PROTEIN COMPONENTS OF CHICKEN ERYTHROCYTE NUCLEI*

by

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Several attempts have been made in recent years to identify the major chemical components of cellular nuclei. MAYER AND GULICK[2], MIRSKY AND POLLISTER[3], and DALLAM *et al.*[4] found that besides the two long-known components: desoxyribonucleic acid (DNA) and histone, a third, a non-histonic protein, was present. A similar statement has been made by the STEDMANS[5,6], who postulated that the non-histonic protein was responsible for the Feulgen reaction of chromosomes. The latter claim has been contested by several authors[7-11].

The purpose of the present work was to fractionate and to analyse protein components of chicken erythrocyte nuclei making use of crystalline desoxyribonuclease of KUNITZ[12,13], which is protease free.

EXPERIMENTAL

Material. Chicken blood was obtained from the local slaughter house and was worked up in 500 ml portions. Nuclei were prepared according to the method of DOUNCE AND LAN[14]. The two final washings were done with isotonic saline without phosphate.

An aliquot was analysed for the phosphorus containing compounds according to the method of SCHMIDT AND THANNHAUSER[15]. The results are shown in Table I. The amount of acid soluble phosphorus present in nuclei was rather variable, but significant, accounting for up to 8% (exp. 4). This variation could have been due to an incomplete removal of the phosphate buffer. The quantities of ribonucleic acid were small, but possibly significant, while the amount of phosphoprotein was within the limits of experimental error and is not considered significant.

TABLE I

ANALYSIS OF NUCLEI FOR PHOSPHORUS CONTAINING COMPOUNDS ACCORDING TO SCHMIDT AND THANNHAUSER

Total mg P	*Inorg.* mg P	*Acid Sol* mg P	*Lipid* mg P	*RNA* mg P	*Phospho-protein* mg P	*DNA* mg P
124.0	1.27	7.96	—	—	—	—
132.5	2.23	3.93	16.9	4.8	1.6	—
132.7	1.96	4.35	12.8	4.1	0.4	112.0
130.6	1.23	10.38	13.4	3.0	1.6	102.2

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Fractionation procedure. The major portion of the nuclei was centrifuged down from the last wash fluid, the washings were discarded, the nuclei were placed in a Waring blendor with 500 ml of water and were blended for 3 minutes at full speed. The pH of the mixture was adjusted to 7.0 with 0.1 *N* NaOH. The mixture was brought up to 37°, and magnesium sulfate was added to attain a concentration of 0.025 *M*. Once recrystallized desoxyribonuclease, prepared according to KUNITZ[12,13], was dissolved in water and portions containing 500 gammas of enzyme were added at 30 minute intervals. The pH of the mixture was kept constant by adding 0.1 *N* NaOH when required, and the progress of the reaction was checked by determining the acid soluble phosphorus[16]. Usually, the acid soluble phosphorus became constant after ten portions of enzyme had been added. The mixture was stored at 5° overnight and was centrifuged at the same temperature at 4000 r.p.m. for an hour.

The supernatant was referred to as "neutral extract 1". Residue 1 was treated with 250 ml of 0.1 *N* HCl and left till the next morning at 5°. The mixture was centrifuged at 4000 r.p.m. at 5° and "acid extract 2" was collected. An aliquot of "residue 2" was also analysed (Table II).

TABLE II

NITROGEN AND PHOSPHORUS DISTRIBUTION IN FRACTIONS

Fraction	*Sample*	*N (mg)*	*P (mg)*	*N/P*
Nuclei	1	867.0	290.7	2.9
Nuclei	2	841.7	307.7	2.7
Nuclei	3	707.1	285.3	2.4
Neutral extract 1	1	331.3	194.8	1.7
Neutral extract 1	2	302.9	202.8	1.5
Neutral extract 1	3	251.9	188.2	1.3
Acid extract 2	1	304.2	65.0	4.6
Acid extract 2	2	332.6	67.3	4.9
Acid extract 2	3	268.3	56.2	4.8
Residue 2	1	200.0	44.3	4.5
Residue 2	2	217.0	55.0	3.9
Residue 2	3	205.0	48.5	4.2

Further fractionation of "residue 2" was not quantitative. "Residue 2" was treated with 75 ml of 1 *N* KOH, and left at 5° overnight, after which time the mixture was adjusted with HCl to pH 8.5. It was then filtered with suction through a small Buchner funnel using Whatman filter paper No. 50 covered with a ½ cm pad of celite No. 545. The yellow liquid obtained was referred to as "alkaline extract 3". Residue 3 was discarded.

