

## THE INFLUENCE OF DIETARY WHEY PROTEIN ON TISSUE GLUTATHIONE AND THE DISEASES OF AGING

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**Abstract**—This study compared the effects of a whey-rich diet (20 g / 100 g diet), with that of Purina mouse chow or casein-rich diet (20 g / 100 g diet), on the liver and heart glutathione content and on the survival of old male C57BL / 6 NIA mice. The study was performed during a limited observation period of 6.3 months. In mice fed the whey protein-rich diet between 17 months and 20 months of age, the heart tissue and liver tissue glutathione content were enhanced significantly above the corresponding values of the casein diet-fed and Purina-fed mice. Mice fed the whey protein diet at the onset of senescence at 84 weeks, exhibited increased longevity as compared to mice fed Purina mouse chow over the 6.3 month observation period extending from the age of 21 months (corresponding to a human age of 55 years) to 26–27 months of age (corresponding to a human age of 80 years), during which time 55% mortality was observed. The corresponding mean survival time of mice fed the defined casein diet is almost identical to that of Purina-fed controls. Body weight curves were similar in all three dietary groups. Hence, a whey protein diet appears to enhance the liver and heart glutathione concentration in aging mice and to increase longevity over a 6.3 month observation period.

**Résumé**—Nous avons examiné les effets d'une diète riche en petit lait (20 g / 100 g de diète) sur le contenu hépatique et cardiaque en glutathion et sur la survie de souris mâles âgés C57BL / 6 par comparaison aux effets d'une diète commerciale Purina ou une diète riche en caséine (20 g / 100 g de diète). Notre étude a été réalisée durant une période d'observation limitée de 6.3 mois. Les souris nourries à la diète riche en petit lait entre le 17ème et le 20ème mois de vie ont présenté un niveau plus élevé de glutathion dans le foie et le coeur que les deux (2) autres groupes témoins. Les souris nourries au petit lait au début de la vieillesse, soit à 84 semaines, ont également présentées une plus grande longévité que les souris nourries témoins au cours d'une période d'observation s'étendant de l'âge de 21 mois (correspondant à l'âge humain de 55 ans) à 26–27 mois (correspondant à l'âge humain de 80 ans), période d'observation au cours de laquelle 55% de mortalité est observée. Le temps moyen de survie des souris nourries à la caséine s'est avéré identique à celui des souris nourries au Purina. Les courbes de poids corporel étaient similaires dans les 3 groupes. Donc, une diète riche en petit lait élève le contenu cardiaque et hépatique en glutathion chez la souris âgée et augmente sa longévité mesurée sur une période de 6.3 mois.

*Key words:* Whey protein, tissue glutathione, aging.

### INTRODUCTION

THE FREE RADICAL THEORY of aging proposed by Harman [1] 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 by-products 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 an ubiquitous cellular constituent and is 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. This sulfur-containing tripeptide ( $\gamma$ -glu-cys-gly) is not only central in cellular protection against oxygen radicals, but serves a variety of functions which help maintain normal conditions. For example, adequate intracellular levels of glutathione are necessary for lymphocyte proliferation in the development of the immune response [2]. Glutathione maintains normal tissue hydration in ocular tissue [3], and is the source of the cysteine utilized in the biosynthesis of mercapturic acid conjugates of N-acetylcysteine, end products of a process which serves to detoxify a variety of harmful compounds and xenobiotics [3]. Glutathione binds transitional metals and is an important factor in their elimination [4]. It appears that, whereas data on age-related changes in tissue vitamin E and other antioxidants are, at best, contradictory [5], the tissue glutathione levels are more consistently reported to decline with old age. Thus, the age-related decline in immune

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responsiveness of mice was found to be associated with a 19% drop in spleen lymphocyte glutathione content [6]. Other studies showed that glutathione content of liver, kidney, heart [7], and brain [8] was respectively 30%, 34%, 20% and 30% lower in very old mice (31 months) than in mature mice (17–23 months). A lower glutathione status has been demonstrated in erythrocytes of aging mice [9]. Similarly, in elderly populations the erythrocyte glutathione peroxidase activity was reported to be lower than in young adults [10]. Glutathione concentrations were found to decrease in aging human lenses [11] and aging erythrocytes [12].

