THE NUTRITIONAL VALUE OF BRINE SHRIMP CYSTS: THE ENCYSTED EGGS OF ARTEMIA SALINA

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ABSTRACT

THE NUTRITIONAL VALUE OF BRINE SHRIMP CYSTS:

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By

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Brine shrimp cysts (BSC) have always been considered indigestible because of the outer chitinous shell. When male rats were fed a diet of 10% protein from BSC, the rats lost weight. Despite the poor growth when BSC was the only source of protein, growth was improved when casein was included in the ration. This was evident when rats were fed a 10% protein ration with half of the protein from BSC and the other half from casein. These animals gained more weight than the controls fed 5% protein from casein. A similar response occurred when a 20% protein diet (half from BSC and half from casein) was fed; these rats gained more weight than those fed 10% protein from casein.

Results similar to those with casein were observed with soy protein. All evidence indicated that the cysts were ruptured within the gut, making BSC protein available for digestion.

The role of gut micro-organisms on cyst rupture was examined with germfree rats. Germfree rats fed the same diets (but sterilized with 2.5 megarads gamma irradiation) as conventional animals grew according to the same pattern

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of weight gain as the conventional rats. Those fed a diet of 10% BSC protein lost weight, and those fed a diet of BSC protein mixed with casein or soy protein gained more weight than the casein or soy protein controls. This indicates that the microflora were not responsible for the cyst breakage in the mixed protein diets. Cyst breakage was confirmed by the disappearance of diguanosine tetraphosphate (G_p4G, found only in BSC) as the diet passed through the intestinal tract. Levels of G_p4G in all fecal samples supported the hypothesis that the chitin shell had been ruptured.

The amino acid profile of BSC has shown the eggs to be a good source of essential amino acids, with histidine the most limiting for the growing rat. Brine shrimp cysts contain high levels of thiamin, niacin, riboflavin and pantothenic acid. The mineral content is fairly high: Potassium (8133 ppm), phosphorus (8765 ppm), sodium (5433 ppm), calcium (4781 ppm), magnesium (2735 ppm), iron (681 ppm), zinc (75 ppm), manganese (32 ppm), copper (2 ppm) and selenium (.8 ppm). These data justify further investigation into the useability of BSC as a dietary nutrient source either for animals or man.

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Vala Jean Stults

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CHAPTER I

INTRODUCTION

Hunger is not a new problem to man. Ever since his life on this planet began, he has had to struggle for food. When his hands proved insufficient, he developed tools. When tools became inadequate, he developed machines and now, even before machines become unproductive, he is turning to chemical research in hopes of creating synthetic nourishment.

Today, it is ironic to find mankind at his most advanced stage in history confronted with the subsequent starvation of hundreds of millions of his fellow men. "This is impossible!" a well-fed American might scoff. But he is ignoring the great imbalance of the world's food supply--a grossly unequal distribution of nutriment. He who has these needed nutrients reaps health, while he who experiences their deprivation, suffers.

Unless men combine their efforts and their resources on an international scale and work to understand and solve the problems of the underdeveloped nations, these nations will continue to suffer from famine. It will be so great, that more than likely, within the next twenty-five years, the entire world will bear the consequences. There is great danger in the fact that people do not respond to famine as they do natural disasters. Relief given to the victims of earthquakes or floods is spontaneous, emotional and immediate. However, famines do not happen overnight and assistance given to the hungry must be deliberately planned--a prevention and not a cure. The time to act is before the famine takes on disaster proportions.

Too often, information about severe starvation becomes available to the general public only after the situation is so severe that pictures are likely to be very dramatic. Although organizations like the Ford Foundation and FAO are aware of those parts of the world where food is in short supply, it is a problem to convince the people in the developed world that relief is necessary. The apathy that comes from repeated exposure to pictures of starving individuals plus the proximity of "more urgent problems" results in a reluctance to provide assistance to the individuals facing starvation in underdeveloped countries.

