminal group per 83000 molecular weight) and traces of N-terminal glutamic acid, threonine, and serine. Upon incubation of the protein at 5° either in solution at pH 2 or as a suspension at pH 7, several new N-terminal amino acids appear and some of the N-terminal amino acids originally present increase in amount. This is illustrated in Table III.

Since chromatography of the N-protein and subsequent concentration of the chromatographic fractions normally required about a week to accomplish, the appearance in the chromatographic fractions of N-terminal amino acids greater in both variety and amount than found in the crude N-protein was not unexpected.

As indicated in Table III, the amounts of terminal alanine, the principal amino terminal amino acid, and of serine do not change appreciably upon incubation at 5°. In Table IV are given the amounts of these two N-terminal amino acids found in each of the three main fractions obtained upon chromatography of reprecipitated N-protein. That the values for Fraction 2 fall between those for Fractions 1 and 3, supports the possibility, suggested on the basis of the amino acid analyses, that Fraction 2 may be contaminated with Fraction 1 and that Fraction 3, consequently, represents a more purified form of N-protein than Fraction 2.

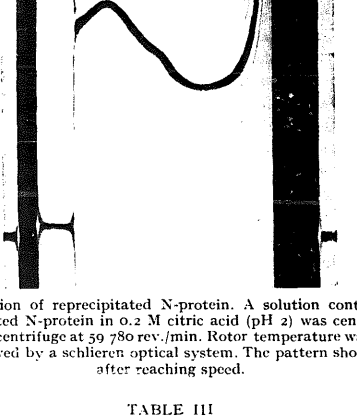

Fig. 5. Ultracentrifugation of reprecipitated N-protein. A solution containing approximately 10 mg/ml of reprecipitated N-protein in 0.2 M citric acid (pH 2) was centrifuged in the Spinco Model E analytical ultracentrifuge at 59 780 rev./min. Rotor temperature was 4.5°. Sedimentation of the protein was followed by a schlieren optical system. The pattern shown was made 248 min after reaching speed.

TABLE III

INCREASE IN END GROUPS UPON INCUBATION AT 5°

Into each of six centrifuge tubes was pipetted a 1.0-ml aliquot of a suspension containing 7.8 mg/ml of freshly prepared, crude N-protein in 0.02 M sodium citrate solution (pH 7). The tubes were centrifuged. The sediments in three tubes were resuspended in 1.0 ml aliquots of 0.02 M sodium citrate (pH 7.0), the remaining three sediments were dissolved in 1.0-ml aliquots of HCl (final pH 2.0). After incubation at 5° for various times, one of the samples at pH 2 and one of those at pH 7 were analyzed for N-terminal amino acids by the FDNB method.

Amino acid	μmoles N-terminal amino acid/g protein					
	Incubated at pH 2			Incubated at pH 7		
	3 h	72 h	240 h	3 h	72 h	240 h
Glutamic acid	0.7	1.0	1.2	0.4	0.5	0.7
Threonine	0.9	1.4	1.5	0.7	0.8	1.0
Serine	1.3	0.8	1.0	0.9	0.9	1.0
Alanine	11.9	11.6	12.6	11.0	11.9	11.2
Phenylalanine	0.0	5.9	7.6	0.0	0.0	0.0
Leucine–isoleucine	0.0	1.0	1.5	0.0	0.7	0.9
Valine	0.0	0.5	0.8	0.0	0.4	0.4

Alanine, the most abundant N-terminal amino acid in Fraction 3, accounts for only one end group per 330000 molecular weight. Since the sedimentation data for reprecipitated N-protein indicate no component with a molecular weight greater than 30000, the bulk of the protein of Fraction 3, which presumably represents N-protein, has no N-terminal amino acid as determined by the FDNB method.

TABLE IV

N-TERMINAL SERINE AND ALANINE OF CHROMATOGRAPHIC FRACTIONS

Amino terminal amino acids other than alanine and serine have not been included (see text).

Amino acid	*μmoles N-terminal amino acid/g protein*		
	Fraction 1	*Fraction 2*	*Fraction 3*
Serine	0.9	0.6	0.1
Alanine	22.6	13.1	3.0

The end group values presented in this paper have not been corrected for destruction during hydrolysis and chromatography. However, several determinations on reprecipitated N-protein were performed in which known amounts of DNP-alanine were added to the DNP-protein prior to hydrolysis. Recovery of the added DNP-amino acid after hydrolysis and chromatography averaged 80 %.

Possibility of protease activity during preparation of N-protein

The finding that N-protein, which itself seemed to have no N-terminal amino acid as determined by the FDNB method, was contaminated by peptides or proteins having a variety of N-terminal amino acids, and that the amount and variety of these amino terminal groups increased upon incubation, suggested the possibility of protease activity during preparation of N-protein. To test this possibility, reprecipitated N-protein was prepared by three methods: one designed to permit maximum activity of intracellular enzymes (incubated sample), another employing DFP, a powerful inhibitor of many common proteolytic enzymes (DFP-treated sample), and a third by the usual method of preparation (control sample). The yield of protein and the number and amount of N-terminal amino acids in the isolated protein were compared for the three isolation methods.