Our previous studies have shown that in mice fed a 20 g whey protein / 100 g diet, the splenocyte immune response to sheep red blood cells is substantially greater than that of mice fed any of the commercially available purified animal plant or algae proteins, of similar nutritional efficiency, at 20 g protein / 100 g diet concentration [13]. The whey protein mixture contains very high levels of cysteine, which is considered to be a rate limiting substrate for the synthesis of glutathione, which is also necessary for lymphocyte proliferation [14,15]. Our studies [16] have further shown that enhancement of host humoral immune response is associated with greater and more sustained production of splenic glutathione during the antigen driven clonal expansion of the lymphocyte in whey protein-fed mice in comparison to mice fed the equivalent casein or even the cysteine-enriched casein diet. Hence the efficiency of dietary cysteine in inducing supernormal glutathione levels is greater when it is delivered in the whey protein than as free cysteine. We have also shown that C3H / HeJ mice fed a whey protein diet are more resistant to infection with *streptococcus pneumoniae* type 3, independently of the weight of the animal at the time of infection and the weight gained before infection [17]. The development of dimethylhydrazine induced colon cancer was found to be inhibited in A / J mice fed a whey protein diet in comparison to mice fed a nutritionally equivalent casein diet or Purina mouse chow [18].

The present study was designed to investigate the effect of long term feeding of a whey protein diet on the tissue glutathione contents and the mortality associated with diseases of aging.

## MATERIALS AND METHODS

### Mice

Male C57BL / 6NIA mice raised by Charles River Canada for the National Institute of Aging with median survival time of 28.9 months [7] were segregated into three equal groups of 18 mice each at 21 months of age, and assigned to eat either of three diets in a specific pathogen-free environment; their mortality from natural causes was recorded. The presented data refer to an observation period of 6.3 months during which 55% mortality was reached for all groups. In another set of experiments, male C57BL / 6NIA, segregated into three equal groups of ten mice, were assigned to eat either of

TABLE 1. VITAMIN AND MINERAL CONTENT OF FORMULA DIETS

The vitamin mixture plus the vitamins contained in the basal diet (Mead Johnson product 80056) provided in milligrams per 100 g diet: ascorbic acid, 53.3; niacin, 5.1; riboflavin, 0.38; thiamin, 0.34; folic acid, 0.063; vitamin B-6, 0.26; biotin, 0.031; pantothenic acid, 1.93; choline, 44; and per 100 g diet; retinyl palmitate, 1295 IU, ergocalciferol, 260 IU; vitamin E (*dl*-tocopheryl acetate), 11.6 IU; vitamin B-12, 0.001 mg; and vitamin K (phylloquinone), 0.06 mg. The mineral content of ions or cations (expressed in milligrams per 100 g diet) and the actual chemical compounds fed were:

Ca, 350 (CaHPO<sub>4</sub>·2H<sub>2</sub>O and CA<sub>3</sub> (C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>)<sub>2</sub>·4H<sub>2</sub>O); P, 260 (K<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O); Fe, 7.9 (FeSO<sub>4</sub>·2H<sub>2</sub>O); Mg, 63.2 (MgO); Cu, 0.31 (CuSO<sub>4</sub>·5H<sub>2</sub>O); Zn, 3.5 (ZnSO<sub>4</sub>·7H<sub>2</sub>O); Mn, 0.48 (MnSO<sub>4</sub>); Cl, 1108 (C<sub>5</sub>H<sub>14</sub>CINO); K, 997 (K<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O); Na 232 (NaCl).

three dietary regimens from 17 to 20 months of age, when they were sacrificed for liver and heart glutathione assay. In two other groups of ten mice each of the same strain and origin, fed Purina chow, the heart and liver glutathione were measured at 10 weeks and 17 months of age.

### Diet

The formula diets contained 20 g / 100 g diet of either whey protein concentrate or casein. Whey protein concentrate is made of proteins that remain soluble in "milk serum" or whey after precipitation of casein at pH 4.6 and 20°C, as in the manufacture of cheese. The whey protein concentrate used in our experiments was Lacprodan-80 (Danmark Protein, Worthington, Ohio) and the casein was given by Bristol-Meyers of Canada. The only variable in these two purified diets was the type of protein. The detailed composition of some common ingredients (vitamins and minerals) in the two defined formula diets is given in Table 1. Diets are prepared in the following way: 20 g of selected net protein, 56 g of product 80056 protein-free powder containing corn syrup, corn oil, tapioca starch, vitamins and minerals (Mead-Johnson Co. Inc., U.S.A.), 18 g cornstarch, 2 g wheat fiber, 0.05 g Nutramigen vit-iron premix (Bristol-Meyer, Ontario, Canada), 2.65 g KCl, 0.84 g NaCl. The amino acid composition of bovine whey protein concentrate and casein is given in a companion article [18]. Other animals were fed Purina mouse chow (estimated 23% protein from various sources). Diets, refrigerated between feeding, were continuously available in powder form in stainless-steel feeders designed to reduce spillage. Drinking water was provided *ad libitum*. The mice were housed five per cage.