Characterization of fractions. Table II indicates the distribution of phosphorus and nitrogen in extracts 1 and 2 and residue 2. Protein nitrogen accounted only for a small percentage of the total nitrogen in "neutral extract 1" as was found by precipitation with trichloroacetic acid. No attempts were made to characterize these soluble proteins because of their low concentrations. It was also realized that a large part of the soluble protein fraction already had been lost during the preparation of nuclei in aqueous saline solutions (see discussion).

The expected N/P ratio for the degradation products of nucleic acids is around 1.7. The lower ratio seen in "neutral extract 1" was due to the presence of other phosphorus containing compounds (see Table I).

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The "acid extract 2" still contained some acid-soluble phosphorus, but proteins were the major component. The N/P ratio was almost twice as high as that of the original nuclei. When "acid extract 2" was adjusted to pH 7 and the concentration of sodium sulfate was brought up to 70% saturation at 37° C, 65% of the total nitrogen and only 19% of the total phosphorus were precipitated. Most of the phosphorus-containing compounds remained in the solution, which showed an N/P ratio around 2.

The precipitate obtained from "acid fraction 2" by 70% saturation with sodium sulfate at pH 7.0 was composed of histones. When heated with a mixture of $HgSO_4$–H_2SO_4 and nitrite[2,17] a clear red solution was obtained, indicating the absence of proteins of the albumin or globulin type. After the mixture had been cooled, no precipitate appeared, indicating the absence of protamines.

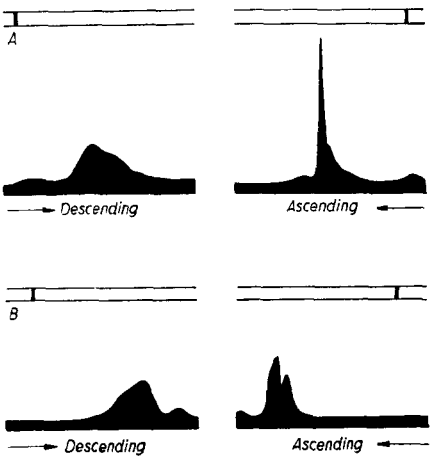

Fig. 1. A. Electrophoretic patterns of histones in 0.1 μ acetate buffer at pH 4.95 after 100 minutes in a field of 5.5 volts per cm. B. Electrophoretic patterns of histones in a 0.1 μ acetate buffer at pH 6.08 after 160 minutes in a field of 6.4 volts per cm.

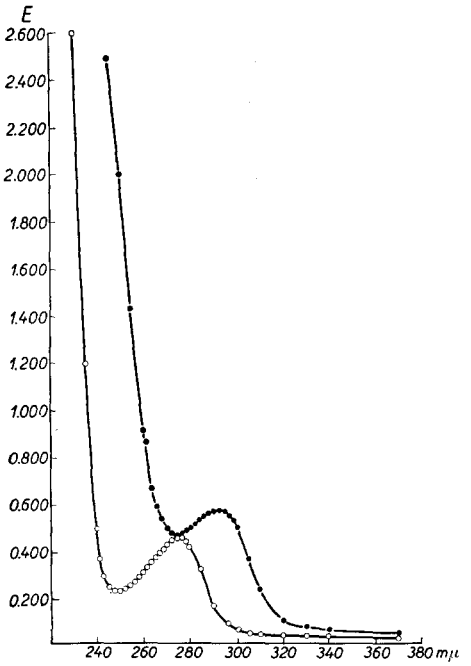

Fig. 2. Ultraviolet absorption spectra of histones from chicken erythrocytes. Concentration of histones 915 μg per ml. ● — in 0.1 *N* NaOH; ○ — in 0.1 *N* HCl. Silica cell, 1 cm, Beckman spectrophotometer.

