THE EFFECT ON RAMAN SPECTRA OF EXTRACTION OF PERIPHERAL PROTEINS FROM HUMAN ERYTHROCYTE MEMBRANES*

S. C. Goheen[1], T. H. Gilman[1], J. W. Kauffman[1] and J. E. Garvin[2]

[1]Biomedical Engineering Center, Technological Institute, Northwestern University, Evanston, Illinois 60201.

[2]Department of Biochemistry, Ward Building, Northwestern University, Chicago, Illinois 60611.

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SUMMARY: We compare the Raman spectra of intact erythrocyte membranes with spectra of membranes from which essentially all peripheral proteins have been extracted. The results indicate that the extraction procedure causes considerable alteration in the environment of peptide bonds and a marked change in the environment of the phenylalanine and tryptophan ring moieties of the integral membrane proteins. We infer from our data that the lipid portion of the bilayer is little affected by extraction. Finally, we have observed that the total decrease in the heights of the protein-associated peaks of the extracted vesicles is generally less than that expected from the mass of protein removed by extraction.

INTRODUCTION

Interpretation of Raman spectra from erythrocyte membranes in terms of specific structures remains incomplete. Since the first Raman spectrum of an erythrocyte membrane preparation appeared in 1972 (1), several spectra of higher resolution have been reported (2,3,4). Recently Verma and Wallach (5) examined the effects of systematic variation of pH and temperature on the Raman spectra of erythrocyte membrane preparations. The observed changes in the spectra were attributed to alterations in both lipid and protein components. However, spectral changes brought about by systematic variation in the composition of erythrocyte membrane preparations have not been reported previously.

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Abbreviations: SDS, sodium dodecyl sulfate; PAS-1, PAS-2 and PAS-3, periodic acid Schiff-staining bands according to numbering system of Fairbanks, et al (8).

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We present here, a comparison of the Raman spectra of vesicles derived from intact erythrocyte ghosts with vesicles derived from erythrocyte membranes stripped of peripheral proteins by the method of Steck and Yu (6). From our data, we conclude that the extraction of peripheral proteins does not substantially disrupt the order and structure of the bilayer. In contrast, the environment of the peptide bonds and the tryptophan and phenylalanine ring moieties of the integral proteins, appears to have been drastically altered by extraction. Also, our data indicate that the contribution of the peripheral proteins to the total Raman spectrum is much less than expected on the basis of their mass.

EXPERIMENTAL

Membrane Preparation

Human erythrocyte membranes were isolated from fresh and outdated human whole blood by a variation of the procedure of Fairbands, et al. (8). Following hemolysis in their procedure, ghosts were washed and pelleted three times in 5 mM sodium phosphate, pH = 8.0 (5P(8)) at 5°C using 3 X 10^5 g-min in a Sorvall GSA rotor.

Ghosts were kept overnight in 5P(8) at 5°C. The suspension was divided into two portions. One of these was subjected to nitrogen cavitation (600 psi, N_2) and pelleted at 6 X 10^6 g-min. in a Beckman 75Ti rotor at 5°C. This fraction will be referred to as standard vesicles.

The remaining portion of the ghosts was incubated at 37°C in 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5P(8) and then chilled at 5°C and centriguged at 3 X 10^5 g-min. and the pellet was resuspended in 7 parts water. The pH was adjusted to 12.0 using 2N NaOH, and constant N_2 gas flow at 5°C. The pH was held at 12.0 for 15 minutes and the sample was neutralized to pH 7.7 with concentrated HCl. The sample was then centrifuged three times at 8 x 10^5 g-min, washing the pellet with distilled wated after each spin. The sample was then placed in 5P(8) and centrifuged at 6 X 10^6 g-min. using a Beckman 75Ti rotor (5°C). This pellet will be referred to as the extracted vesicles.

SDS-Polyacrylamide Gel Electrophoresis

Reagents for polyacrylamide get electrophoresis were basically those of Potempa and Garvin (7). The polyacrylamide gels, 5.6% in acrylamide and 0.12% in bisacrylamide, were prepared according to the method of Fairbanks et al. (8).

