The biological activity of undenatured dietary whey proteins: role of glutathione

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Abstract
This study compared the effects of different sources of whey protein concentrate (20g/100 g diet) and of casein on the spleen, liver, and heart glutathione content of C3H/HeJ mice, and on the immune response of their spleen cells to sheep red blood cells. Body weight curves were similar in all dietary groups. Our data indicate that the humoral immune response is highest in mice fed a dietary whey protein concentrate exhibiting the highest solubility (undenatured conformation) and a greater relative concentration of the thermolabile bovine serum albumin and immunoglobulins. In addition, the mice fed this type of whey protein concentrate exhibit higher levels of tissue glutathione. The presence in the serum albumin fraction of glutamylocysteine groups (rare in food protein) and the specific intramolecular bond as related to the undenatured conformation of the molecule are considered to be key factors in the glutathione-promoting activity of the protein mixture.

Résumé
Notre étude compare l'effet des protéines du petit lait (20 g/100 g de diète) de plusieurs sources et de la caséine sur le contenu en glutathion de la rate, du foie, et du cœur de la souris ainsi que sur la réponse immunitaire des cellules spléniques de souris C3H/HeJ provoquée par l'injection d'érythrocytes de moutons. Les courbes de croissance des souris se sont avérées similaires dans tous les groupes. Nos données indiquent une réponse humorale immune plus importante chez les souris nourries avec un concentré de petit lait de grande solubilité (non dénaturé) contenant un concentration élevée d'albumine bovine sérique thermolabile et d'immunoglobulines. De plus les souris nourries avec ce type de concentré protéique ont présenté des niveaux tissulaires élevés de glutathion. La présence dans l'albumine sérique de glutamylocystéine, un peptide rare dans les protéines diététiques et les liaisons spécifiques intramoléculaires réliées à la conformation non-dénaturée de ces molécules, sont donc considérées comme des facteurs positifs permettant l'accumulation de glutathion dans ces circonstances.

Introduction
Our studies have shown that the humoral immune response (number of plaque-forming cells to sheep red blood cells) is significantly higher in mice fed a 20 g whey protein concentrate/100 g diet than in mice fed formula diets of similar nutritional efficiency containing 20 g/100 g diet of any other type of commercially available semipurified food protein, such as casein, soy, wheat, corn, egg white, fish, beef protein, *Spirulina maxima*, *Scenedesmus* algae protein, or Purina mouse chow [1]. The im-
munoenhancing effect of whey protein concentrate is maintained when the proteins are replaced in formula diet by a pancreatic hydrolysate (oligopeptides with mol. wt. <1000) of undenatured whey protein concentrate [2, 3]. This observation appears to obviate the likelihood of milk protein allergy or some other manifestation of oral immunization. We have further shown that the immunoenhancing activity of dietary whey protein concentrate is related to greater production of splenic glutathione in the whey protein-fed animals during the oxygen-requiring antigen-driven clonal expansion of the lymphocyte [3]. It was then theorized that this might reflect the ability of the lymphocytes of whey protein diet-fed mice to offset potential oxidative damage, thus responding more fully to the antigenic challenge [4, 5]. In fact, the capacity of a cell to recover from an oxidative insult is considered to be represented by its ability to regenerate intracellular stores of glutathione [6]. Our studies also showed that administration of S-(n-butyl) homocysteine sulfoximine, which reduces splenic glutathione in half, significantly reduced the humoral immune response of whey protein-fed mice. This was taken as further evidence for the important role of glutathione in the immunoenhancing effect of dietary whey protein [3]. A recent observation has revealed to us that the described biological activity of whey protein concentrate, already shown to be unrelated to its nutritional quality, is actually dependent on the undenatured conformation of the ingested proteins. This discovery was made accidentally when a batch of whey protein concentrate that was sent to us in 1988 by the usual supplier (Lacprodan from DanMark Protein S.A., Denmark) failed to exhibit the immunoenhancing effect previously described while exhibiting the same nutritional efficiency. Upon analysis, it became apparent that this preparation was less water soluble than the previous samples of undenatured whey protein concentrate exhibiting high biological activity.

