Enzymatic Hydrolysis of Proteins for Increased Solubility

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The relation of protein solubility to the degree of denaturation and to the extent and the mechanism of enzymatic hydrolysis is discussed, and a model based on Linderstrom-Lang's theory for the initial enzyme attack on the protein molecule is presented. In the practical use of protein hydrolysates in foods, the formation of bitter peptides is the most serious problem. According to the literature reviewed, the bitterness seems to be related to the content of hydrophobic amino acids in the protein rather than to the enzyme applied. Consequently, the majority of reports describes ways of attacking the bitterness problem, such as the application of a reverse osmosis cell or the extended hydrolysis by peptidases. The literature on the engineering aspects of protein hydrolysis comprises important work on continuous hydrolysis of fish protein concentrate and soy protein isolate.

In the last few decades an increasing number of unconventional proteins have for technical and economic reasons found their way into our food. Several important factors must be considered in that context: The nutritional value, the organoleptic quality, and the functional properties of the protein, plus, of course, the economic and legal aspects of protein addition. Often these factors are conflicting, and we could briefly state the protein industry's problem as giving the raw protein the desired functional and organoleptic properties without an unacceptable loss of nutritional value and at a reasonable cost.

Proteins for use in liquid foods and beverages must possess a high solubility in the system. However, solubility is an important functional property which is sometimes difficult to achieve. Although many technically important proteins in their native state are soluble in certain food systems, a true solution is rarely obtained in practice (egg albumen being an important exception). During the manufacture of proteins, such as soybean protein isolate, a partial denaturation occurs, which renders the protein more or less insoluble. In fact, by varying the processing conditions, a range of isolates with different solubility properties is obtained (Wolf, 1970). In addition, the food system itself may give rise to solubility problems. Many soft drinks have a pH in the range of 3–4, where the majority of proteins are only slightly soluble. Furthermore, the stability of native proteins in the acid pH range is often limited, as exemplified by soy protein (Nash et al., 1971).

The obvious way of attacking these problems is degradation of the protein by a proteolytic enzyme, whereby the solubility should increase and the nutritional value be retained. In the literature on protein hydrolysates for food use, general agreement seems to exist on this qualitative statement.

DENATURATION AND PROTEIN SOLUBILITY

Solubility of small molecules, such as salts or simple organic compounds, is expressed in grams per 100 ml of solution at standard temperature, which gives us a comprehensible and unambiguous figure. On the other hand, solubility of commercial proteins is often given as a percentage, such as the nitrogen solubility index or NSI (American Oil Chemists' Society, 1969a), and the protein dispersibility index or PDI (American Oil Chemists' Society, 1969b), which means that a certain *fraction* of the protein is soluble under the *specific* conditions used in the above-mentioned methods. The NSI or PDI are commonly used in the protein industry, and they are extremely valuable as a practical estimate of the extent of denaturation (Wu and Inglett, 1974; Wolf, 1970). However, these methods are only empirical (Wolf, 1970) and not quantitatively related to the denaturation, as shown earlier by Fukushima (1959).

Protein denaturation is defined by Tanford (1968a) as a major change from the original native structure without alteration of the amino acid sequence. It is important to realize that denaturation does not automatically imply insolubility. As an example it may be mentioned that, when glycinin (the major protein in soybeans) is rapidly heat denatured, the quaternary structure is destroyed and the protein molecules break up into several subunits, of which some slowly form a soluble and later insoluble aggregate, whereas the rest remains in solution (Wolf and Tamura, 1969). The conversion into an insoluble form is often caused by a polymerization due to an irreversible disulfide interchange reaction (Tanford, 1968b; Wolf and Tamura, 1969). In contrast, the actual denaturation is normally reversible (Tanford, 1968a).

Furthermore, native protein often shows a complex solubility behavior. For example, the solubility of the native soybean globulins strongly depends on the pH (Wolf, 1970), temperature (Wolf, 1972), and salt content (Anderson et al., 1973; van Megen, 1974), and in certain food systems the saturation level is reached and precipitation of native protein occurs.