According to Borgstrom (1), population increases suggest that the world would need a four-fold increase in food production by the year 2000. Protein is one of the major nutrients upon which a major effort must be placed, even though it is true that in some parts of the world calories are equally important. Existing protein sources must be expanded and new protein sources must be accepted and utilized beneficially. Tannenbaum (2) defines "new" protein sources as ones which exist but have not yet been used for human consumption or those sources which either have or are, at least, thought to have potential. He lists fish protein concentrat, single-cell protein and leaf protein concentrate as the new sources of protein with the greatest potential. It is my opinion that developing "new" protein sources should not be limited to those for human consumption. They should also include sources of protein to feed livestock, poultry and any other animals raised for the purpose of feeding people.

Brine shrimp cyst is just such a protein source, and it deserves a place in any list of new or potential protein sources. In order for a protein to have potential for human consumption, it must be 50% crude protein (dry wt.) (2). Artemia salina eggs easily meet this requirement, and thereby exceed the requirement of livestock, as a food source. Their value is also enhanced by a high vitamin and mineral content. Brine shrimp cysts are also dehydrated and therefore no special production techniques are required and they would store well in tropical areas. These crustaceas exist in salt ponds on every continent of the world and in many regions which are now experiencing severe famine such as the arid regions of Africa where errosion and depletion decrease the amount of arable land. Brine shrimp do not compete with man for fresh water. Ideal conditions for the culture of artemia are ponds containing H_2O unfit for human consumption (high sodium, chloride, sulphate and carbonate).

There is no single solution to the problem of feeding the 7 billion people anticipated to live on this planet by the year 2000 (1). It is imperative that every possibility be examined to the fullest extent and that present knowledge be applied to creating situations in which all contributions, however small, may be used to their fullest extent. The study of the nutritional value and the use of brine shrimp cyst as a protein source for domestic animals may appear to be an indirect approach but is nonetheless justifiable from the point of view of the immediacy, vastness and severity of the need for all possible protein sources. The ever-increasing gap between the "haves" and "have nots" must be filled. It is the hope of the author that this dissertation will serve, at least as a beginning.

> and he gave it for his opinion, that whoever could make two ears of corn or two blades of grass to grow upon a spot of ground where only one grew before, would deserve better of mankind and do more essential service to his country, than the whole race of politicians put together.
>
> -Jonathan Swift
> Gulliver's Travels

CHAPTER II

REVIEW OF LITERATURE

General Description

Brine shrimp cysts are the dry dormant stage or the encysted bastula of the microscopic crustacean *Artemia salina*. The eggs are .2mm in diameter (3) are cup-shaped when dry and become rounded upon hydration, just before hatching. The eggs which are released in the spring and summer hatch, and those which are released in autumn become encysted with a hard chitinous shell, thus equipping them for the dormant stage (4).

The chitinous shell has been the subject of much study, both morphological and chemical. Figure 1, from the work of Morris and Afzelius (5), delineates the structural components of the shell. The shell is divided into two major areas, the chorion and the embryonic cuticle. The chorion, the outer-most division, contains pigment, pores, and is of maternal origin (5). It is about 1 micron thick and contains approximately 50% protein (5).

The pores in the cortical layer of the chorion contain a waxy substance which is also in a thin continuous layer surrounding the cyst (5). The open areas in the

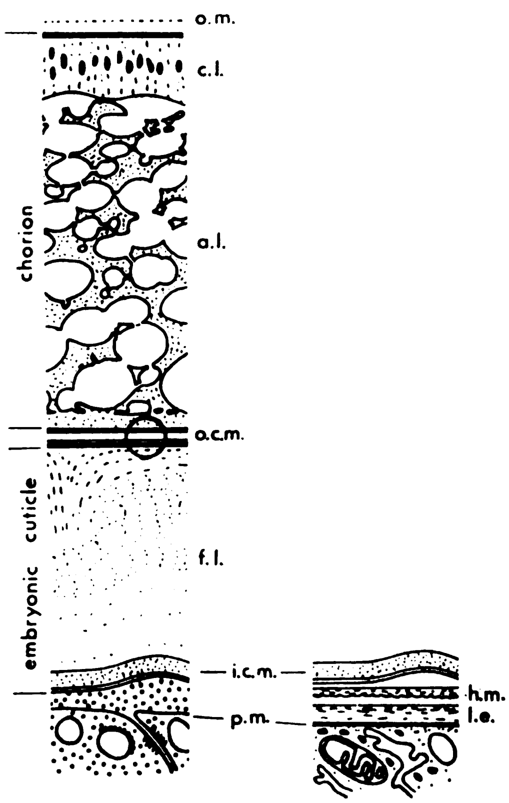

Figure 1. Shell and outer membranes of encysted embryos (5).