The electrophesis buffer was 0.024 M in sodium phosphate, 0.0008 M in EDTA, and 1% in SDS. Samples were solubilized in 3% SDS and kept at room temperature. Other than the Pyronin Y tracking dye and sucrose, there were no other additions to the solubilization solutions. 65 µg. of protein were layered on each gel. Protein was determined by the method of Lowry (9) using albumin as standard.

Electrophoresis was continued until the Pyronin Y dye front had migrated 9.1 cm. The gels were then stained for amino acids and carbohydrate by the coomassie blue and periodic acid shiff procedures respectively (8).

Thin Layer Chromatography

Thin layer chromatography was done on Silica gel G plates, using $CHCl_3$: CH_3OH:H_2O:NH_4OH in a volume/volume mixture of 65:25:4:0.4 for development.

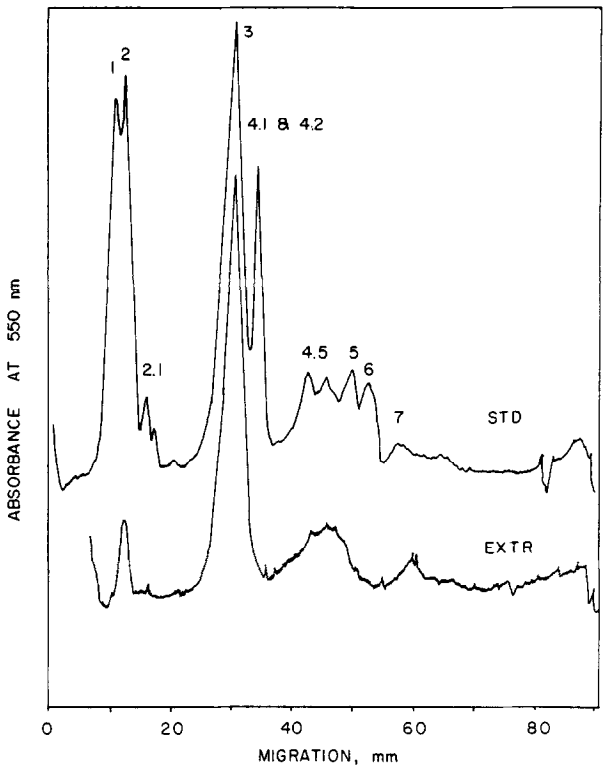

FIGURE 1

Effect of extraction at pH12 on the electrophoretic profiles of human erythrocyte membrane preparations. Electropheresis was conducted in 5.6% polyacryalamide gels, 1% in SDS, staining with Coomassie blue. Scans were made at 550 nm on a Beckman DU 2400 spectrophotometer fitted with a Gilson Linear Transport attachment No. 222. STD: typical scan from a gel used to separate proteins of vesicles derived from ghosts, EXTR: typical scan from a gel used to separate proteins of vesicles extracted at pH 12 to remove peripheral proteins.

The plates were then stained for lipid phosphorus by spraying with Molybdenum Blue. Crystalline phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and lysolecithin dissolved in $CHCl_3$ were run separately and as a mixture for standards. Total lipids were extracted from the prepared vesicles following the method of Folch (10). Lipid phosphorus was determined using the Bartlett procedure as dexcribed by Dittmer and Wells (11).

It was found that 0.01 μM P was the limit of detectability. In order to maintain good resolution, 0.1 μM P was applied per spot of total lipid, so that any components comprising 10 mole percent of the total phospholipid would be detected.

Raman Spectroscopy

The Raman spectrometer has been described elsewhere (12). The temperature in the vicinity of the sample was kept at 5°C by boiling liquid N_2 with a heating coil and allowing the cool N_2 gas to flow over the sample, through an