Electrophoretic studies revealed that this fraction was not homogeneous. Fig. 1 shows typical electrophoretic patterns obtained with this histone fraction; it indicates the presence of several, apparently closely related, histone-type proteins. The heterogeneity was most pronounced at the higher pH values (around 6). A similar conclusion that more than one kind of histone is present in one tissue was reached by MIRSKY AND RIS[18] in respect to thymus. Their evidence is based on the finding that the tryptophan content of histone released by HCl is less than the tryptophan content of the total thymus histone.

The ultraviolet absorption spectra of the histone fraction were investigated (Fig. 2). The curve obtained with acid solutions agrees well with that published by ALLGEN[19]. The content of tryptophan and tyrosine, calculated from the curve obtained with alkaline solutions according to GOODWIN AND MORTON[20], was 0.36 and 3.62% respectively. The results are discussed below.

The "alkaline extract 3" contained only a small amount of phosphorus. Direct electrophoretic examination of "extract 3" was not possible since it was too dilute in respect to

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protein. However, by adjusting it to pH 4.5 the protein was precipitated almost quantitatively, and was concentrated by redissolving at pH 8.5.

The protein thus obtained was examined electrophoretically. It was possible to obtain patterns in the pH range from 6 to 8, and in acid, below pH 3.5. Between pH 6 and 3.5 the protein was almost completely insoluble. Fig. 3 shows the electrophoretic patterns obtained above and below the isoelectric point. Both show one major component and are rather symmetrical. Fig. 4 indicates the mobilities at different pH values. The approximate isoelectric point is somewhat below pH 5.

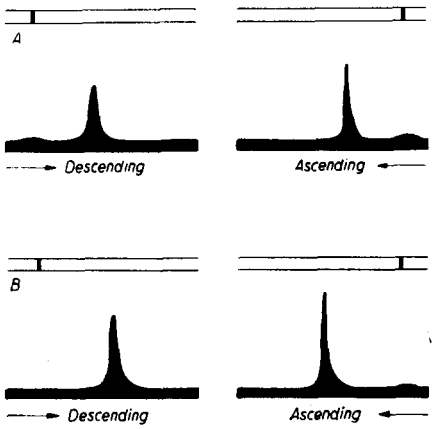

Fig. 3. A. Electrophoretic patterns of the "protein from alkaline extract 3" from chicken erythrocytes, in 0.1 μ phosphate buffer at pH 7.73 after 150 minutes in a field of 3.2 volts per cm. B. Electrophoretic patterns of the same protein in a 0.1 μ glycine-HCl buffer at pH 2.65 after 150 minutes in a field of 3.8 volts per cm.

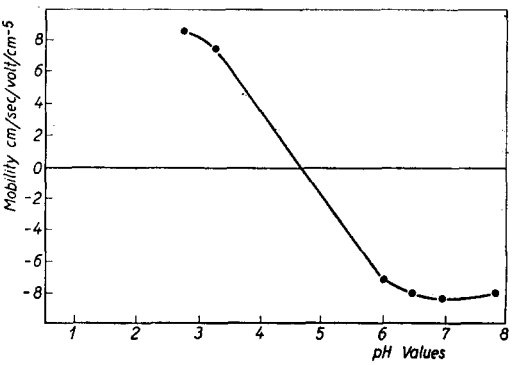

Fig. 4. pH-mobility curve of chicken erythrocytes "protein from alkaline extract 3".