The solubility behavior of a partially denatured protein can be summarized as shown in Figure 1. The *precipitate* consists of the irreversibly denatured protein and of precipitated native protein. The former must be the concentration-independent fraction, simply because the aggregate is virtually insoluble under normal conditions, whereas the amount of the latter fraction must depend on the total protein concentration. The *supernatant* contains a saturated solution of native protein plus the soluble denatured protein. The latter may be of minor significance in practice, because the major part of it has a tendency to aggregate and precipitate on continued heating (Wolf and Tamura, 1969).

ENZYMATIC HYDROLYSIS AND PROTEIN SOLUBILITY

Hydrolysis of Native Proteins. Native, globular proteins are generally resistant to proteolysis and only in a few cases do proteolytic enzymes attack native proteins as such, e.g., in the formation of other proteolytic enzymes from their precursors (Green and Neurath, 1954). The relative resistance to proteolysis is generally explained by

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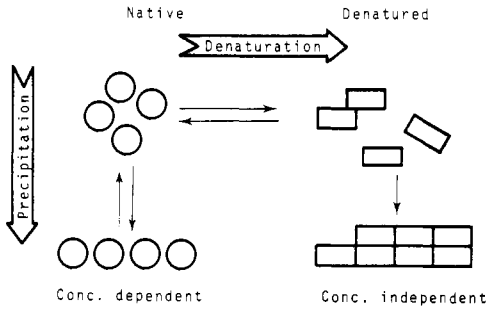

Figure 1. Solubility of globular proteins in relation to denaturation. Native protein is denoted by circles, denatured protein by rectangles.

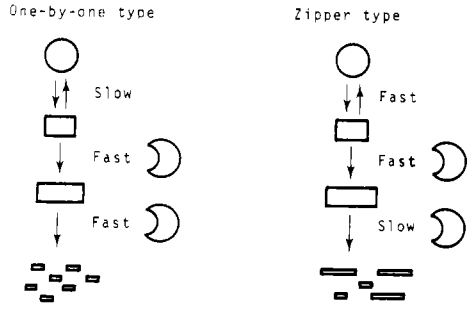

Figure 2. The Linderstrom-Lang hydrolysis model. Native protein is denoted by circles, denatured protein by rectangles, peptides by small rectangles of different length according to the chain length. The crescents symbolize enzymatic attack.

the compact tertiary structure of the protein which protects most of the peptide bonds. On the other hand, in the denatured form the peptide bonds are exposed and available for enzymatic cleavage. In solution the native protein molecule, because of its flexibility, is in a dynamic equilibrium with a number of more or less distorted forms, of which some are so different that they must be considered denatured and thereby accessible to enzyme attack. The breaking of one or a few peptide bonds destabilizes the molecule, an irreversible unfolding occurs, by which more peptide bonds are exposed, and the polypeptide chains are extensively degraded to intermediate peptides in accordance with the kinetic characteristics of the enzyme–substrate system.

Linderstrom-Lang (1952, 1953) proposed that the initial hydrolysis of a native globular protein could be described by two extreme types of reactions: the "one-by-one" and the "zipper" type (Figure 2). If the initial denaturation step is rate determining, it will appear as if the molecules are broken down, one by one, to peptides. No appreciable amounts of intermediary products will be found in the system. A number of globular proteins will under certain circumstances approach the one-by-one type, e.g., egg albumen, when it is hydrolyzed in the native state by pepsin (Christensen, 1953).

If the initial attack is of a much higher rate than the subsequent degradation to peptides, it will appear as if all the native protein is denatured and unfolded in the very beginning of the hydrolysis. The large number of exposed peptide bonds leads to the formation of a wide range of peptides in the subsequent reaction. Linderstrom-Lang used the word zipper to describe this type of reaction. As stated by Rupley (1967) in his review of Linderstrom-Lang's theory: "It is not entirely clear whether he referred to the zipping about of the enzyme or to the unzipping effect that cleavage of one bond often has on the protein structure". Zipper reactions are found in a few cases, as

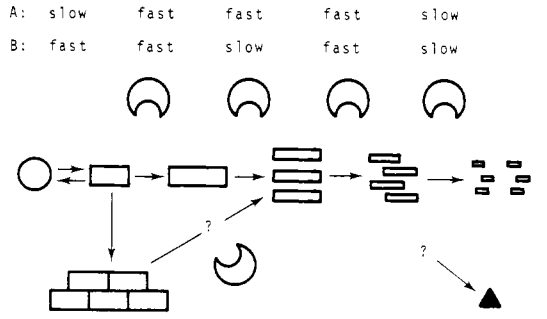

Figure 3. A qualitative model for enzymatic hydrolysis of partially denatured globular proteins: (A) one-by-one reaction; (B) zipper reaction. The triangle denotes the insoluble residue which remains after prolonged hydrolysis. All other symbols are as in Figure 2.

in the hydrolysis of ribonuclease by *Subtilisin Carlsberg* (Richards and Vithayathil, 1959).