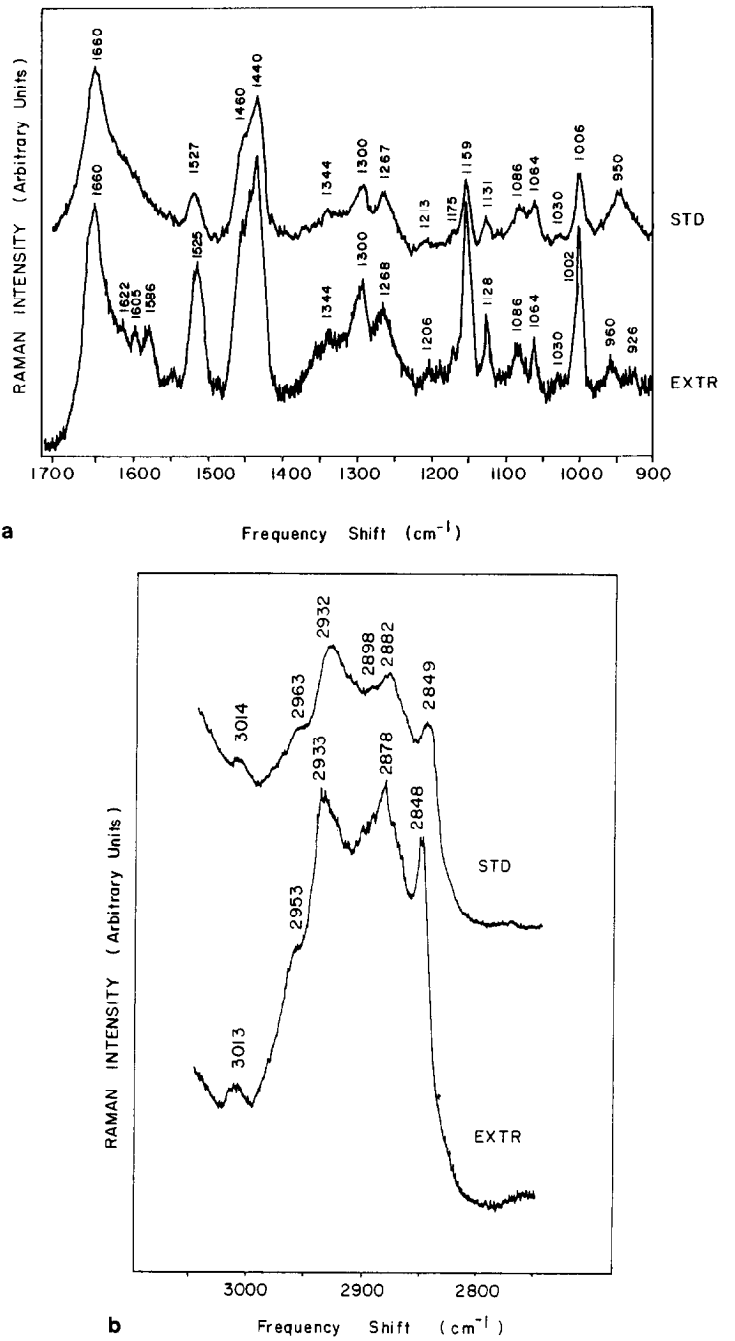

FIGURE 2

Raman spectra of human erythrocyte vesicles (STD) and vesicles from which peripheral proteins have been removed (EXTR) (a) 900-1700 cm^{-1} (b) 2750-3150 cm^{-1}. Excitation was by argon ion laser with ca. 500 mW power to the sample at 514.5 nm. Resolution is ca $\pm$ 2 cm^{-1} with an 8.0 cm^{-1} bandpass; time constant, 10 sec; counting rate, 3 X 10^4 photon counts / sec; scanning speed, 10 cm^{-1}/min. temperature controlled enternally at 5°C.

insulated quartz jacket. Temperature was regulated with a rheostat. A thermocouple placed inside the sample chamber was used to measure temperature. Figures were drawn from the highest resolution spectra taken from at least 4 independently prepared samples. No significant variations were observed in the spectra of any of the similarly prepared membrane samples.

RESULTS AND DISCUSSION

Figure 1 shows typical electropherograms obtained from standard and extracted human erythrocyte membranes stained with Coomassie blue. The scan from the standard membrane preparation shows the usual profile described by Fairbanks et al. (8), while the scan from the extracted vesicles shows that virtually all of the peripheral proteins (bands 1,2, 4.1, 4.2, 5, and 6) are absent leaving only the integral proteins (bands 3, 4.5 and 7) as described by Steck and Yu (6). Companion electropherograms obtained from preparations stained with Schiff's periodic acid method for glycoproteins showed the presence of PAS-1, PAS-2 and PAS-3 in both the standard and extracted preparations. These results show that our standard preparations contained the usual complement of integral and peripheral proteins whereas the extracted preparations contained almost exclusively integral proteins.