o.m. - outer membrane
c.l. - cortical layer
a.l. - alveolar layer
o.c.m. - outer cuticle membrane
f.l. - fibrous layer
i.c.m. - inner cuticular membrane
p.m. - plasma membrane
h.m. - hatching membrane
l.e. - larval exoskeleton

alveolar layer seem to be empty and perhaps relate to the flotation mechanism of the cyst (5). When newly layed, the cysts drift to the surface of the water, and are blown into piles along the shore by prevailing winds.

Between the chorion and the embryonic cuticle there are two very thin triple-layered membranes, which prevent the entrance of substances into the embryo, (5). The membrane complex is selectively permeable to ions and is believed to be important in regulating salt balance during hydration changes (5). The fibrous layer of the embryonic cuticle contains chitin and protein (5). It is this layer which cracks open and is shed upon hatching of the egg. The other compound layer of the embryonic cuticle, the inner cuticular membrane, is the last layer of the shell and separates from the shell during hatching moving outward with the larva (5) as a protective coating during emergence. Further discussion of the hatching process will not be considered here as the subject of this paper is the intact cyst.

Chemically the cyst shell is composed of protein, pigment and chitin. In all types of chitin the protein portion is attached to the chitin by aspartic acid and histidine (6). Rudall (6) presents the estimate of the number of links between amino acids (histidine or aspartic acid) and chitin as being one amino acid for every 200 - 400 glucosamine residues.

According to Rudall (6), the linkage between protein and chitin is by covalent bonds, thereby making the chitin protein complex a glycoprotein. The chitin itself is a polymer of glucosamine and poly-N-acetyl-glucosamine residues (Figure 2). Rudall (6) suggests that it is 82.5% poly-N-acetyl-glucosamine, 12.5% polyglucosamine and 5.0% water with one glucosamine placed in the chain every 6 or 7th poly-N-acetyl-glucosamine. The elemental composition of chitin, according to Tosher and Hackman (7), is C - 47.3%; H - 6.4%; N - 6.9%; and, these three from 100 would give O - 39.4%. Overall, the shell of the brine shrimp egg makes a very effective protective coating against the elements of almost any environment, enabling the egg to remain in a dormant but viable phase for years.

The extent to which the brine shrimp cyst can resist various environmental conditions has been tested for temperature (8, 9); organic solvents (10); various degrees of irradiation (9, 11); anerobic conditions (8, 12) and digestive enzymes (5, 9, 11, 13).

Both Bowen (9) and Hempel-Zawitkowska (8) found that freezing brine shrimp eggs did not decrease viability. Hempel-Zawitkowska (8) examined the effects of temperature in relation to salinity of freezing medium and dessication, and his results indicate that both dry and hydrated eggs could tolerate temperatures ranging from -10°C to -196°C. The dehydrated eggs maintaining the greatest viability.

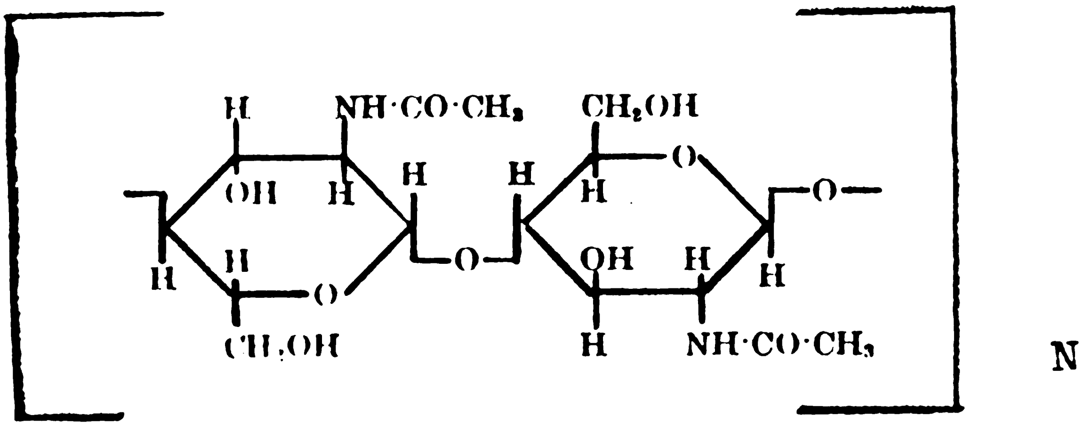

Figure 2. Poly-N-acetyl-glucosamine.

