HYDROLYSIS OF PROTEINS DURING DIALYSIS AND ULTRAFILTRATION

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During the course of studies with crystalline glutamate dehydrogenase (EC 1.4.1.3) it was observed that dialysis of solutions of this enzyme resulted in the loss of significant amounts of protein. At pH 8 to 9 and temperature of 4°C, as much as 20% of protein, as determined with a Folin reagent [1], was not recovered after 12 hr of dialysis. The procedure caused almost total enzyme inactivation. Passage of intact molecules through the Cellophane casing (Visking division of Union Carbide Corporation) would appear to be excluded, since at the concentration used the molecular weight has been estimated to be about 2×10^6 [2–4]. The dialysate, which was found to contain ultraviolet-absorbing, ninhydrin-positive [5] material, was concentrated *in vacuo* after freezing, subjected to electrophoresis in 1.5 M HCOOH at 8000 V for 2 hr on Whatman 3 mM paper strips; these were then treated with 0.25% ninhydrin in acetone [6]. After overnight development at room temperature, a number of ninhydrin-positive areas, some corresponding to known amino acids, were readily apparent. Treatment of the concentrated dialysate, before paper electrophoresis, with 6 N HCl at 110°C for 24 hr resulted in a 3–4 fold increase of total ninhydrin value and additional ninhydrin-positive areas on the paper after electrophoresis, suggesting that peptides were present as well as amino acids.

Essentially the same results were obtained when solutions of glutamate dehydrogenase (previously passed through a Sephadex G-25 column) were ultrafiltered *in vacuo* through sacs of dialysis casing at 4°C. Ultracentrifuge runs of the protein remaining inside the sac showed a single peak with a sedimentation coefficient identical to that of untreated enzyme. Measurements carried out at different times showed an increase of ninhydrin value, but never above 5–10% of the initial value. The filtrate contained similar ninhydrin-positive material as the dialysate but in considerably greater amount. After total hydrolysis, the concentrate of filtrate contained up to 30–35% (expressed as leucine equivalents in the total hydrolysates) of the protein originally placed in the sac. Solutions of the same concentration of glutamate dehydrogenase maintained under the same conditions of pH and temperature and for the same time, either in glass tubes or in dialysis casing sacs, did not show any ninhydrin-positive material other than the single area corresponding to the protein itself. Control experiments with water or dilute buffer solutions passed through unwashed casing demonstrated some ultraviolet-absorbing, ninhydrin-positive material to be in the casing. However, the experiments described above were conducted with casing which was cleaned by the procedure of Hughes and Klotz [7], followed by exhaustive washing by means of ultrafiltration with EDTA solutions and glass-distilled water. (In some experiments, moreover, the casing was also boiled again for 15 min in 50% ethanol and washed with glass-distilled water [8].) These samples of casing were completely free of soluble ultraviolet-absorbing, ninhydrin-positive

material. A few drops of chloroform were usually added to prevent bacterial growth.

Degradation of protein during ultrafiltration was not unique for glutamate dehydrogenase. Gel-filtered samples of bovine serum albumin, hemoglobin, ceruloplasmin and crystalline aldolase (EC 4.1.2.13) reacted in a similar manner. In each case, the protein recovery inside the bag was partial and varied between 85% and 75%; ninhydrin values showed a slight increase with time; in the case of aldolase, the activity recovered after ultrafiltration was about 40–50% of the initial value, which corresponded to a specific activity drop of 35–45%. The ultrafiltrates contained amino acids separable by paper electrophoresis and detectable by the ninhydrin reaction. Quantitative estimates indicated 10–25% of the original sac content to be in the ultrafiltrates, as judged by measuring leucine equivalent values of total hydrolysates. Protein-free supernatants prepared with perchloric acid from solutions of each protein contained no amino acids detectable by electrophoresis or ninhydrin determination, in addition to Sephadex gel-filtration. Fig. 1 shows a characteristic electrophoretic pattern obtained with ultrafiltrates of the above-mentioned proteins.

In order to confirm that these amino acids were indeed derived from the protein solution added to the dialysis sac, experiments were conducted with a mixed protein fraction extracted from ^{14}C-labelled algae (*Chlorella pyrenoidosa*). The radioactive protein, which was rather insoluble in water after isolation, was dissolved in 0.01 N NaOH and an insoluble residue removed by centrifugation. The supernatant solution was neutralized with HCl and ultrafiltered. The collected ultrafiltrable material was concentrated and high voltage electrophoresis was carried out on small aliquots. The neutralized supernatant, prior to ultrafiltration, was used as control. Fig. 2 shows the presence of several radioactive peaks, some of which were fast-moving, and corresponded to known amino acids as shown by ninhydrin staining. In addition, a few other radioactive peaks were monitored in a region where peptides would be present after electrophoresis; the concentration of these fragments was presumably too low to be detected by ninhydrin reaction. On the other hand, all the radioactivity of the control sample was found in one peak at the origin, thus indicating that the degradation took place during the ultrafiltration step.