Figure 2 shows typical Raman spectra taken from preparations of standard and extracted erythrocyte membranes in the regions 900 cm^{-1} to 1700 cm^{-1} (fig. 2a) and 2750 cm^{-1} to 3050 cm^{-1} (fig. 2b). Previsously reported spectra from intact erythrocyte ghost preparations (not vesicles) (3,4) are similar to our spectrum of standard vesicles suggesting that conversion to vesicles does not introduce significant Raman-detectable alterations in the membrane architecture. Lippert et al. (2) obtained a spectrum, assumed to be of spectrin-depleted ghosts, which is nearly identical to our extracted vesicle spectrum. Although at first glance there appears to be general similarity between the spectra from our standard and extracted preparations, important qualitative and quanititative differences are present which will be developed in the rest of this paper. Table I gives tentative peak assignments for standard and extracted vesicles.

In order to compare the contributions of the various moieties to each

TABLE I

Tenative Assignments of Raman Peaks From Erythrocyte Vesicles.

FREQUENCY (cm^{-1})		TENATIVE ASSIGNMENTS			
Standard	Extracted	Protein	Lipid	Other	Carotenoid / Water
	926w	υ(C-C) [20]			
950	960W	υ(C-C) [2,3,4,20]			
1006s	1002s	Trp & Phe ring mode [2,3,4,20]			
1030	1030	Phe [4,21]			
1064	1064	υ(C-C)	υ(C-C of trans chain [2,4,21]		
1086	1086		υ(O-P-O) and υ(C-C) of random chain [2,4,21]		
1131	1128		υ(C-C) of trans chain [2,4,21]		
1159s	1156s	CH_3 rock [4]		υ(=C-C=) [3,19,20]	
1175		Tyr, Phe [4,21]			
1213	1206	Tyr, Phe [2,4,21]			
1267s,sh	1268 s,sh	Amide III [3,4,20] CH twist [2]			
1300s	1300s	Amide III [4,19]	CH_2 wag [2,3,4,20,21]		
1344sh	1344sh	Trp [18], Phe [4], CH_2 def. [4,20,21]			
1377		Trp [18]			
1440s 1460sh	1440s 1460sh	CH_2 and CH_3 bend [2,3,4,20,21]	CH_2 and CH_3 bend [2,3,4,20,21]		
1527s	1525 s	Trp [3]		υ(=C-C=) [3,19,20]	
	1586sh	Phe, Arg [21]			
	1605sh	Tyr, Phe [2,21] Trp [20] Amide I [3,20]			H_2O [3,20]
	1622sh	Tyr, Phe [21] Amide I [3,20]			H_2O [3,20]
1660s	1660s	Amide I [2,3,4,20,21]	υ(C-C), cis [4]		H_2O [3,20]
2849s,sh	2848s,sh		$\upsilon(CH_2)$ sym, [3,4,20]		
2882s	2878s	$\upsilon(CH_2)$ sym. [4]	$\upsilon(CH_2)$ antisym [4]		
2898sh		$\upsilon(CH_2)$ [20]	$\upsilon(CH_2)$ [20]		
2932s	2933s	υCH_2) antisym [3,4,] $\upsilon(CH_3)$ sym [4]	$\upsilon(CH_3)$ sym [4]		
2963sh	2953sh	$\upsilon(CH_3)$ asym. [4,20]	$\upsilon(CH_3)$ asym [4,20]		
3014	3013		υ(CH) olefinic [4]		

Relative intensities are indicated as follows: s=strong; w=weak; sh=shoulder; υ=stretching; def=deformation; sym=symmetric; antisym=antisymmetric; asym= asymmetric. References next to the assignments signify assignments in other membranes.

spectrum we have chosen the 1440 cm^{-1} CH_2 and CH_3 wag intensity as an internal reference since this peak does not appear to be significantly affected by extraction. We estimate that the amino acid side chains of the peripheral proteins which have been removed contribute only about 12% to the height of the 1440 cm^{-1} peak in standard preparations, 88% being contributed by lipids. The choice of the 1440 cm^{-1} peak as a reference is further supported by the finding of Steck and Yu (6) that there is almost no loss of lipid phosphorus from the pellet during their pH 12 extraction. In addition, we found by thin layer chromatography, no differences in the relative amounts of phosphatidylcholines, phosphatidylethanolamines, and sphingomyelins as judged by intensities of spots from standard and extracted vesicles. More importantly, since no lysophosphatidylcholines at all were observed, we assume that loss of fatty acids (primarily responsible for the 1440 cm^{-1} peak) from our extracted samples was neglibible. Furthermore, there appears to be little spectral evidence for alterations in lipid conformation between the standard and extracted samples (discussed later). All these data support the use of the intensity of the 1440 cm^{-1} peak as a reliable internal standard.