### Glutathione assay

Ninety milligrams of mouse heart or liver were homogenized in 5-sulfosalicylic acid (5% w / v). Homogenates were centrifuged for 5 minutes in a microfuge at 10,000 × g. The assay was carried out using the supernatants on the same day, according to the method of Anderson [19]. Values are expressed as μmol / g / wet tissue.

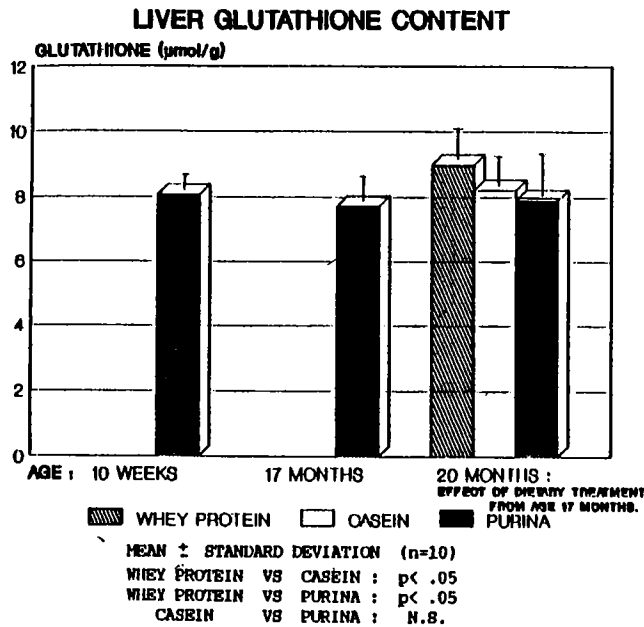


FIG. 1. Effect of age and dietary treatment on liver glutathione content.

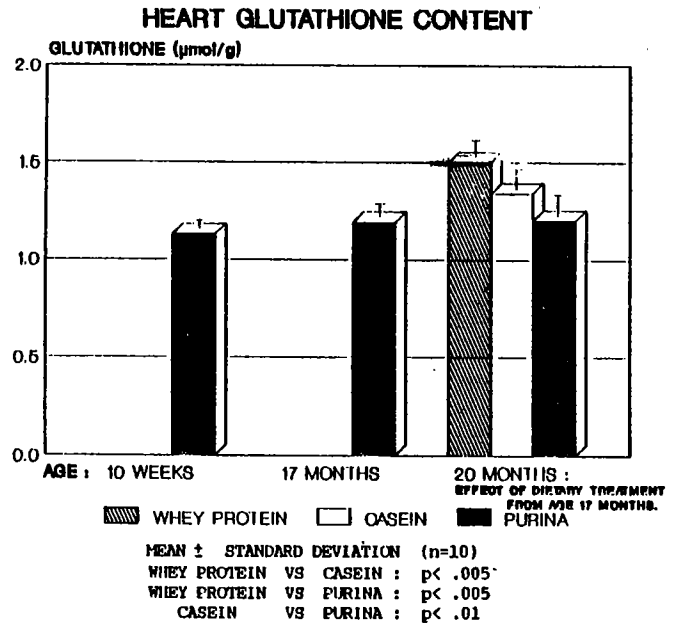


FIG. 2. Effect of age and dietary treatment on heart glutathione content.

#### Immunization for plaque assays

The method used for assaying IgM plaque-forming cells was essentially the one described by Cunningham and Szenberg [20] with certain minor modifications [21]. The mice were injected intravenously with  $5 \times 10^6$  sheep red blood cells and assayed for plaque-forming spleen cells on day 5, when the response was shown to peak [21].

#### Statistics

Statistical evaluation of differences between dietary groups was done by unpaired Student's t-test or by generalized Wilcoxon test.