This was true whether or not they were frozen after dessication or frozen in an osmotically dehydrating saline solution. They suggest that this is due to the dehydration of the eggs plus the amounts of trehalose and glycerol contained in the eggs (8).

Iwanami (10) examined the effects of soaking brine shrimp cyst in seventeen different organic solvents (e.g., acetone, benzene, chloroform, ethyl ether, etc.) for 10 days. His study established the fact that viability of the soaked cysts was equal to that of non-soaked eggs in all cases (10).

Snipes and Gordy (11) found brine shrimp cyst to be more resistant to destruction by gamma irradiation (5×10^6r) when in water than in dry air. Evidently the water protects against damage or increases healing of damage caused by irradiation (11). Bowen (9) in searching for a method of storing cysts which would decrease the naturally occurring decline in viability tested x-irradiation. Her results indicated that although dry cysts remained viable after 50kr x-irradiation the nauplii would not mature and that hydration in this case did not protect against damage in the development of the nauplii. She suggested that no damage appeared until after emergence because of the fact that the _Artemia_ blastulae differentiate with cleavage (9). She also found that freezing increased the period of time that cysts would remain viable (-19°C to -24°C for 2 years) (9).

Warner, Stocco and Beers (12), Dutrieu (14), and Hempel-Zawitkowska (8) have each demonstrated that the brine shrimp cyst was quite resistant to anoxia. The metabolic activities are so low in the gastrula that very little oxygen is required for survival (14). Dutrieu (14) held eggs anaerobically for periods up to 5 months with no adverse effects upon hatching or development.

It has long been assumed that brine shrimp cyst were not labile to digestive enzymes. Both Proctor (15, 16) and Loeffler (17) have shown passive dispersal of intact viable cysts in the droppings of birds scattered over large geographic areas. Horne (13) tested the impermeability of the cysts with a variety of digestive enzymes *in vitro*. She incubated (32°C) cysts in mixtures of pepsin, trypsin, lipase and chitinase for 2-hour periods. No difference in hatchability occurred regardless of the enzyme or mixtures of enzymes used (13). Apparently, nature has very successfully provided the brine shrimp (by virtue of its indestructable shell) with a guarantee of future generations.

The fact that the metabolism of the encysted embryo is different from embryonic stages of other developing animals should be obvious by now, especially in view of the fact that they have the ability to exist under anoxic conditions. It would, therefore, not be surprising to find their metabolism involving unusual or different metabolites or processes. One of these compounds distinct to the metabolism of brine shrimp cysts is p^1, p^4 diguanosine 5'-tetraphosphate ($G_{p4}G$). This compound has been extensively studied by Warner (12). He has been involved in research on the presence and function of $G_{p4}G$ in brine shrimp cysts. They contain very high quantities (1.2% to 3.0%) depending upon the stage of development and source.

Warner (12) has used mature ovarian cysts from Ogden, Utah. The nucleotide studied is used as an energy supply and a source of purines by Artemia. It is one of the major contributing factors to the ability of the cysts to remain dormant for long periods of time and to survive harsh climatic conditions (12).

In the embryonic development of most organisms there are a number of isozymes of lactate dehydrogenase (LDH). However, in Artemia, Ewing and Clegg (18) have shown that there is only one macromolecular form of the enzyme. It appears that a single form of LDH is more common to invertebrates than vertebrates (18).

Emerson (19) examined free amino acid levels of brine shrimp cyst at different stages of hatching. He found that free amino acid content was a function of the salinity of the hatching media and of the period of embryonic development (19). From his data he concluded that upon hydration there were marked increases in the free amino acids: alanine, glycine, threonine, histidine and lysine; and decreases in: aspartic acid, serine and arginine (19). Distilled water as the medium resulted in a decrease of all free amino acids (19). In 0.5M NaCl, the period of free amino acid build-up starts immediately and peaks at 16 hours; but in 1M NaCl, it does not begin for 16 hours and peaks at 32 hours at a higher level than .5M NaCl produced (19). The reason for the lag in free amino acid levels is not known, but Emmerson suggested that it is related to decreased ammonia excretion which was also noted in the 1M NaCl medium (19).