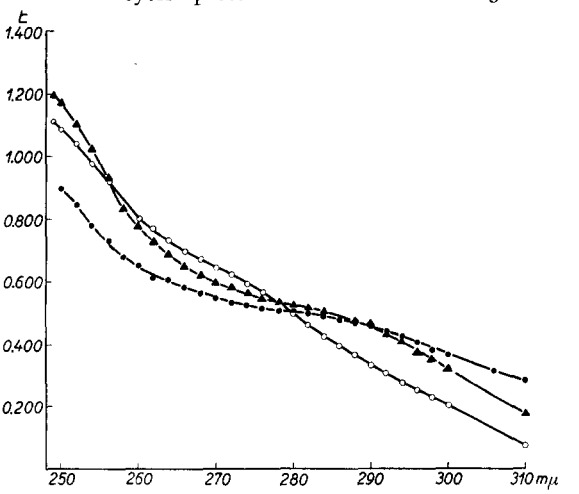

Fig. 5. Ultraviolet absorption spectra of "the proteins from alkaline extract 3" in 0.1 N NaOH ●— erythrocytes; ○— thymus; ▲— liver. Concentration of protein 250 μg per ml.

The protein from "alkaline extract 3" gave the usual color reactions for proteins, including the reaction for tryptophan. It also showed a positive Molisch reaction. When treated with the hot $HgSO_4$–H_2SO_4 reagent in presence of nitrite, a red precipitate formed, but the liquid remained colorless, indicating the absence of histones. The protein contained 15.2% nitrogen and 0.4% phosphorus; this was probably all lipid phosphorus since 80% of it was extractable with 7:3 alcohol-ether mixture.

After 30 minutes hydrolysis in 1 N HCl, reducing sugar was detected. It was determined according to SOMOGYI[21] and, if expressed as glucose, it accounted for approximately 1% of the original substance. The ultraviolet spectrum was atypical for protein (Fig. 5).

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In order to exclude the possibility that the protein of the "alkaline extract 3" was derived from stroma of the erythrocytes, which was present in our preparation of nuclei, two types of experiments were performed. First, human erythrocytes were treated in the identical manner as chicken erythrocytes. We were unable to isolate either histones or the alkali soluble protein from stroma of the human erythrocyte.

A second type of experiments was performed on nuclei prepared from calf thymus and perfused rabbit liver*. These nuclei were microscopically clean. They could have been contaminated with small amounts of cytoplasmic material but not with the stromata of erythrocytes. In both cases (thymus and liver) the proteins from "alkaline extract 3" were isolated and were found to have very similar solubility properties to the analogous protein from chicken erythrocytes.

The electrophoretic patterns of proteins obtained from liver and thymus (Fig. 6) indicated less homogeneity than the patterns of the corresponding protein from chicken erythrocytes. The mobilities of the major components of each of the three preparations (erythrocytes, thymus, liver) were similar, but not identical.

All three proteins contained carbohydrate. The amount of reducing sugar (expressed

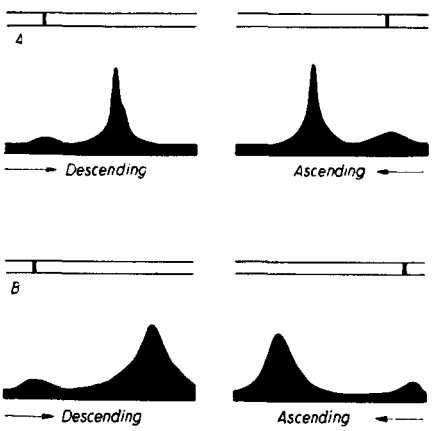

Fig. 6. A. Electrophoretic patterns of liver "protein from the alkaline extract 3" in 0.1 μ glycine-NaOH buffer at pH 9.87 after 120 minutes in a field of 4.9 volts per cm. B. Electrophoretic patterns of thymus "protein from the alkaline extract 3" in 0.1 μ barbiturate buffer at pH 7.96 after 200 minutes in a field of 5 volts per cm.