## RESULTS

#### Tissue glutathione

After three months on either dietary treatment, glutathione content was found to be higher in the liver and heart of whey protein fed mice compared to their casein-diet or Purina fed counterparts (Figs. 1 and 2). The glutathione values in the hearts and livers of mice fed Purina laboratory chow were almost identical at 17 and 20 months of age. Thus, no age-related decline is observed during this period of time. Moreover, the glutathione values at 17 and 20 months of age of Purina fed mice are similar to those of 10 week old mice. The whey protein diet appears to enhance the glutathione content of heart and liver above "normal" values, after 3 months. The mean  $\pm$  SD body weight changes over the three month period, expressed as percentage of initial weight, of mice fed either the whey protein diet, casein diet, or Purina diet was  $98.90 \pm 17.7$ ,  $100.38 \pm 15.99$  and  $99.30 \pm 18.50$ ,

TABLE 2. TIME AT WHICH 55% OF MICE FED ONE OF THREE DIETARY REGIMENS (FROM 21 MONTHS OF AGE TO 26-27 MONTHS OF AGE) WERE DEAD.

Dietary treatment	Days of feeding <sup>a</sup>
Casein	$92.2 \pm 55.2^b$
Whey	$125.0 \pm 41.6^c$
Purina	$92.7 \pm 31.7^d$

<sup>a</sup>Mean of 10 mice per group  $\pm$  standard deviation. Survival time for  $d < c$  ( $p < 0.05$ ). If the two control diet groups with near identical survival time are pooled together:  $b, d < c$  ( $p < 0.05$ ).

respectively. Thus, no significant differences were noted in body weight between the various dietary groups. Food consumption was also similar, varying from  $3.0 \pm 0.3$  g / 24 hrs in the whey protein diet group to  $3.3 \pm 0.4$  g / 24 hrs in the Purina fed mice.

#### Survival studies

Mice which were fed a 20 g whey protein / 100 g diet, *ad libitum* at the onset of senescence, exhibited delayed mortality in comparison with mice fed Purina laboratory chow. The 6.3 month observation period extended from the age of 21 months to 27.3 months of age, during which 55% mortality was reached for all groups (Table 2). The corresponding human age range derived from the survival curves for males in the industrialized world would be 55 to near 80 years of age [22, 23]. The mean survival time during the limited observation period of mice fed the casein-containing diet is

almost identical to that of Purina fed controls (Figure 3, Table 2). No significant difference was noted among dietary groups in average body weight changes throughout the experiment (Table 3). We have not included weights taken during the last 2 months because significant pathological changes were noted in the animals during the later months that could have influenced body weight independently of the nutritional status. Average food consumption for the 6.3 month observation period in the whey protein-diet group was  $2.8 \pm 0.4$  g / 24 hrs and  $3.0 \pm 0.4$  g / 24 hrs in the casein-diet group. The greater amount of spillage of the Purina powder precluded a realistic appraisal of food consumption in this particular group. Throughout the study repeat bioassays of spleen plaque-formation were done to document the consistent physiologic effects of the diets on immune function as reported previously, as well as the stability of these effects. The mean  $\pm$  S.D. values obtained after three weeks of feeding with the whey protein diet were  $150 \pm 12.5$  plaque-forming cells per spleen  $\times 10^{-3}$ . This was consistently confirmed for the first 6–7 months of the study; however, in the following two months (June and July), the immunoenhancing effect previously observed in mice fed the whey diet was no longer seen. Corresponding values during this period were  $40 \pm 10$ , significantly lower ( $p < 0.001$ ) than the values obtained during the first 6 months of study. During this period, extending from the 8th to the 9th month of study, the time of death of the remaining whey protein diet-fed mice became similar to that of their casein diet or Purina-fed counterparts. In a previous study we had accidentally discovered that when the whey protein batch was obtained during the hot summer months the immunoenhancing property was lost, although the nutritional quality of the protein was preserved. Indeed, a retrospective check has revealed that the whey protein batch in the June-July formula diets used for the current experiment, was received by surface transport from Denmark through the U.S. in particularly hot and humid weather. It is also possible that a different fractionation technique was used in the industrial preparation of this whey protein concentrate used in the later part of the study. This protein is known to display the greatest tendency to denature under heat, thus exposing its free sulfhydryl groups. It is thus possible that the proportion of undenatured protein, originally 90%, had dropped significantly under these conditions. In fact solubility, which decreases with heat denaturation of whey protein,