Zasloff (20) has described the mechanisms of protein synthesis for Artemia and has shown that the 40 S ribosomal subunits of the brine shrimp cyst can bind formylmethionyl-transfer RNA non-enzymatically, or, in other words, in the absence of an initiation factor. Binding is largely dependent on the adenine-uridine-guanine (AUG) triplet in the 40 S subunit, and in the 80 S couple. It occurs at the peptidyl or donor site of the 80 S couple or the initiation site of the 40 S subunit (20).

Zasloff (21) previously showed an initiation factor for binding f-met-TRNA does exist (21); therefore, the reaction can occur with or without an initiation factor. Zasloff (21) suggested that this would be advantageous to the dormant cyst as it would decrease the amount of energy required for this part of protein synthesis.

Occurrence

The cysts and nauplii of *Artemia salina* are found in almost every body of salt water in the world.

Proctor (14, 15, 22) studied the dispersal of these organisms in North America; Loeffler (16) in Europe; Al-Uthman (23) in Iran, Krisna and Baid (24) in India, and Cole and Brown (25) have compiled a lengthy list of lakes throughout the world which contain brine shrimp and clearly could yield brine shrimp cyst.

Due the fact that *Artemia* can adapt to different conditions of temperature and salinity, discrepancies exist as to the chemical features of their natural habitat. Cole and Brown (25) compared the Cl, SO_4 and CO_3 contents, and the Na:K ratios of various lakes containing *Artemia salina*. Total salinity ranged from 3% to 30%. In their study, the majority of the lakes containing brine shrimp were high in chlorides with the rest being found in sulfate lakes. There are very few reports of brine shrimp cyst in carbonate lakes (25). The Na:K ratio was highest in the Cl^- lakes with most of the habitats similar to sea water (25).

Because of the varied habitat of *Artemia*, it is very important to specify their source. Salinity is very important to a number of metabolic processes and would directly or indirectly affect experimental results.

Protein Quality

The value of brine shrimp cysts as a protein source has not been examined previously. Wickins (26) has tested the adequacy of brine shrimp nauplii as food for prawns. He found that the nauplii from eggs obtained from the San Francisco area supported good growth (26).

The product bearing the greatest similarity to the way in which brine shrimp eggs were used in this study is crustacean meal. Borgstrom (27) reviews the food value of crustacean meal and indicates a protein content of most curstaceans (whole) varying from 9% - 15%. He presents efficiency ratios, (gm. wt. gain/gm. protein eaten), for shrimp, clams and oysters of 2.2, 2.1, and 1.3 (27). For dry drustacean meals the protein content is higher (35%) (27); and when mixed with soy protein meals, the crustacean meals (shrimp or crab) showed good utilization by hogs (27).

Fish meals have a protein content, (50 - 75%) equal to that of brine shrimp eggs (58). Fish meals are more common than crustacean meals and will be used for further comparison of nutrient content to that of brine shrimp eggs in this dissertation.

The measures used in this study to evaluate protein quality are the well established methods of PER and NPU (26). PER (protein efficiency ratio) according to the Food and Nutrition Board of the National Research Council is a biological method developed by Osborne and Mendel (29) which assumes that the rate of growth of weanling rats under standardized conditions (30) is a reliable measure of the quality of the protein in their diet. PER is calculated by dividing the 28-day weight gain by the same 28-day protein intake. Length of assay period, protein level and sex of animal will all affect PER results and must be standardized (29). Weanling male rats fed ad libitum for four weeks give the most reproducible PER values (29). PER has been criticized since the composition of the weight gained by the test animals may differ when different forms are assayed. The results also vary with protein level fed, and no allowance is made in the calculation for the maintenance requirement of the test animal. For this reason NPU (net protein utilization) was also used as it evaluates carcass N to insure that the weight gain was not due to a disproportionate retention of water or fat (29). This method is based on weight gain but has the advantage of including nitrogen retained by the test animals during the assay (29).