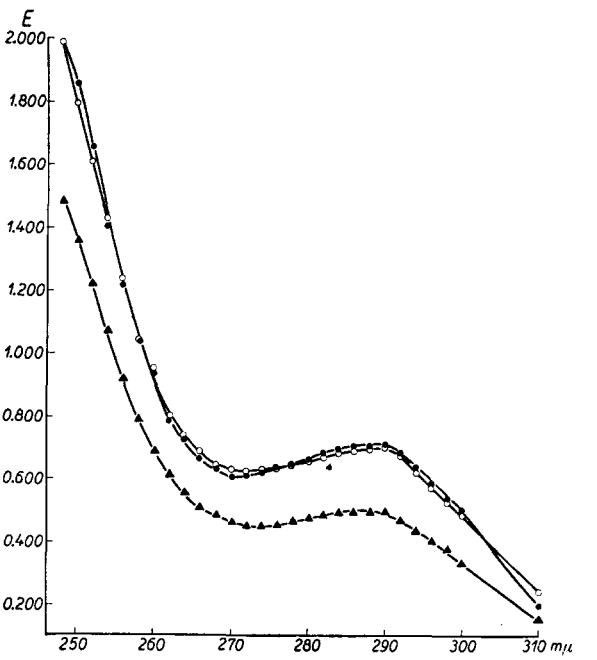

Fig. 7. Ultraviolet absorption spectra of liver "protein from alkaline extract 3" in 0.1 *N* NaOH, concentration unknown. ▲— after the first washing with trichloroacetic acid; ○— after the second washing with trichloroacetic acid; ●— after the seventh washing with trichloroacetic acid.

* This was made possible through the kindness of Dr J. M. LUCK of Stanford University. Thymus nuclei were prepared using saline and the tissue press of CUNNINHAM *et al.* (Unpublished). Liver nuclei were prepared in 2 % citric acid. We are greatly indebted to Drs J. M. LUCK, L. CUNNINHAM, L. NODA AND D. W. KUPKE for advice and help during these experiments.

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as glucose), determined after acid hydrolysis, was very similar in all three proteins and accounted for approximately 1% of the original weight. Liver and thymus proteins contained 14.7% nitrogen. All three proteins showed similar atypical spectra in the ultraviolet (Fig. 5).

Working with liver protein, it was noticed that a substance (or substances) responsible for the unusual spectrum of the fraction could be removed by precipitating the protein with trichloroacetic acid and subsequently dissolving it in 0.1 *N* NaOH. The results of this experiment are shown in Fig. 7. No significant difference between the second and the seventh washing with trichloroacetic acid was found. The relative content of tyrosine and tryptophan was calculated[20] from the curve obtained on the protein after the seventh washing and was found to be 1 tryptophan to 2.7 tyrosine. A similar proportion, 1 to 2.4, was found in the products of hydrolysis after the liver protein was partially digested with a mixture of trypsin and chymotrypsin *a*.

Washing with trichloroacetic acid produced little change in the spectrum of the protein from erythrocytes. No attempts were made to purify the thymus protein by this method.

DISCUSSION

In 1943 it was noticed[22] that under appropriate conditions of pH and salt concentration the nuclear material can be extracted from isolated nuclei without a visible rupture of the nuclear outline. This observation led to the conclusion that a selectively permeable membrane may be absent in nuclei, and therefore the composition of isolated nuclei would depend on their previous exposures to extracting media. DOUNCE *et al.*[23] proved that nuclei prepared in aqueous media lose a considerable amount of protein and ribonucleic acid. The conclusion of DOUNCE has been confirmed by MIRSKY *et al.*[24] and also in this laboratory[25].

On the other hand it has been shown[25] that after exhaustive washings the DNase and the RNase of thymus nuclei reach a constant value per nucleus, indicating that a part of the soluble proteins within the nucleus is not in equilibrium with the wash-fluid. It seems likely that these soluble, but protected proteins were released into "neutral extract 1" after the DNA had been digested. The results presented in this paper by no means account for all proteins originally present in chicken erythrocyte nuclei. On the contrary, it is realized that only the "bound" or "structural" proteins were present in nuclei at the onset of our fractionation.