The present study was designed to define the effect of changes in the molecular conformation of whey protein concentrate on the immune response and glutathione formation of the host, and to explore the factors and the mechanism of the observed effect of dietary whey protein concentrate on glutathione formation and on humoral immunity.

Table 1. Vitamin and mineral content of formula diets

<table>
<thead>
<tr>
<th>Vitamin/mineral</th>
<th>Content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>20,000</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>2,000</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>12,000</td>
</tr>
<tr>
<td>Thiamin</td>
<td>90</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>140</td>
</tr>
<tr>
<td>Niacin</td>
<td>20</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>50</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>25</td>
</tr>
<tr>
<td>Biotin</td>
<td>5</td>
</tr>
<tr>
<td>vitamin C</td>
<td>100</td>
</tr>
</tbody>
</table>

Materials and methods

Animals
Male C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME.) at seven weeks of age and were maintained five per cage in a temperature-controlled 12 h light-dark cycle room.

Diets
The detailed composition of the common ingredients (vitamins and minerals) in all of the defined formula diets is given in Table 1. Diets were prepared in the following way: 20 g of selected pure protein, 56 g of product 80056 protein-free diet powder containing corn syrup, corn oil, tapioca starch, vitamins, and minerals (Mead-Johnson Co. Inc., IN.), 18 g corn-starch, 2 g wheat bran, 0.05 g Nutramigen vit-iron premix (Bristol-Meyers, Ont.), 2.65 g KCl, 0.84 g NaCl. The only variable in the various purified diets was the type of protein. The formula diets contained 20 g/100 g diet of either of the following types of bovine whey protein concentrate: Product X, especially prepared for us by the “Service de recherche sur les aliments du Ministère de l’agriculture du Québec” in St.-Hyacinthe, Que. (Table 2); Promod (Ross Laboratories, Columbus, OH.); Alacen 855 (New Zealand Dairy); Lacprodan-80 (produced in 1989 by Danmark Protein, Worthington, OH.); Sapro (Saputo, Montréal, Que.); Savorporo-75 (Golden Cheese, CA.); Bioisolates (Lesueur Isolates,
Table 2. A schematic representation of the process to produce Product X

<table>
<thead>
<tr>
<th>Raw milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Skimmed at 35°C</td>
</tr>
<tr>
<td>→ cream</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Skimmed milk pasteurized</td>
</tr>
<tr>
<td>at 63°C for 30 min</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>At 38°C</td>
</tr>
<tr>
<td>Addition of rennet (20 ml/100 kilos), allowing the agitation to resolve at low speed</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>→ curd</td>
</tr>
<tr>
<td>Whey</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Filtered with cheese cotton to remove debris (45 min)</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>At 40°C</td>
</tr>
<tr>
<td>Ultrafiltration (Romecon UFSI, polysulphone membrane, cut off 50,000 pore diameter 60/1000 of an inch, surface 2–3 m²)</td>
</tr>
<tr>
<td>Dialfiltration to wash out salts and lactose</td>
</tr>
</tbody>
</table>

Whey Protein Concentrate

↓ (Pasteurized at 63°C for 30 min)

At 40°C

Lyophilization

↓

Whey Protein Concentrate Powder

Minneapolis, MN.). An additional group of mice was fed a diet containing 20 g of casein free of whey protein coprecipitate/100 g diet. All purified proteins were vitamin free. The net protein content of the different protein powders was taken into consideration when preparing the various 20 g protein/100 g diets. Diets were continuously available in powder form from stainless steel feeders, 1.5 inches high and especially designed to reduce spillage and spoilage. Mice were placed on the various diets and immunologic studies or spleen glutathione assays commenced 3 weeks later.

**Solubility measurements**

Heat denaturation unfolds and exposes the poorly soluble hydrophobic amino acid residues of whey proteins to water. The extent of denaturation is normally assessed by loss of solubility at pH 4.6 [7, 8]. In our studies we evaluated solubility by the following method: after dispersion of a 4% protein solution in distilled water at room temperature and pH adjustment at 4.6 acetic acid/sodium acetate buffer, the solution was stirred. Percent solubility was computed as the portion of total protein filtered through a Durieux red III filter paper. Total nitrogen was determined by the micro-Kjeldahl method.