Figure 3 compares the relative intensity of each resolved peak of the extracted spectrum with the relative intensity of the same peak in the standard spectrum. Therefore, if extraction removes about 50 % of the protein mass, as reported by Steck and Yu (6), it would be predicted, all other things being equal, that the peaks assigned to protein moieties would yield a I_{12}/I_N ratio of approximately 0.5. Examination of the points assigned to protein moieties (shaded points) shows that this prediction is not realized in a single instance. Thus, the first conclusion is that the peripheral proteins contribute less to Raman signal than would be expected from their mass alone. Perhaps even more striking however, is the observation that the ring vibrations at ca. 1005 cm^{-1} and ca. 1030 cm^{-1} actually increase in intensity upon extraction. These paradoxical increases to I_{12}/I_N ratios of 1.8 and

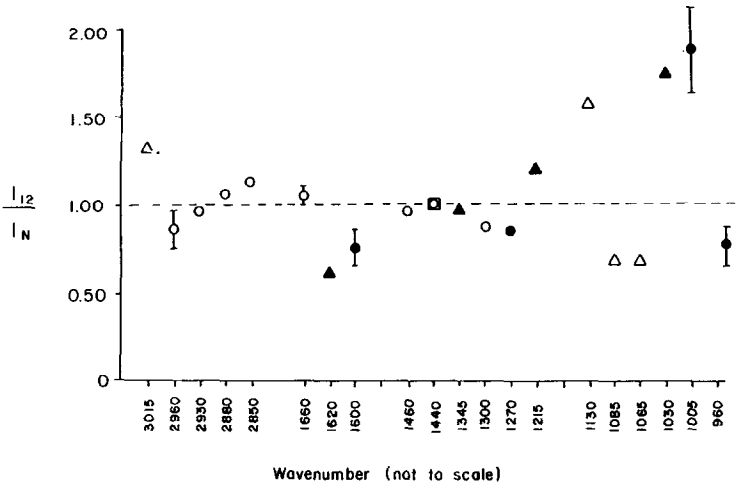

FIGURE 3

Relative intensity ratios of Raman peaks at various wavenumbers. I_{12} is the ratio of the peak height of a Raman line from the extracted membrane to the peak height of the 1440 cm^{-1} line of the same extracted membrane preparation. Δ indicates data taken from only one extracted membrane. ○ indicates data taken from four independently prepared extracted samples. Error bars are indicated for standard deviations except where they are within the confines of the symbol. Shaded points (▲●) indicate peaks which have previously been assigned predominately to proteins vibrations. Carotenoid peaks at ca. 1590 cm^{-1} (I_{12}/I_N = 2.30, $\sigma = \pm$ 3.10) and ca. 1155 cm^{-1} (I_{12}/I_N) = 3.88, $\sigma = \pm$ 2.34) are not shown.

1.7 respectively must be due to some radical alterations in the environments of the tryptophan and phenylalanine ring moieties. This conclusion is supported by evidence for an altered environment about a large portion of the peptide bonds as indicated by the splitting of the 950 cm^{-1} peak and the emergence in the Amide I region of peaks at 1622, 1605 and 1586 cm^{-1}. Thus we infer extensive rearrangements of the integral proteins upon extraction of the peripheral proteins from the membrane.