MIRSKY AND POLLISTER[3] reported that the histone fraction from liver nuclei contained 0.09% of tryptophan, and that from fowl erythrocytes 0.02%. In the footnote, added in proof, these authors state that the correct values are higher than those reported. Our histone fraction showed 0.36% of tryptophan, and 3.62% of tyrosine. The latter agrees well with 3.49 as reported by DALY, MIRSKY AND RIS[17], but the tryptophan value is much higher. The same criterion for purity of the histone fraction (solubility in $HgSO_4$–H_2SO_4 reagent) was used in both laboratories, the difference apparently being due to the different analytical methods used.

The protein extractable with alkali was found in all three types of nuclei investigated. It seems quite probable that this type of protein is common to all types of nuclei. This assumption is further corroborated by the independent work of THOMAS and co-workers, who have isolated from several species of nuclei and from fish sperm heads

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an alkali soluble protein which also contained lipid[26] and carbohydrate[27]. These authors used different technique of isolation, but the electrophoretic properties and properties of solubility of their material appear to be identical with the respective properties of the protein described in this paper.

It is not possible to say at this time what relationship there may be between the "protein from the alkaline extract 3" and the "residual protein of MIRSKY[28]". The residual protein has been located in chromosomes while the location of alkali-soluble protein can be traced only to the nucleus and no further. Electrophoretic studies are available only for the alkali-soluble protein. Both have relatively large amounts of tryptophan. Neither of them could be extracted from nuclear material by neutral or mildly acid solvents.

The evidence presented in this paper does not justify the conclusion that the "protein from alkaline extract 3" is a single chemical substance. The electrophoretic homogeneity favors such a conclusion, but the chemical composition and the ultraviolet spectra do not.

SUMMARY

The "difficulty extractable" and the "structural" proteins of chicken erythrocyte nuclei were separated into three distinct fractions by first digesting the nuclei with crystalline desoxyribonuclease and removal of the neutral extract; then extracting the residue with acid, and finally extracting the residue with alkali. The first, neutral extract, contained small amounts of probably very numerous proteins. The second fraction, representing the acid extract, contained only histones and no other type of proteins. It has been shown that more than one individual histone were present in chicken erythrocyte nuclei. The third fraction, represented by alkaline extract, contained an electrophoretically homogeneous protein. This protein contained lipid and carbohydrate, was isoelectric at a pH value between 4 and 5 and showed a high content of tryptophan. Non-identical, but very similar proteins were isolated from nuclei of calf thymus and rabbit liver, indicating that such proteins are probably characteristic for all nuclei.

RÉSUMÉ

Les protéines "difficiles à extraire" et les protéines "structurales" des noyaux d'érythrocytes de poule ont été séparées en trois fractions distinctes, d'abord par digestion des noyaux par la désoxyribonucléase crystalline et séparation de l'extrait neutre, puis par extraction du résidu à l'acide et enfin par extraction du résidu à l'alcali. La première fraction, l'extrait neutre, contenait de faibles quantités de probablement plusieurs protéines. La seconde fraction, l'extrait acide, contenait seulement des histones et pas d'autres types de protéines. Il a été montré que les noyaux d'érythrocytes de poule contenaient plus d'une histone. La troisième fraction, l'extrait alcalin, contenait une protéine électrophorétiquement homogène. Cette protéine contenait du lipide et de l'hydrate de carbone, elle était isoélectrique à une valeur du pH comprise entre 4 et 5 et avait une teneur élevée en tryptophane. Des protéines non identiques mais très semblables ont été isolées du thymus de veau et du foie de lapin, ce qui fait penser que de telles protéines sont probablement caractéristiques pour tous les noyaux.