Most proteins will show an intermediate behavior between the one-by-one and the zipper type. As Christensen (1953) demonstrated in his important work on denaturation and proteolysis, the hydrolysis behavior depends not only on the nature of the substrate and enzyme, but also on such parameters as the pH, temperature, and concentration of substrate and enzyme. These results point out interesting possibilities of *controlling* the outcome of a protein hydrolysis by varying the hydrolysis parameters.

Hydrolysis of Denatured Proteins. Several workers demonstrated that most proteins are more easily hydrolyzed in their denatured than in their native form (Fukushima, 1969, 1959; Christensen, 1953; Evans, 1946). This is also obvious from the Linderstrom-Lang hydrolysis model, if the system behaves according to the one-by-one reaction. Some of the studies have been done on reversibly denatured protein, others on irreversibly denatured protein. However, we do not know how the aggregation influences the kinetics of the hydrolysis. In both cases, there is a large number of available peptide bonds, and if the aggregates are small so that the reaction is not controlled by diffusion, it is assumed that this influence is of minor importance.

Hydrolysis of Partially Denatured Protein. By combining the qualitative statements mentioned above, a model for the hydrolysis of a partially denatured globular protein can be established (Figure 3). The model is a slight extension of the Linderstrom-Lang model. The subsequent hydrolysis to very small peptides is incorporated as well as the hydrolysis of the irreversibly denatured protein. In the latter case, the question marks signify our present lack of knowledge of the course of proteolysis. The insoluble residue is the ill-defined material which has been reported to remain undissolved after prolonged hydrolysis (Pour-el and Swenson, 1973a) or to build up in continuous hydrolysis experiments (Iacobucci et al., 1974).

In the light of our present knowledge of the solubility of the various compounds formed during the hydrolysis it is now possible to establish a qualitative connection between the course of hydrolysis and the composition of the soluble, respectively insoluble, fraction as shown in Figure 4. The difference in the hydrolysate composition between the one-by-one and the zipper type at a low degree of hydrolysis is remarkable. The functional properties may be very different in the two cases.

It should also be mentioned that the composition of the soluble fraction of the one-by-one type depends on the proportion between native and denatured protein in the initial material. As shown by Christensen (1953), the peptides will be larger in size if the protein is denatured

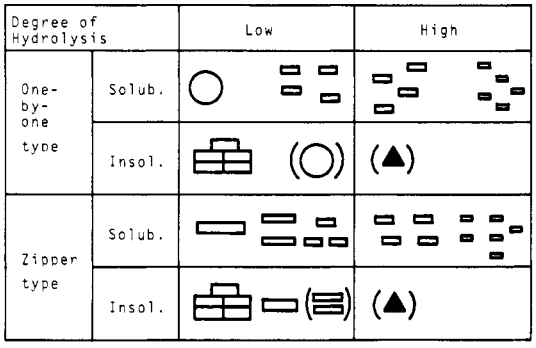

Figure 4. The qualitative composition of protein hydrolysates according to hydrolysis mechanism and DH (degree of hydrolysis). Symbols are as in Figures 2 and 3.

prior to hydrolysis. This is evident from the previous discussion, because the hydrolysis shifts toward a zipper type with the large number of available peptide bonds in the initial hydrolysis.