The supplementary effect of one protein on another when combined in a diet is also observed here. Many studies have been carried out with the supplementation of certain amino acids to various protein sources (wheat, rice, corn, etc.) to improve the biological value of the protein (31).

Tannenbaum (2) discusses the successful combination of two different protein sources (in his case, animal plus vegetable) to yield better utilization of each. In this study the mixture of casein plus brine shrimp cysts (animal protein plus animal protein) or soy protein plus brine shrimp cysts (vegetable protein plus animal protein) produce better growth than brine shrimp cysts fed alone. Bender (31) in a discussion of the advantages and disadvantages of amino acid supplementation pointed out that if a protein supplement was not pure protein then it should not only increase both the quality and quantity of protein in the diet but be a source of vitamins and minerals as well. He also emphasized that any protein supplement should provide both the first and the second limiting amino acids (31). The combination of soy or casein with brine shrimp cysts offers the above advantages, and therefore, should be given serious consideration in any search for new world protein resources.

Germfree Research

Germfree research has developed to such an extent that it would neither be practical nor realistic to give a complete review here. For the most part, germfree animals have been used as models by which the mechanism of various biological reactions have been studied (e.g., immune response, lipid metabolism, tumor development, etc.), (32, 33, 34, 35, 36). Those characteristics of germfree life which are different from conventional life have been identified and classified by morphology and function (e.g., gastrointestinal tract, lymphatic tissue, organ size, etc.) (32, 33, 34, 35, 36). Only those studies concerning the growth rate of the germfree rat have been reviewed here. The expense and time involved in raising germfree rats have limited their use, and thus, the standard or "normal" data for them has been restricted. By virtue of their differences from conventional rats, it has been standard procedure, in most studies, to have a germfree group of rats and a conventionalized group (contaminated age mates or litter mates noted here as x-germfree) given the same treatment and possibly even a third group of conventional age-mates given the same treatment as the germfree and x-germfree. Because of this, each research group has, by virtue of all of the control animals already involved, had neither the time nor inclination to run long extensive studies by which normative data could be derived. Instead, they used their simultaneously treated x-germfree or non-germfree conventionals as the norm to which the germfree data were compared. Unfortunately, the variables used are so numerous between different investigators that it is impossible to compare one distinctive feature or trait from one experiment to the others which may have included it.

Consider, as an example, weight gain, a trait important to this dissertation. It cannot be compared throughout the literature because no standards have been elucidated either for diet or for expected rate of gain in germfree rats as there has been for conventional rats (37).

The following is a list of those factors which are dissimilar and thereby have prevented meaningful comparisons between past reports of weight gain in the literature:

1. different protein levels used.
2. different protein sources used.
3. other different dietary constituents used in different proportions which affect growth.
4. consistency of diet (i.e., liquid vs. solids).
5. method of sterilization, stream vs. irradiation.
6. length of time used for experiment.
7. different ages of animals at start of experiment.
8. different measures taken (i.e., weight gain and food intake).
9. breed of rat.

Both soy protein (38) and casein (29) have been shown to produce good growth in germfree rats, despite the suggestion of Thompson and Trexler (40) that germfree rats absorb protein less well than conventional rats because of the lack of proteolytic enzymes provided by the gastrointestinal bacteria under normal conditions.

Oace (38) found a purified diet containing soy protein produced 292 g gain in conventional rats and 260 g gain in germfree rats fed the same diet for 8 weeks. However, in the same experiment, a purified casein diet produced 8 week weight gains in germfree rats which do not appear to be different from conventional controls (germfree: 200 g vs. conventional: 207 g). Unfortunately, no statistical comparisons were reported between the germfree rats and their conventional controls. Wostman and Kellog (39) reported that a 24% casein diet produced as good growth in germfree Wistar rats as in conventional Wistar rats.

Combe (41) observed that food intake and weight gain were the same for germfree and conventional rats; but fecal N excretion was greater in the germfree rats than in the conventional controls. Also, urinary N excretion was greater in the conventional controls than in the germfree rats. This renders further support for the supposition that apparent N digestion is less under germfree conditions. The difference cannot be too great, however, otherwise germfree rats would not grow as well as conventional rats.