**Gel electrophoresis**

Polyacrylamide gel electrophoresis of the whey protein concentrate samples was carried out with 20% polyacrylamide at pH 8.8 (Laemmli buffer system) after the samples were reduced with 10% 2-mercaptoethanol. An eight-slot gel was run and samples were applied so that each slot received 40 μg of the sample on a dry weight basis. Electrophoresis was performed at 200 volts for 45 min.
Immunization for plaque assays
The diet-fed mice were immunized by an intravenous injection of \(5 \times 10^6\) washed sheep red blood cells obtained weekly from Institut Armand-Frappier, Laval des Rapides, Quebec.

Plaque forming cell (PFC) assay
The method used for assaying IgM plaque forming cells was as described by Cunningham and Szenberg [9] with minor modifications. Spleen cell suspensions were prepared by gently tamping the spleen through a 50-mesh stainless steel screen, and collecting the cells in balanced salt solution (BSS) supplemented with 10% heat-inactivated calf serum (Grand Island Biological Company, Montreal, Que.). The spleen cells were washed and made up to 15 ml with BSS. Sheep red blood cells were washed twice and made up to a 20% concentration. Guinea pig serum (Grand Island Biological Company, Montreal, Que.) was a source of complement was diluted 1/15 with BSS. All stock solutions were kept on ice water until used. The test consisted of mixing 0.05 ml of spleen cells, 0.15 ml of sheep red blood cells, 0.75 ml of the complement solution in a test tube at 37°C. The whole mixture was immediately withdrawn and put into slide chambers, sealed with warm paraffin wax, and incubated at 37°C for 45–60 min. The number of plaque-forming cells was counted and the total number of plaque-forming cells per spleen estimated by multiplying the number of plaque-forming cells in each sample (0.05 ml spleen cells) by 300. Plaque-forming cells have been expressed per total organ rather than per 10^6 spleen cells, since this appears to reflect more accurately the functional status of the spleen per se.

Mice were normally assayed for the plaque-forming cell response to sheep red blood cells on the fifth day after immunization, when the response was shown to peak.

Spleen, heart, and liver glutathione content
Ninety milligrams of mouse tissue were weighed using a Mettler PM-300 balance; samples varied from 90 mg by less than 5 mg (<5%). The samples were homogenized in 5-sulfosalicylic acid (5% w/v). Homogenates were centrifuged for 5 min in a microfuge at 10,000 \(\times\) g. The assay was carried out using the supernatants on the same day according to the methods of Anderson [10]. Values are expressed as \(\mu\)mol/g/wet tissue.

Statistics
Values were compared among the dietary groups using either Student’s t-test, when two groups were being compared, or the analysis of variance (ANOVA) for more than two groups. Each dietary group comprised at least ten mice.

Results
Data in Table 3 indicate that humoral immune response as measured by the plaque assay is highest \((p < 0.004\) by ANOVA) in mice fed Product X, which exhibited the highest level of solubility. The serum albumin content of Product X, at 10% of total whey protein, is almost twice the corresponding values found in any other whey protein concentrate examined \((p < 0.05\) by ANOVA). In addition the level of immunoglobulins is also substantially higher in Product X \((p < 0.05\) by ANOVA). No intergroup difference was noted in body growth, food consumption, or serum protein (data not shown).

On days 2, 3, 4, and 6 after immunization, the spleen glutathione levels in Product X diet-fed mice were 13% \((p < 0.01)\), 8% \((p < 0.05)\), 21% \((p < 0.01)\) and 20% \((p < 0.01)\) higher than the corresponding values in Lacprodan-80 diet-fed mice, and they were 12% \((p < 0.01)\), 7% \((p < 0.05)\), 20% \((p < 0.001)\), and 20% \((p < 0.001)\) higher than the corresponding values in casein diet-fed mice (Figure 1). No intergroup difference was noted in body growth, food consumption, and serum protein (data not shown). After three weeks of dietary treatment, no significant differences were seen among dietary groups in splenic glutathione levels of unimmunized mice \([3.10 \pm 0.30\) (mean \pm standard deviation), 3.09 \pm 0.29, 3.07 \pm 0.33, \(\mu\)mol/g in Product X diet-fed, Lacprodan-80 diet-fed, or casein diet-fed mice respectively]. These values were maintained up to 5 days later. Hence the values reported in Figure 1 as percentage of values in the corresponding unimmunized mice are considered to be significant variations from control values.