Degree of Hydrolysis. A brief comment should be made on the term degree of hydrolysis. In accordance with most reports in the literature, the definition below is suggested in preference to more empirical ones based on solubility in trichloroacetic acid or the like.

$$\text{DH} = \text{degree of hydrolysis}$$

$$\text{DH} = \frac{\text{no. of peptide bonds cleaved}}{\text{total no. of peptide bonds}} \times 100\%$$

$$= \frac{\text{av mol wt of AA residues}}{\text{equiv wt of end groups formed}} \times 100\%$$

BITTER PEPTIDE FORMATION

Even a quick survey of the literature on protein hydrolysates for food use reveals that the formation of bitter peptides during hydrolysis of certain proteins is regarded as the main problem. Casein is noted for giving rise to an intensely bitter taste when hydrolyzed with endopeptidases, and some of the peptides which are responsible for the bitterness have been characterized (Clegg et al., 1974a; Minamiura et al., 1972a,b). Soybean protein hydrolysates have also generally been reported as bitter, and the composition of some of the bitter peptides has been determined (Shiraishi et al., 1973; Arai et al., 1970). Many other proteins have been investigated, and a comprehensive study (Petritschek et al., 1972) revealed that the bitter taste depends on the protein rather than on the endopeptidase applied, although different enzymes gave different intensities of bitterness when compared with caffeine solutions. Matoba and Hata (1972) stated that the bitterness was caused by peptides with a high content of hydrophobic amino acids, regardless of their primary structure. In agreement with the Japanese group, Ney (1971/72, 1972) proposed a semiquantitative relationship which states that the higher the content of hydrophobic amino acids in a certain protein, the more pronounced its *tendency* to form bitter peptides. This theory has recently been supported by other workers (Clegg et al., 1974a; Schalinatus and Behnke, 1974, 1975a,b). Also, the observation (Noguchi et al., 1975) that the addition of glutamic acid peptides to bitter tasting protein hydrolysates effectively masks the off-taste indirectly confirms the theory.

The observation that different enzymes acting on the same substrate give rise to different intensities in bitterness (Petritschek et al., 1972) is not in conflict with Ney's theory. The bitterness seems to be more pronounced if the hydrolysis is very extensive, whereas limited hydrolysis occasionally prevents the formation of bitter taste. The commercial products known as "soy albumens" are thus partially hydrolyzed soybean proteins (Smith and Circle, 1972).

Unfortunately comparisons of various enzymes with regard to the formation of bitter taste (Petritschek et al., 1972; Murray and Baker, 1952) have *not* been carried out at a constant degree of hydrolysis, which makes conclusions as to the role of the enzyme rather difficult. It is plausible that the enzyme specificity has some influence; otherwise the remarkable capacity of pepsin in forming bitter hydrolysates (Petritschek et al., 1972) cannot be readily explained, but the field is still not adequately studied.

METHODS OF PRODUCING SOLUBLE PROTEIN HYDROLYSATES

Most of the work done on the preparation of soluble protein hydrolysates for food use has naturally concentrated on the problem of preventing the formation of bitter peptides, or on removing them if already present. One method is the use of an exopeptidase. This has been applied in the degradation of the bitter peptides in casein hydrolysates (Clegg and McMillan, 1974; Clegg, 1973; Sullivan et al., 1973). The method, however, required an enzymatic treatment of a fairly long duration.

The addition of 2–3% polyphosphate to the protein solution during hydrolysis is reported to have a positive effect on the formation of bitter peptides (Tokita, 1969), but the mechanism is unknown.

Roozen and Pilnik (1973) applied a reverse osmosis cell as an enzyme reactor for the hydrolysis of soybean protein. Their results, however, are not definite with regard to the claim that the peptide size can be controlled by the properties of the membrane, since only one type of membrane was used. The very extensive and meticulous work of Iacobucci et al. (1974) and Myers et al. (1974) on soybean protein hydrolysis in a continuous membrane reactor showed in fact that the peptide size was controlled by the reaction kinetics and not by the fairly high cut-off value (10 000–20 000 daltons) of the membrane.

In their studies Iacobucci et al. (1974) and Myers et al. (1974) used a thermostable acid proteinase which was able to degrade the protein to very small peptides with an average chain length of approximately three amino acid residues, in other words, a very high DH. The confinement of the enzyme in the membrane reactor permitted a large ratio of enzyme to substrate, whereby high productivity was obtained. In 1 h 94% of the protein was converted to hydrolysate. The nutritional value of the hydrolysate was, as expected, equal to that of the original substrate. The whole process was carried out on a pilot plant scale.

Among other significant works on soybean protein hydrolysate, the process developed by Pour-el and Swenson (1973a,b) should be mentioned. They hydrolyzed soybean protein isolate and concentrate under quiescent conditions and obtained a nonbitter hydrolysate, which forms a clear solution in acid beverages.