In contrast, there is little spectral evidence for structural changes in the lipids of the bilayer. Examination of the points assigned to lipids in Fig. 3, (open points) especially in the region ca. 2900 cm^{-1} (four open circles) shows little difference from I_{12}/I_N = 1.0. This evidence is quite reliable since model systems have shown that the CH_2 stretch region at 2900

cm^{-1} as well as the C-C stretch region at ca. 1100 cm^{-1} are sensitive to structural variations (13,14,15,16,17). However, the variations observed in the CC stretch region on extraction are unusual (See open triangles in Fig. 3, also Fig. 2). In model systems, the I_{1065}/I_{1085} and I_{1130}/I_{1085} peak height intensity ratios decrease simultaneously with hydrocarbon chain disorder (18) whereas in the extracted vesicle spectrum, the I_{1130}/I_{1085} ratio increases while the I_{1065}/I_{1085} ratio remains unchanged relative to the standard vesicle spectrum (Data derived from Fig. 2). We postulate that this together with the lack of relative intensity change in the 2900 cm^{-1} region indicates that there may be some protein vibrations which contribute to the 1100 cm^{-1} region. This explanation could also account for the narrowing of the 1130, 1085 and 1065 cm^{-1} peaks in the extracted spectrum. Furthermore, it appears that similar protein peaks could have been subtracted out of the CH stretch region at ca. 2860 cm^{-1}. Fig. 2 shows that resonance enhanced peaks assigned to carotenoids appear in both spectra at ca. 1160 cm^{-1} and 1520 cm^{-1}. We attach no importance to differences in intensity of these peaks since their heights are altered by some unexplained factors in the bloods of origin (2).

The data described above suggest that the systematic alteration of the composition of a biomembrane provides a useful strategy in advancing the interpretation of its Raman spectra. We have adduced evidence that removal of the peripheral proteins from the erythrocyte membrane modifies the lipid portion of the remaining bilayer only slightly while the environment of the integral protein peptide bonds and tryptophan and phenylalanine ring moities are substantially altered. Furthermore, we have found that the peripheral proteins contribute much less than the expected intensities to the protein portions of the spectrum. It appears that these findings have general application in the interpretation of Raman spectra of biological membranes.

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REFERENCES

1. Bulkin, B. (1972) Biochim. Biophys. Acta 274 649-651.

2. Lippert, J., Gorczyca, L. and Miklejohn, G. (1975) Biochim. Biophys. Acta. 382, 51-57.

3. Wallach, D. and Verma, S. (1975) Biochim. Ciophys. Acta 382,542-551.

4. Milanovich, F., Shore, B., Harney, R. and Tu, A. (1976) Chem. Phys. Lipids 17, 79-84.

5. Verma, S. and Wallach, D. (1976) Proc. Nat. Aca. Sci. 73, 3558-3561.

6. Steck, T. and Yu, J. (1974) J. Supramol. Str. 1, 220-232.

7. Potempa, L. and Garvin, J., (1976) Biochem. Biophys. Res. Comm. 72. 1049-1055.

8. Fairbanks, G., Stech, T. and Wallach, D. (1971) Biochem. 10, 2606-2617.

9. Lowry, O., Rosenbrough, N., Farr, A. and Randell, R. (1951) J. Biol. Chem. 193, 265-275.

10. Folch, J., Lees, M., and Sloane - Stanley, G.H. (1957) J.Biol. Chem. 226 497-509.

11. Dittmer, J. and Wells, M. (1969) Methods in Enzymology 14, ed. J. M. Lowenstein, Acad. Press, New York 482-528.

12 Lis, L. Kauffman, J. and Shriver, D. (1975) Biochim. Biophys. Acta 406, 453-464.

13. Gaber, B. and Peticolas, W. (1977) Biochim. Biophys. Acta 465, 260-274.

14. Mendelsohn, R., Sunder, S. and Bernstein, H. (1975) Biochim. Biophys. Acta 413, 329-340.

15. Mendelsohn, R., Sunder, S. and Bernstein, H. (1976) Biochim. Biophys. Acta 419, 563-569.

16. Lis, L., Goheen, S., Kauffman, J. and Shriver, D. (1976) Biochim. Biophys. Acta 443, 331-338.

17 Goheen, S., Lis, L. and Kauffman, J. (1977) Chem. Phys. Lipids, in press.

18. Bulkin, B. and Krishnamachari, N. (1972) J. Am. Chem. Soc. 94, 1109-1112.

19. Gill, D., Kilponen, R. G. and Rimai, L. (1970) Nature 227, 743-744.

20. Verma, S., Wallach, D. and Schmidt-Ullrich, R. (1975) Biochim. Biophys. Acta 394, 633-645.

21. Rothschild, K., Andrew, J., DeGrip, W. and Stanley, H. (1976) Science 191, 1176-1178.