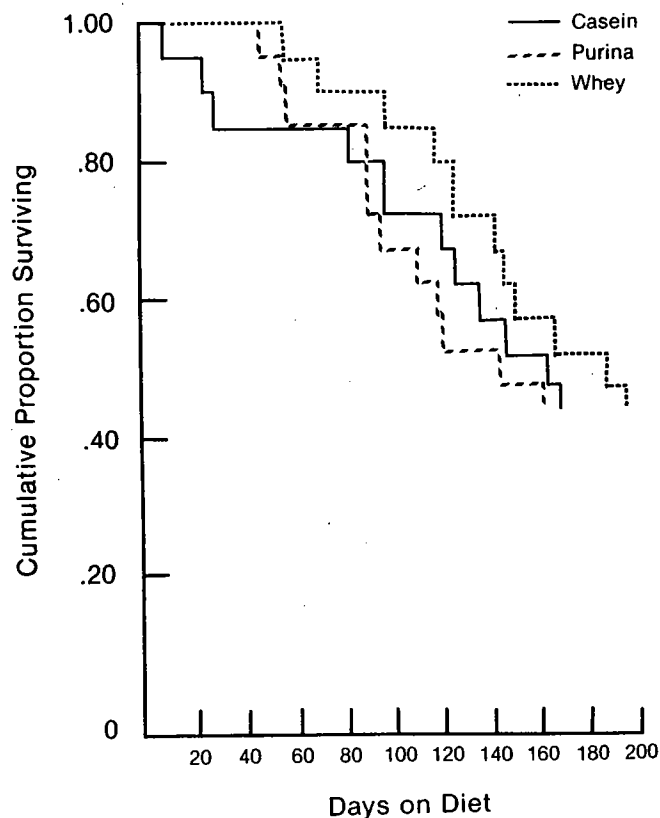


FIG. 3. Effect of three dietary regimens initiated at 21 months of age on the survival curve of mice. On the vertical axis is the cumulative proportion of surviving animals. On the horizontal axis the number of days on diet.

was found to be 80% in a 3% protein solution of the whey protein powder used in the later part of the study. This value is significantly lower than the 94.5% solubility of the previous samples at the same 6.4 pH. If we are correct in our hypothesis that the whey protein was denatured during months 8 and 9, then at least in terms of the immunoenhancing effect of the diet, this study could be regarded as a single direction cross-over from test to control diet. As we are now investigating the nature of this phenomenon it was felt appropriate not to include the 8th and 9th month treatment data of the aging study as they were obtained under different, as yet ill defined, experimental conditions. The necessity to terminate the study before the 100% mortality was reached

TABLE 3. AVERAGE BODY WEIGHT OF SURVIVING MICE ON DIFFERENT DIETARY REGIMENS, FROM AGE 21 MONTHS

Diets		Mouse age in months				
		21	22	23	24	25
Casein	Weight in g <sup>a</sup>	34.9 $\pm$ 2.6	33.7 $\pm$ 4.9	33.8 $\pm$ 5.4	32.4 $\pm$ 6.8	31.1 $\pm$ 6.8
	As % of initial weight	100	97	97	93	89
Whey protein	Weight in g	33.2 $\pm$ 3.0	34.0 $\pm$ 4.5	33.5 $\pm$ 4.1	33.9 $\pm$ 5.0	30.7 $\pm$ 3.3
	As % of initial weight	100	102	101	102	93
Purina	Weight in g	35.4 $\pm$ 3.7	36.5 $\pm$ 4.1	34.8 $\pm$ 3.7	35.1 $\pm$ 3.4	33.8 $\pm$ 1.5
	As % of initial weight	100	103	98	99	95

<sup>a</sup>Mean  $\pm$  standard deviation.

may thus have contributed in rendering not significant a statistical analysis of cumulative proportion of surviving mice by the generalized Wilcoxon test.