A treatment of soybean protein concentrate with dilute sulfuric acid at 105 °C and pH 3 for 15 min is described and patented by Chiang and Sternberg (1972). During this pretreatment the protein is denatured and partially hydrolyzed, and it is claimed that in the subsequent enzymatic hydrolysis higher yields and better taste of the hydrolysate are obtained, compared with hydrolysates made from untreated protein material.

Sugimoto et al. (1971) described a process for the manufacture of a lemon flavored soft drink enriched with soybean protein hydrolysate. Hydrolysis (8–10 h) at 50

°C with an acid protease from the fungus *Trametes sanguinea* gave a hydrolysate with an almost bland taste and favorable solubility properties.

Turning to other proteins we find the work of Clegg and her group on casein hydrolysis (Clegg, 1973; Clegg and McMillan, 1974). By hydrolyzing the casein with papain a bitter tasting hydrolysate is formed. Subsequent hydrolysis with exopeptidases from pig kidney tissue yields a hydrolysate with a bland taste and a very high DH. The hydrolysate is intended for dietetic treatment of children suffering from cystic fibrosis, where the digestion of protein in the small intestine is impaired (Clegg and McMillan, 1974). The process has recently been carried out on a pilot plant scale, using skim milk as the substrate (Clegg et al., 1974b).

The hydrolysis of fish protein does not present serious problems with bitter peptide formation. This is presumably the reason why fish protein hydrolysates are on the verge of being produced on large scale for food use. In this connection the work done at Massachusetts Institute of Technology on the solubilization of fish protein concentrate with various enzymes should be mentioned. The hydrolysis experiments were carried out as batch reactions (Cheftel et al., 1971) as well as continuous reactions (Cheftel, 1972; Archer et al., 1973). One of the interesting observations from the study of the kinetics of the continuous processes was that the enzyme is adsorbed on the insoluble substrate during hydrolysis, and that the remaining free enzyme can be removed with little or no difference in yield of soluble protein (Archer et al., 1973).

CONCLUSIONS

This brief summary of some of the aspects of soluble protein hydrolysates for food use leads to the conclusion that in future research in this field emphasis should be placed on the more general problems, such as the bitter peptide formation and the hydrolysis kinetics. Without a better understanding of these problems, enzymatic hydrolysis will remain at the fairly empirical stage. But it is also important that the work is performed with proteins which are of commercial interest, although they are more complicated to work with than the purified and well-characterized proteins. Then protein hydrolysates should have a fair chance of becoming an attractive means of improving the nutritional and organoleptic quality of our food.