Table 3 shows that glutathione in the liver of unimmunized mice fed Product X diet for 3 weeks is higher than the corresponding values in Promod,
Table 3. Physical-chemical characteristics and biological activity of different types of whey protein concentrate

<table>
<thead>
<tr>
<th>Undenatured conformation (pH 4.6)</th>
<th>Protein composition (% of total whey proteins)</th>
<th>Effect of 3 weeks dietary treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-LG¹</td>
<td>Alpha-La²</td>
</tr>
<tr>
<td>Product X</td>
<td>99.5%</td>
<td>57.8 ± 0.9</td>
</tr>
<tr>
<td>Promod</td>
<td>97%</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Alacen 855</td>
<td>97.1%</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>Lacprodan-80</td>
<td>96%</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>Sapro</td>
<td>95%</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Savorpro-75</td>
<td>98%</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>Bioisolate</td>
<td>90.1%</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>Casein</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; 20 g protein/100 g diet
¹β-LG, β-Lactoglobulin
²Alpha-La, Alpha-Lactalbumin
³S.A., Serum Albumin
⁴Number of plaque-forming cells / spleen 5 days following immunization with 5 × 10⁶ sheep red blood cells
⁵Effect of 3 weeks of dietary treatment in immunized mice
-- Not done
*Please see text for statistical significance

Fig. 1. Spleen glutathione levels expressed as percent of values in unimmunized mice fed the corresponding diets for 3 weeks. Effect of 3 weeks dietary treatment with 20 g/100 g diet of either Product X, Lacprodan-80, or casein. D-day following immunization with 5 × 10⁶ sheep red blood cells (SRBC). Each value represents the mean of 10 mice. See text for statistical analysis.

Alacen or Lacprodan-80 diet-fed mice (p < 0.01 by ANOVA). Glutathione in the heart of unimmunized mice fed Product X diet for 3 weeks is higher than the corresponding values in Promod, or casein diet-fed mice (p < 0.05 by ANOVA). No intergroup difference was noted in body growth, food consumption, or serum protein (data not shown).

Discussion

Our data are consistent with previous findings demonstrating the correlation between enhancement of plaque-forming cells response and spleen glutathione levels in immunized mice fed whey protein in comparison to mice fed the casein diet, and the central role of glutathione in the immunoenhancing effect of dietary whey protein [3]. The current findings indicate that the previously described biological activity of dietary whey protein is restricted to the undenatured form of the protein, and it is not related to its nutritional efficiency. The differences in solubility of the protein mixture as measured by filtration may reflect conformational changes biologically more significant than the actual numbers appear to indicate. Indeed specific thermolabile proteins, crucial to the biological activity of the protein mixture, may be involved. In addition, partial unfolding of some molecules, undetected by solubility changes, could initiate further unfolding and/or
Table 4. Protein composition of cow and human milks

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cow milk</th>
<th>Human milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/L)</td>
<td>Mol. wt.</td>
</tr>
<tr>
<td>Caseins</td>
<td>26</td>
<td>19,000–25,000</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>3.2</td>
<td>18,300</td>
</tr>
<tr>
<td>β-lactoglobulin**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfa-lactalbumin</td>
<td>1.2</td>
<td>14,160</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.4</td>
<td>66,267</td>
</tr>
<tr>
<td>IgG</td>
<td>0.6</td>
<td>155,000</td>
</tr>
<tr>
<td>IgA</td>
<td>0.1</td>
<td>400,000</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>trace</td>
<td>80,000</td>
</tr>
</tbody>
</table>

*Disulfide bond


In columns 3 and 5 the numbers of Glu-Cys groups per molecule of protein are given.

other biologically significant alterations during the process of digestion in the gastrointestinal tract. The type of protein present in the whey protein concentrate is of obvious importance. Our analysis shows that the relative concentration of the heat-labile bovine serum albumin and immunoglobulin is highest in Product X, which exhibits greater biological and glutathione-promoting activity.