## DISCUSSION

Meaningful studies on the effect of dietary intervention on longevity should be carried out using long-lived, robust, wild-type animals with no genetically induced deficiencies that require dietary supplements. Two "control" diets should be utilized: the standard laboratory chow, used by the progenitors of the test animals and providing optimal nutrition, and an adequate formula diet differing from the test diet only in the variable under investigation. Ideally these two "control" diets should exhibit similar nutritional efficiency. Finally, food consumption and growth should be similar in all dietary groups, because covert calorie restriction is the most effective method of extending life span in laboratory animals [24, 25]. Two such studies investigated the effect on longevity of dietary whey protein in nutritionally adequate and similar diets. In lifetime feeding studies, survival was reported to be better in hamsters fed 10, 20, or 40 g whey protein / 100 g diet in comparison with those fed commercial laboratory diet containing an estimated 24% protein from various sources. Hamsters fed the 20% level of whey protein survived the longest [26]. Survival of hamsters during the first 20 weeks of study was better in animals fed the 20 g whey protein / 100 g diet than those fed a corresponding methionine and cysteine enriched casein diet [27]. Our study shows that the mean survival time, at the time at which 55% of mice were dead, is increased by about 30% in mice commenced on the whey protein diet at the onset of senescence, in comparison with "controls" fed the nutritionally equivalent Purina mouse chow. The change from a situation of immunoenhancement and delay in time of death seen in the whey protein fed group during the first 6 months of study, to a state of relative immunodepression and accelerated time of death during the subsequent final period, was concomitant with the use of a new batch of whey protein. Our final results show no significant difference between dietary groups in total life span. It is difficult to predict what might have been the final outcome had the whey protein group continued to eat their original type of undenatured whey protein. Assuming that the sudden loss of a positive biological property may have produced, by rebound, a temporary specific adverse effect, we can postulate that the longevity of whey protein fed mice might have increased if the diet had remained unaltered throughout the remainder of the experiment. Our data on tissue glutathione, compatible with the glutathione theory of aging, may explain the effect of whey protein on survival. In C57 mice treated from 17 to 20 months of age, the whey protein diet appears to have enhanced the liver and heart glutathione levels above values observed in control mice fed either the casein diet or Purina mouse chow; these latter two groups exhibit similar survival times during the observation period. As the major organ involved in the elimination and metabolism of xenobiotics,

the liver has the greatest content of reduced glutathione [28]. Conjugation with nucleophilic reduced glutathione protects DNA and other nucleophilic cell components from attack by carcinogen. Although glutathione is synthesized in virtually all cells from precursor amino acids, the liver is unique in three major aspects of glutathione regulation: it has the ability to convert methionine to cysteine and it efficiently exports glutathione mainly into plasma at a rate that accounts for nearly all of its hepatic biosynthesis [28]. As mentioned in the introduction, glutathione plays a critical role in detoxification reactions. In this regard, an important function of glutathione is the reduction of hydrogen peroxide ( $H_2O_2$ ), which is a by-product of oxygen requiring metabolism. Undoubtedly, the status and efficiency of this reaction, coupled with other endogenous mechanisms for scavenging oxygen radicals, are important in modulating the aging process. The previously reported enhancement of immune responses [13, 16, 17], resistance to infection [17], and to colon cancer [18] in whey protein diet-fed mice is consistent with the apparent greater resistance of mice on a whey protein diet to diseases of aging. This enhanced resistance could well be mediated by higher tissue glutathione levels. In addition to its reported effect on the immune system and cancer development, it is possible that enhanced myocardial glutathione resulting from a whey protein diet could increase the tolerance of the heart to injury by free radicals generated during post-ischemic reperfusion [29, 30]. It is thus apparent that, though normal long-lived mouse strains do not benefit from simple dietary supplementation of most antioxidants [31], a particular case can be made for glutathione. A reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation was reported in mice [6]. In the aging mosquito, correction of glutathione deficiency has been shown to increase longevity [32], and compensatory elevation of whole body glutathione was found to increase the life span of the housefly [33]. A glutathione enhancing whey protein diet delays the outcome of fatal, age-related, diseases in hamsters [26, 27] and possibly in mice. It has been recently reported that the *in vitro* life span of human diploid fibroblasts with increased or decreased glutathione levels was extended or shortened respectively [34].

It is difficult to isolate aging changes in clinical physiology from age-related diseases. The two areas are interrelated and to some extent interdependent, one upon the other. On the basis of available clinical and experimental data a theory on a broad biological role of whey protein and glutathione formation can be hypothesized. Whey-predominant formulas appear to be crucial for normal metabolism and well-being of premature human babies, even in a protected environment. Thus, premature infants fed a casein-predominant formula have a higher incidence of metabolic acidosis than infants fed human milk or whey-predominant formula [35, 36], who also exhibit greater nitrogen retention [37]. In the mature baby or in the weaned experimental animal the source of dietary protein is relevant primarily to the provision of essential amino acids in the strict nutritional sense. Under

standard external conditions, the physiological functions of normal life in the adult experimental animals and humans do not appear to be influenced by dietary protein type in nutritionally adequate diets. However, when challenged by abnormal stimuli such as injections of sheep red blood cells, pneumococci or carcinogen, the whey protein-fed young adult animals exhibit an abnormally high protective response. On the other hand, in the old experimental animal kept in a protected environment, the spontaneous diseases of aging are delayed by whey protein feeding.

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