LITERATURE CITED

American Oil Chemists' Society, "Official and Tentative Methods", 3rd ed, Method Ba 11-65, 1969a.
American Oil Chemists' Society, "Official and Tentative Methods", 3rd ed, Method Ba 10-65, 1969b.
Anderson, R. L., Wolf, W. J., Glover, D., *J. Agric. Food Chem.* **21**, 251 (1973).
Arai, S., Yamashita, M., Kato, H., Fujimaki, M., *Agric. Biol. Chem.* **34**, 729 (1970).
Archer, M. C., Ragnarsson, J. O., Tannenbaum, S. R., Wang, D. I. C., *Biotechnol. Bioeng.* **15**, 181 (1973).
Cheftel, C., *Ann. Technol. Agric.* **21**, 423 (1972).
Cheftel, C., Ahern, M., Wang, D. I. C., Tannenbaum, S. R., *J. Agric. Food Chem.* **19**, 155 (1971).
Chiang, P. C., Sternberg, M. Z. (to Miles Laboratories Inc.), German Offen. Patent 2 220 299 (Nov 9, 1972).
Christensen, L. K., *C.R. Trav. Lab. Carlsberg, Ser. Chim.* **28**, 37 (1953).
Clegg, K. M. (to The University of Strathclyde), British Patent 1 338 936 (Nov 28, 1973).
Clegg, K. M., Lim, C. L., Manson, W., *J. Dairy Res.* **41**, 283 (1974a).
Clegg, K. M., McMillan, A. D., *J. Food Technol.* **9**, 21 (1974).
Clegg, K. M., Smith, G., Walker, A. L., *J. Food Technol.* **9**, 425 (1974b).
Evans, R. J., *Arch. Biochem.* **11**, 15 (1946).
Fukushima, D., *Bull. Agric. Chem. Soc. Jpn.* **23**, 7 (1959).
Fukushima, D., *Cereal Chem.* **46**, 405 (1969).
Green, N. M., Neurath, H., *Proteins* **2B**, 1171–1198 (1954).
Iacobucci, G. A., Myers, M. J., Emi, S., Myers, D. V., IV IUFoST Congress, Madrid, Spain, Sept 1974.
Linderstrom-Lang, K. U., "Proteins and Enzymes", Lane Medical Lectures, Vol. VI, Stanford University Press, Stanford, Calif., 1952, pp 53–72.
Linderstrom-Lang, K. U., *Bull. Soc. Chim. Biol.* **35**, 100 (1953).
Matoba, T., Hata, T., *Agric. Biol. Chem.* **36**, 1423 (1972).
Minamiura, N., Matsumura, Y., Fukomoto, J., Yamamoto, T., *Agric. Biol. Chem.* **36**, 588 (1972a).
Minamiura, N., Matsumura, Y., Yamamoto, T., *J. Biochem.* **72**, 841 (1972b).
Murray, T. K., Baker, B. E., *J. Sci. Food Agric.* **3**, 470 (1952).
Myers, D. V., Ricks, E., Myers, M. J., Wilkinson, M., Iacobucci, G. A., IV IUFoST Congress, Madrid, Spain, Sept 1974.
Nash, A. M., Kwolek, W. F., Wolf, W. J., *Cereal Chem.* **48**, 360 (1971).
Ney, K. H., *Z. Lebensm. Unters. Forsch.* **147**, 64 (1971/72).
Ney, K. H., *Z. Lebensm. Unters. Forsch.* **149**, 321 (1972).
Noguchi, M., Yamashita, M., Arai, S., Fujimaki, M., *J. Food Sci.* **40**, 367 (1975).
Petritschek, A., Lynen, F., Belitz, H.-D., *Lebensm. Wiss. Technol.* **5**, 77 (1972).
Pour-el, A., Swenson, T. C. (to Archer Daniels Midland Co.), U.S. Patent 3 713 843 (Jan 30, 1973a).
Pour-el, A., Swenson, T. C. (to Archer Daniels Midland Co.), U.S. Patent 3 741 771 (June 26, 1973b).
Richards, F. M., Vithayathil, P. J., *J. Biol. Chem.* **234**, 1459 (1959).
Roozen, J. P., Pilnik, W., *Process Biochem.*, 24 (July 1973).
Rupley, J. A., *Methods Enzymol.* **11**, 905–912 (1967).
Schalinatus, E., Behnke, U., *Nahrung* **18**, 697 (1974).
Schalinatus, E., Behnke, U., *Nahrung* **19**, 433 (1975a).
Schalinatus, E., Behnke, U., *Nahrung* **19**, 447 (1975b).
Shiraishi, H., Okuda, K., Sato, Y., Yamaoka, M., Tuzimura, K., *Agric. Biol. Chem.* **37**, 2427 (1973).
Smith, A. K., Circle, S. J., *Soybeans: Chem. Technol.* **1**, 372 (1972).
Sugimoto, H., van Buren, J. P., Robinson, W. B., *J. Food Sci.* **36**, 729 (1971).
Sullivan, J. J., Mou, L., Rood, J. I., Jago, G. R., *Aust. J. Dairy Technol.*, 20 (March 1973).
Tanford, C., *Adv. Protein Chem.* **23**, 123 (1968a).
Tanford, C., *Adv. Protein Chem.* **23**, 275 (1968b).
Tokita, F., *Z. Lebensm. Unters. Forsch.* **138**, 351 (1969).
van Megen, W. H., *J. Agric. Food Chem.* **22**, 126 (1974).
Wolf, W. J., *J. Agric. Food Chem.* **18**, 969 (1970).
Wolf, W. J., *Soybeans: Chem. Technol.* **1**, 101 (1972).
Wolf, W. J., Tamura, T., *Cereal Chem.* **46**, 331 (1969).
Wu, W. J., Inglett, G. E., *J. Food Sci.* **39**, 218 (1974).

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