The factors and mechanisms of the observed effect of some undenatured whey proteins on glutathione formation is a matter of interest. Glutathione (L-gamma-glutamyl-L-cysteinylglycine) is dependent upon the supply of cysteine which is derived from dietary protein. At the present time, the optimal strategy for obtaining “normal” glutathione tissue levels in healthy animals is by providing the animal with a diet containing adequate levels of protein. Little or no increase in tissue or organ glutathione has been found when animals on a diet with 20–25% protein are given supplemental sources of sulfur amino acids [11]. Recent studies in rats showed that whereas a growth-retarding low casein diet caused decreased hepatic glutathione concentration, neither excess casein (30 and 45%) nor excess sulfur amino acids could increase glutathione concentrations above the levels found when rats were fed a diet adequate in protein (15%) [12]. Whereas concentrations of glutathione are dependent on the supply of cysteine, administration of cysteine is not an ideal way to increase glutathione concentration because cysteine is rapidly metabolized and furthermore, it is toxic [13, 14]. On the other hand, tissue glutathione concentration may be increased by administration of gamma-glutamylcysteine: glutathione increased in the kidney by about 50%, 40–60 min after s.c. injection in mice, returning to control values 2 h later [15]. The administered gamma-glutamylcysteine is transported intact and serves as substrate of glutathione synthetase [16]. Advances in amino acid sequencing of food proteins allowed us to investigate the occurrence of glutamylcysteine groups in whey protein and the possible relation to glutathione promotion. Indeed, whey protein concentrate from bovine milk contains substantial amounts of glutamylcysteine groups, unlike casein, which does not increase tissue glutathione when fed to mice [1] (Table 4). The glutamylcysteine groups are located primarily in the serum albumin fraction (Table 4). Although the amino acid sequence of bovine milk immunoglobulins is not totally known, the high levels of cysteine and glutamine in bovine immunoglobulin suggest the possi-
bility of glutamlycysteine groups as described in human milk immunoglobulin (Table 4). Glutamlycysteine groups are extremely rare in animal and plant edible proteins. An extensive search of all available data on amino acid sequencing of edible proteins [23–57] reveals that the Glu-Cys group with disulfide link is indeed limited to some of the whey protein as indicated in Table 4, and to the ovomucoid fraction of egg white which contains 2 of these groups in a 30,000 mol. wt. molecule [26]. This molecule represents about 8% of total egg white proteins which are 75% ovalbumin [26]. This literature search included 81 different animal and plant proteins from several species as indicated in the title of the references [23–57]. In the natural state the milk whey proteins have a definite configuration which, when exposed to heat above a certain critical level, is disrupted [58]. In addition to heating, other processing treatments, e.g., pumping, mixing, aeration, vacuum evaporation, and spray drying further promote denaturation [59]. The half-cystine residues, frequent in some of the whey proteins [18] are connected by intramolecular disulfide bonds which contribute to the spatial conformation of the molecule and partly block unfolding of the molecule [60]. It is interesting to note that the cystine residues of all glutamlycysteine groups in β-lactoglobulin and serum albumin are connected by intramolecular disulfide bonds [18]. It is also relevant to note that pancreatic trypsin does not hydrolyse the disulfide cross-linkage characteristic of the native whey proteins [61]. It is our hypothesis that the glutathione-promoting activity of dietary whey protein concentrate is dependent on the glutamlycysteine groups contained in serum albumin fractions, in the β-lactoglobulin and possibly in the immunoglobulin G fraction. The preservation of the disulfide bond (which involves the cysteine) may be crucial to the release, upon digestion, of intact glutamlycysteine peptide for absorption by the intestinal mucosa. Denaturation, on the other hand, by unfolding the protein molecule, exposes the glutamlycysteine sequence to the digestive enzymes with subsequent release of either of the single amino acids or other peptide combinations. This would be consistent with the lesser glutathione-promoting activity of the denatured form of whey protein concentrate which contains the same amount of cysteine. It is conceivable that the single glutamlycysteine group in the small β-lactoglobulin molecule is more vulnerable during the intestinal transit than some of the 6 groups in the folded, larger serum albumin molecule. Whether glutamlycysteine enters the cells as a cysteine-mixed disulfide is not known. If this were the case, it could more readily cross cell membranes [62].