Washed erythrocytes from 60 ml of chicken blood were divided into three equal portions. Each of these served as starting material for one of the isolation procedures.

The control sample was prepared as previously described. Total time required for the isolation procedure was about 4 h.

The procedure for preparation of the incubated sample was identical with that of the control except that twice during the isolation the sample was allowed to incubate at 37° for several hours. After treatment of the erythrocytes with digitonin, the lysed cell suspension was allowed to incubate 2 h at the elevated temperature before continuing with the isolation of the nuclei. The gel formed from these nuclei, after brief treatment in the Waring blendor, was put at 37° for 3.5 h prior to removal of the DNA and lipoprotein by addition of ethanol.

The procedure for preparation of the DFP-treated sample was, again, essentially the same as for the control. In this case, however, DFP was added to a final concen-

tration of 2 mM to several of the solutions used in the isolation procedure. The aqueous solutions of DFP were prepared from a 0.5 M solution of DFP in dry isopropanol immediately prior to using. Three of the 0.15 M sodium chloride solutions used in the preparative procedure contained DFP: the solution in which the washed erythrocytes were suspended during treatment with digitonin, the solution in which the sedimented nuclei were resuspended immediately after treatment with digitonin, and the solution in which the isolated nuclei were finally suspended.

TABLE V

YIELDS AND N-TERMINAL GROUPS OF N-PROTEIN ISOLATED UNDER CONDITIONS OF VARYING FAVORABILITY FOR PROTEASE ACTIVITY

For experimental details see text.

	Control sample	*Incubated sample*	*DFP treated sample*
Yield of protein (mg)	15.9	17.2	11.6*
Amount of N-terminal amino acid (μmoles/g)			
Glutamic acid	0.5	0.6	0.6
Threonine	1.0	0.8	0.6
Serine	0.5	0.4	0.6
Alanine	10.2	12.7	11.0
Methionine (?)		0.6	

* Roughly a third of the sample was lost through breakage of a centrifuge tube.

The yield of protein and the N-terminal amino acid analysis for the protein prepared by each of the three isolation methods is presented in Table V. The results indicate no significant level of protease activity during isolation of N-protein.

DISCUSSION AND CONCLUSIONS

A search through the literature dealing with proteins of the cell nucleus has revealed but a single reference to a protein displaying the solubility behavior of N-protein. This reference is the previously mentioned paper by DAVIDSON[1] in which the isolation and characterization of the neutral precipitate are described. N-protein differs from the neutral precipitate in amino acid composition and probably also in its chromatographic behavior. Because the two protein fractions are so similar to one another and so different from other nuclear proteins in solubility behavior, however, they may well have an analogous function, the differences reflecting a species or organ specificity. The name, neutral precipitable protein (abbreviated N-protein throughout this work) was chosen to indicate the similarity to the neutral precipitate described by DAVISON.

The procedure for isolation of N-protein involves the use of digitonin and of solutions of low pH, either of which might possibly act as a denaturing agent. The possibility that N-protein prepared as described is denatured can, therefore, not be dismissed. This possibility has been kept in mind when estimating molecular weights from the experimentally determined sedimentation constants; even if the proteins of interest have a highly unfolded structure, such as is characteristic of denatured

proteins, the true molecular weights still probably fall within the indicated range. The possibility also exists that the N-protein represents a part of some larger structure which has been broken into subunits during the isolation procedure. Should this be the case, then the molecular weight values given for the N-protein cannot, of course, reflect the molecular weight of the larger structure.

Nothing can be said concerning the function of N-protein. This, however, is true of most of the nuclear proteins, including the histones, which have been intensively studied for over three quarters of a century. Lacking is a generally applicable method whereby, given a specific protein, its function in the living organism may be determined. The opposite approach, that of seeking out the proteins which perform various processes known to occur in the living organism has, to date, proved more feasible and has provided us with most of our knowledge of protein function.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the guidance of Professor H. FRAENKEL-CONRAT throughout the course of this work. Thanks are also due to Professor H. K. SCHACHMAN and Miss J. MILLER for performing the ultracentrifuge analysis and to Dr. A. TSUGITA for assistance in performing the amino acid analyses. This investigation was aided by a grant from the National Science Foundation. The author was a Predoctoral Fellow, National Science Foundation, 1957–1960.

REFERENCES

1 P. F. DAVISON, *Biochem. J.*, 66 (1957) 703.
2 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
3 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
4 N. UI, *Biochim. Biophys. Acta*, 25 (1957) 493.
5 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
6 S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
7 S. WINKLER, *Z. Physiol. Chem.*, 228 (1934) 50.
8 G. H. BEAVEN AND E. R. HOLIDAY, *Advan. Protein Chem.*, 7 (1952) 319.
9 A. L. LEVY, *Methods of Biochem. Anal.*, 2 (1955) 360.
10 A. L. DOUNCE AND T. H. LAN, *Science*, 97 (1943) 584.
11 N. E. TOPP AND K. W. PEPPER, *J. Chem. Soc.*, (1949) 3299.
12 M. IKAWA AND E. E. SNELL, *J. Biol. Chem.*, 236 (1961) 1955.