The observations described above do not contradict the tentative hypothesis that some components in Cellophane dialysis casing, which cannot be removed by washing procedures, can promote the splitting of peptide bonds in proteins, under relatively mild

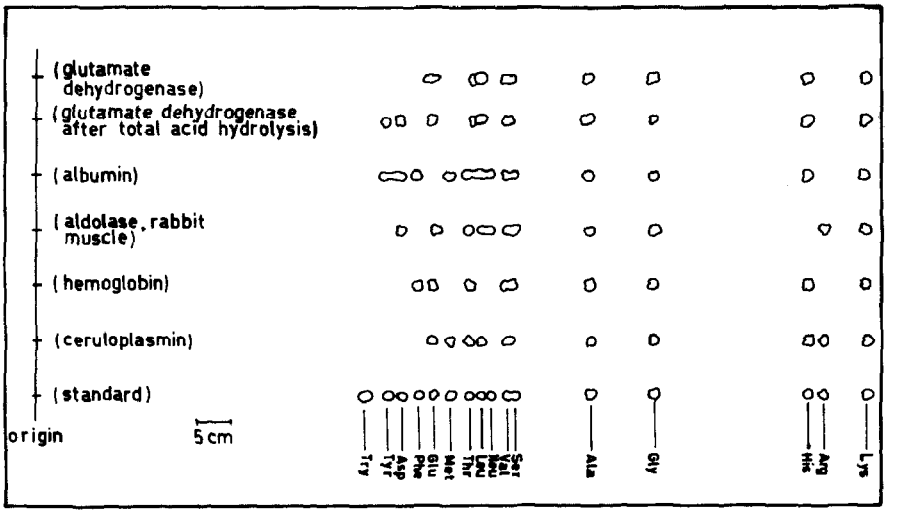

Fig. 1. High voltage electrophoresis patterns of 0.1% aqueous solutions of several proteins after ultrafiltration. Experimental details are given in the text. In each case controls were run in parallel, using comparable amounts of each protein solution prior to ultrafiltration: 10 μl of a solution, containing 0.5 μmole of each amino acid per ml, were used as a standard.

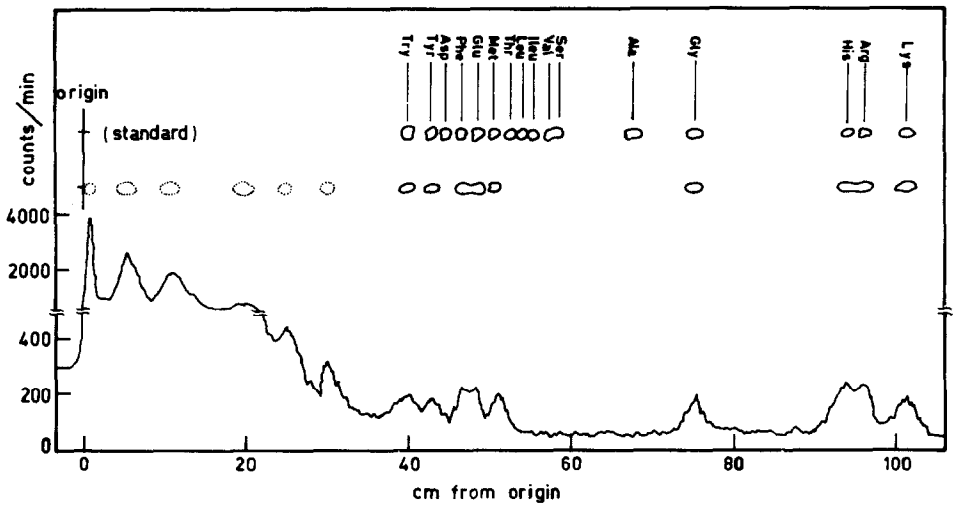

Fig. 2. Effect of ultrafiltration on a solution of ^{14}C-labelled protein fraction, extracted from *Chlorella pyrenoidosa*. Experimental conditions for the high voltage electrophoresis were the same as in fig. 1; the fully boxed areas indicate ninhydrin-positive components. 20 µl (118,000 counts/min) of concentrated, ultrafiltered solution were applied to the paper. After staining with ninhydrin, the radioactive strips were cut and scanned in a Vanguard Autoscanner.

conditions. In addition, other factors are probably involved. Stirring protein solutions with pieces of casing, all other conditions being the same as for ultrafiltration, yielded at most traces of ninhydrin-positive material. Dialysis of glutamate dehydrogenase resulted in more free amino acids than did dialysis of other proteins, for which, however, a significant degradation was achieved by means of a more drastic procedure, such as ultrafiltration, which, by virtue of the pressure which forces the molecules into the pores, probably involved a more thorough contact of the protein with the casing.

The single component observed at the ultracentrifuge in dialysed or ultrafiltered solutions of glutamate dehydrogenase, and the small variation of ninhydrin value during ultrafiltration of all proteins tested, indicate that degradation products are probably very small and pass rapidly through the membrane. The inactivation observed with glutamate dehydrogenase is probably dependent also on enzyme instability at slightly alkaline pH [9].

A detailed study, aimed at identifying the unknown component present in the casing, has not been crried out: the rather drastic treatment to which the casing was subjected prior to use makes it seem unlikely that its nature is enzymatic. It seems to us, however, that these results are of significance, for instance in view of the commonly observed loss of activity occurring with many enzymes after prolonged dialysis. Although oxidation has sometimes been shown to be responsible for these losses, it appears that cleavage of peptide bonds also can occur.

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