The two phases in the production of whey protein concentrate involve firstly the thermal treatment of milk (pasteurization) prior to and during the manufacture of cheese. The second step involves the concentration of the proteins from whey through various phases of ultrafiltration, and freeze or spray drying or lyophilization to obtain a powder that may contain 80–90% whey proteins. If denaturation of a whey protein during the first phase is irreversible, it is evident that the denatured protein would be lost from whey by precipitating with the caseins in the formation of cheese. During the subsequent treatment of whey with the objective of concentrating the remaining proteins, more protein could be denatured but not lost from the whey protein concentrate. The extent of denaturation produced during the second phase of whey protein concentrate production is normally assessed by the loss of solubility at a pH of 4.6. With a denaturation temperature of 78°C, β-lactoglobulin is the least denaturable of the serum proteins. On the other hand, with a denaturation temperature of 64°C, bovine serum albumin is denatured almost as easily as alpha-lactalbumin. Since its denaturation is not as reversible as that of alpha-lactalbumin, it appears to be the most easily denatured serum protein. It precipitates between 40°C and 50°C as a result of hydrophobicity-directed unfolding [63]. Whereas extensive investigations of this protein isolated from bovine blood serum have been made, the configuration of bovine serum albumin isolated from milk has not been investigated [63]. Immunoglobulins in milk are also very heat labile, especially below a pH of 6 [63].

Our Product X whey protein concentrate was prepared in the most lineat way compatible with accepted standards of safety with regard to bacterial contamination. The extremely high solubility index indicates that the proteins present are essentially undenatured, hence demonstrating the lineancy of the ultrafiltration process. Although the proteins contained in the concentrates from the other sources examined were mostly in undenatured form, as indicated by the relatively high solubility of the concen-
trates, the content of serum albumin and immunoglobulins in these mixtures is below the level apparently necessary to produce a biological activity. These very thermolabile proteins are denatured, hence precipitated and partially lost from whey when high pasteurization temperatures are utilized. Conversely, the relatively high concentrations of the thermosensitive serum albumin and immunoglobulins resulting from the low pasteurization of milk in Product X, may reflect more closely the pattern of raw milk. These data lend support to the hypothesis that the thermolabile Glu-Cys containing proteins such as serum albumin in undenatured conformation are crucial elements for the biological activity of whey protein concentrate.

We have also tested another, more rapid, method of low level milk pasteurization (72°C for 13 s) with demonstrated antibacterial effect comparable to that obtained with the method described in Table 2. The characteristic features of relatively high serum albumin and immunoglobulin levels noted in Product X (Table 3) are more pronounced in the whey concentrate obtained from milk pasteurized at 72°C for 13 s: β-lactoglobulin 52 ± 1%; alpha-lactalbumin 6 ± 1%; serum albumin 14 ± 1%; immunoglobulins 28 ± 2%.

Enhancement of glutathione levels in the tissues may well represent the common denominator underlying the beneficial effect observed by a diet of whey protein, as demonstrated by increased longevity in hamsters [64] and mice [65], increased resistance to carcinogens [66] and pneumococcal infection [67] in mice.

Mammalian cells have evolved numerous mechanisms to either prevent or treat injurious events that might result from normal oxidative byproducts of cellular metabolism. Foremost among these endogenous protective systems is the glutathione redox system. Glutathione is a ubiquitous tripeptide (L-gamma-glutamyl-L-cysteinyl-glycine) present in high concentrations (generally in the millimolar range) in most mammalian cells as reduced glutathione [68, 69]. Glutathione peroxidase eliminates H₂O₂ generated by mitochondrial O₂ consumption employing glutathione as a hydrogen donor to reduce H₂O₂ to water. Therefore glutathione plays a critical role in the defense against oxygen toxicity by breaking the chain of reactions leading from superoxide anion to the very active hydroxyl radical through intermediate H₂O₂. Undoubtedly the efficiency of this reaction is important in modulating the aging process [69].