ZUSAMMENFASSUNG

Die "schwierig extrahierbaren" und die "Struktur"-Proteine von Erythrocytenkernen des Huhns wurden in 3 verschiedene Fraktionen getrennt: zuerst durch Spaltung der Kerne mit kristalliner Desoxyribonuclease und Entfernung des neutralen Extraktes, dann durch Extrahieren des Rückstands mit Säure und schliesslich durch Extrahieren des Rückstands mit Alkali. Der erste, neutrale Extrakt enthielt kleine Mengen von wahrscheinlich sehr zahlreichen Proteinen. Die zweite Fraktion — der Säureextrakt — enthielt nur Histone und keine anderen Proteine. Es wurde gezeigt, dass mehr als ein individuelles Histon in den Erythrocytenkernen des Huhns vorhanden ist. Die dritte Fraktion — der alkalische Extrakt — enthielt ein elektrophoretisch homogenes Protein. Dieses Protein enthielt Lipoid und Kohlenhydrat, war bei einem pH-Wert zwischen 4 und 5 isoelektrisch und zeigte einen hohen Gehalt an Tryptophan. Nicht identische, aber sehr ähnliche Proteine wurden aus den Kernen von Kalbsthymus und Kaninchenleber isoliert, was darauf hinweist, dass solche Proteine wahrscheinlich für alle Kerne charakteristisch sind.

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REFERENCES

1 M. LASKOWSKI AND V. KUBACKI ENGBRING, *Federation Proc.*, 11 (1952) 246.
2 D. T. MAYER AND A. GULICK, *J. Biol. Chem.*, 146 (1942) 433.
3 A. E. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 117.
4 R. D. DALLAM, W. R. KIRKHAM, T. Y. WANG, L. E. THOMAS AND D. T. MAYER, *Federation Proc.*, 9 (1950) 164.
5 E. STEDMAN AND E. STEDMAN, *Nature*, 152 (1943) 267.
6 E. STEDMAN AND E. STEDMAN, *Symposia Soc. Exptl. Biol.*, 1 (1947) 232.
7 J. BRACHET, *Experientia*, 2 (1946) 142.
8 H. G. CALLAN, *Nature*, 152 (1943) 503.
9 T. CASPERSON, *Nature*, 153 (1944) 499.
10 R. E. STOWELL, *Stain Technol.*, 21 (1946) 137.
11 H. RIS AND A. E. MIRSKY, *J. Gen. Physiol.*, 32 (1949) 489.
12 M. KUNITZ, *Science*, 108 (1948) 19.
13 M. KUNITZ, *J. Gen. Physiol.*, 33 (1950) 349, 363.
14 A. L. DOUNCE AND T. H. LAN, *Science*, 97 (1943) 584.
15 G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
16 M. LASKOWSKI, *Arch. Biochem.*, 11 (1946) 41.
17 M. M. DALY, A. E. MIRSKY AND H. RIS, *J. Gen. Physiol.*, 34 (1951) 439.
18 A. E. MIRSKY AND H. RIS, *J. Gen. Physiol.*, 31 (1947) 7.
19 L. G. ALLGEN, *Acta. Physiol. Scand.*, 22 (1950), supp. 76.
20 T. W. GOODWIN AND R. A. MORTON, *Biochem. J.*, 40 (1946) 628.
21 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61.
22 M. LASKOWSKI AND D. L. RYERSON, *Arch. Biochem.*, 3 (1943) 227.
23 A. L. DOUNCE, G. H. TISHKOFF, S. R. BARNETT AND R. M. FREER, *J. Gen. Physiol.*, 33 (1950) 629.
24 V. ALLFREY, H. STERN, A. E. MIRSKY AND H. SAETREN, *J. Gen. Physiol.*, 35 (1952) 529.
25 K. D. BROWN, G. JACOBS AND M. LASKOWSKI, *J. Biol. Chem.*, 194 (1952) 445.
26 T. Y. WANG, M. J. CARVER, R. H. RAMSEY, A. J. FUNCKES AND L. E. THOMAS, *Federation Proc.*, 11 (1952) 306; M. J. CARVER AND L. E. THOMAS, *Arch. Biochem. Biophys.*, 40 (1952) 342.
27 L. E. THOMAS, personal communication (1952).
28 A. E. MIRSKY AND H. RIS, *J. Gen. Physiol.*, 34 (1951) 475.

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