In addition to detoxification of endogenous toxins, glutathione plays a crucial and unique role in the elimination of exogenous toxins and xenobiotics. Accordingly, the liver, having the greater content of glutathione [69], is the major organ involved in the elimination and detoxification of xenobiotics. Glutathione readily binds transition metals and is an important factor in their elimination [70]. Among the reactions associated with the -SH group of glutathione are those which convert β-ketoaldehydes (which are toxic) to the corresponding alpha-hydroxyacids [71]. Glutathione inactivates electrophilic drugs and carcinogen metabolites by formation of glutathione conjugates [72]. The reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, and thus good candidates for detoxification by reaction catalyzed by glutathione [68]. This peptide plays a role in protection against tissue damage resulting from exposure to ozone [73] and can also protect against radiation [74]. It has been reported that resistance to x-irradiation varies synchronously with glutathione content. Cellular sensitivity is increased when glutathione level is low [75–77]. A damage-producing product of ionizing radiation is considered to be the hydroxyl radical which reacts rapidly with most organic molecules, including DNA [78]. Thiols react with hydroxyl radicals almost exclusively by donation of a hydrogen atom from the SH bond. A second way in which thiols can protect DNA is through chemical repair of DNA [78].

The free radical theory of aging [79] hypothesizes that the degenerative changes associated with aging might result from toxic effects of the free radicals produced during cellular metabolism. Aging is thus considered to be caused by the byproducts of normal physiological metabolic processes of life. One approach taken to verify the free radical theory of aging has been to determine whether any age-related changes occur in cellular antioxidative protective mechanisms. One such principal mechanism is glutathione, which is a ubiquitous cellular constituent and the most abundant thiol-reducing agent in mammalian tissues. The ubiquitous nature of the aging process makes glutathione an interesting object of aging-related research. It appears that, where-
as data or age-related changes in tissue vitamin E and other antioxidants are, at best, contradictory [79] the tissue glutathione levels are more consistently reported to decline with old age. Thus glutathione contents of liver, kidney, heart [80], and brain [81] were respectively 30%, 34%, 20%, and 30% lower in very old mice than in mature mice. A lower glutathione status has been demonstrated in erythrocytes of aging mice [82] and humans [83]. Glutathione peroxidase activity was also reported lower in the elderly population [84].

More specifically, some characteristic age-related diseases appear to be preceded by or associated with a drop in glutathione content in the organ or systems involved. The immune system loses its effectiveness with age. A major factor in this loss of functions is a progressive age-related failure of T-lymphocytes to respond to proliferating stimuli [85]. The age-related decline in immune responsiveness of mice was found to be associated with a 19% drop in spleen glutathione content [86]. Our experiments indicate that the immunoenhancement by whey protein feeding is dependent upon the glutathione-promoting activity of this diet [3]. Even non age-related conditions of immunodepression have been found to be characterized by decreased levels of glutathione. Systemic glutathione deficiency has been recently observed in symptom-free HIV seropositive individuals [87]. Intracellular glutathione appears to play an important role with the defense of endothelial cells against oxidized low density lipoproteins, hence in the pathogenesis of arteriosclerosis [88].

Osteoarthritis has many characteristics of a free radical-produced disease. The breakdown of the polymer hyaluronic acid, which acts as a joint lubricant, may be caused by oxygen free radicals produced by neutrophils that accumulate in the affected joint. In addition to its action as free radical scavenger, glutathione could be implicated as a source of cysteine [73], because cysteine serves as the active sulfate for the biosynthesis of chondroitin sulfate in the cartilage [89]. In addition, oxygen-derived free radicals stimulate osteoclastic bone reabsorption in rodent bone in vitro and in vivo [90].

One aspect of aging is the formation of cataracts. This process in part involves lipid peroxidation. Severe glutathione depletion in the lens by administration of buthionine sulfoximine produces cataract formation in young mice [91].

Many hypotheses have been proposed to explain the physiology of Alzheimer's disease. The increased prevalence of Alzheimer's disease with age and the fact that the neuropathological injuries are similar in Alzheimer's disease and in normal cerebral aging suggest that the so-called Alzheimer's disease is in fact accelerated aging. As mentioned earlier, the free radical theory of aging postulates that aging can be induced by the deleterious effect of free radicals. Indeed, the level of lipid peroxidation is higher in the brain of subjects affected by Alzheimer's disease than in the brain of the non-demented elderly [92], although an age-related rise in blood lipid peroxides has been reported with fully adequate vitamin E status [93]. A significant decrease in the level of glutathione peroxidase has been reported in erythrocytes of patients with Alzheimer's disease together with evidence of lipid peroxidation [94].

Finally, a recent multicentre study has demonstrated that glutathione concentrations were significantly lower in the putamen, globus pallidus, substantia nigra, and frontal cortex of Parkinsonian brains. A direct positive correlation between the severity of cellular deficit and the glutathione content could be calculated, whereas the distribution of ascorbate was relatively even with no significant differences between controls and Parkinsonian brains [95].

Our current and previous studies demonstrate that it is feasible to produce a moderate but sustained increase in tissue glutathione levels in young and old mice by the simple method of administering undenatured whey protein concentrate containing most of the bovine serum albumin originally present in the raw milk. The present discovery obviates the toxic effects of other known methods for increasing the intracellular levels of glutathione. Most cell types have no direct system for transport of intact glutathione into cells [74]. The methods involving the use of acetyl-cysteine [13], gamma-glutamyl-cyst(e)ine [15], athiazolidine [96], or glutathione esters [97] offer an interesting possibility for short-term intervention. However, their long-term effectiveness in producing sustained elevation of cellular glutathione has not been shown, nor has the possible toxicity of their long-term use been disproved. Our previous studies [63] indicate that sustained elevation of tissue glutathione levels can be obtained by
the administration of undenatured whey protein concentrate (tested up to three months). This discovery could provide a method for efficiently increasing cellular glutathione levels for any purpose for which elevated glutathione levels are desired such as for drug detoxification, arteriosclerosis, Alzheimer's and Parkinson diseases; cellular protection against oxygen and its metabolites such as peroxides, free radicals or foreign compounds, carcinogens, irradiation, immunodeficiency states, etc.

The main similarities between egg white and milk whey are unique features in the plant and animal kingdom: the highest cysteine content of any food protein (2.4 g %) and the presence of glutamylcysteine (Glu-Cys) groups (glutathione precursor) with disulfide bonds. The Glu-Cys groups of egg white proteins are contained in a minor protein constituent (ovomucoid) and their total amount in g/litre of egg white is comparable to that found in milk whey. It may also be noteworthy that from time immemorial, whey from raw milk and/or undenatured raw egg white have been administered to children and to the sick as prophylactic or therapeutic measures in folk medicine. This is probably one of the very few instances in which uncooked, i.e., undenatured animal products are fed to humans, in modern times. Whey protein concentrate was administered in our experiment as the protein component of a normal mouse diet containing all the other ingredients, such as energy, vitamins, minerals, and trace metals including selenium.

The increments of tissue glutathione described in our experiments were moderate but sustained throughout the feeding period. It is noteworthy that the effects of a small increase in cellular glutathione may be greater than expected. For example, there are many reports of human and murine tumor cell lines selected in vitro for resistance to a variety of chemotherapeutic agents. In a number of these cell lines, cellular glutathione is increased consistently by 2-fold compared to the drug-sensitive parental cell line, despite the fact that the level of drug resistance is often much greater, e.g., as much as 30-fold [97, 98]. Given the fact that cellular glutathione is very tightly regulated, that a 2-fold increase may be maximal, and that the effect of small increments in glutathione may be amplified by a variety of glutathione-utilizing enzymes (e.g., glutathione peroxidase, glutathione-S-transferase), the reproducible change in glutathione concentration observed in animals fed Product X diet is likely to have biological importance. The chronic nature of this augmentation may contribute significantly to this effect.

In conclusion, our data lend support to the concept that the concentration of serum albumin and possibly the immunoglobulins, as well as the undenatured conformation of the molecules, are crucial factors in determining the biological activity of dietary whey protein concentrate. The relative content of the active and thermolabile proteins must, in fact, be close to the values found in the whey from raw milk as easily determined by gel electrophoresis. The increasing use of high temperature milk pasteurization in recent years appears to have caused a drop in the levels of serum albumin and immunoglobulin and in the biological activity of most currently produced whey protein concentrates.

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