Steroids

Teshima and Kanazawa (42) have examined the sterol content of *Artemia*. They fed four different sterols to adult brine shrimp and in each case the sterols were converted to cholesterol. They were unable to detect steroidogenesis from acetate and suggested that conversion is the primary source and that cholesterol is the primary steroid in brine shrimp. Cysts were not examined but it is reasonable to presume that they too contain cholesterol as the major form of steroid.

Vitamins

It has been well established that fish meals are high in B Vitamins (27, 28). Therefore, it is not unreasonable to expect that brine shrimp eggs would also provide ample amounts of these.

The only vitamins in brine shrimp cyst which have undergone any study are Vitamins A and C. Both the carotenoid pigment content and the biological value of those pigments have been examined. Krinsky (43) established that the brine shrimp nauplii and eggs contained similar pigments (Canthaxanthin and echinenone) and that these were derived from beta carotene. The scheme he presented was (Figure 3).

β-carotene → echinenone → canthaxanthin

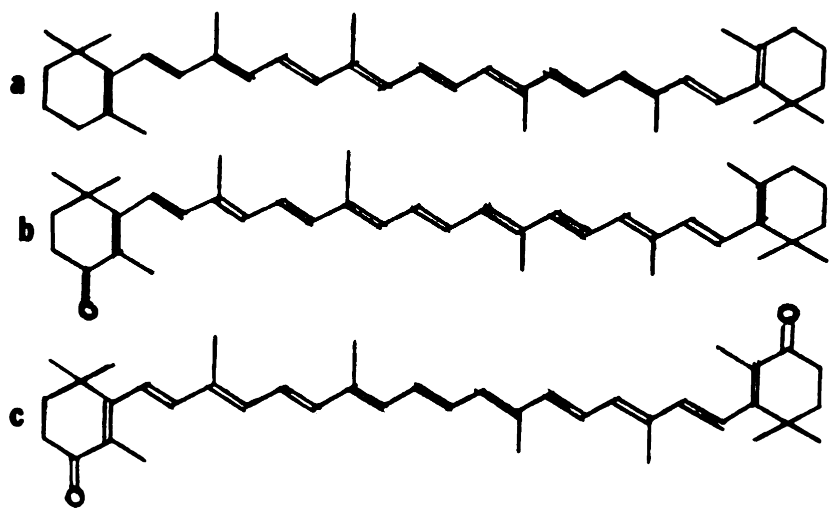

Figure 3. a) β-carotene, b) Echinenone and c) Canthaxanthin Structural Formula

Canthaxanthin, the major carotinoid in brine shrimp has no Vitamin A activity (44). Fisher, Kon and Thompson (45) have examined the Vitamin A content of a number of marine species but found none in the lower crustacea such as Copepoda and Cladocera. They did, however, find carotinoid with no Vitamin A activity.

The ascorbic acid content of brine shrimp cysts was determined by Mead and Finemore (46). Their work showed that it was all in the form of ascorbic acid sulfate and suggested that in the dry cyst, it was a storage form for both ascorbic acid and sulfur.

Minerals

Wickins' report on the "Food Value of Brine Shrimp" (26) contains reference to mineral content of brine shrimp eggs. In his study, San Francisco eggs were compared to Utah eggs in content of cadmium, lead, chromium, zinc and copper. The Utah eggs contained more zinc and copper (26); the other minerals seemed to be the same, regardless of source.

Pesticides

The last information to be reviewed here is that of the pesticide levels in brine shrimp cysts. The pesticides were not measured in this study, but the author feels it deserves mention in any case where animals are to be fed a diet, parts of which may have been environmentally contaminated. Wickins (26) reports DDT levels in brine shrimp eggs from San Francisco of 0.22 μg/g in 1967 and 0.067 μg/g in 1970 on a dry weight basis. He also reports from another set of egg samples: DDE - 0.002 μg/g, TDE - 0.050 μg/g, DDT - 0.024 μg/g, and PCB - 0.12 μg/g for San Francisco eggs (26). These values according to his comments could cary " 25 and 60% according to the amount of processing for the sample."

According to <u>Pesticide Reference Standards and Supplemental Data</u> (47) the following LD_{50} values exist for rats in mg per kg body weight:

DDE - 750-880 mg/kg

TDE (DDD) - 3400 mg/kg

DDT - 113 mg/kg

PCB - 400-10,000 mg/kg

Taking the highest DDT content mentioned above (.22 μg/g) and calculating the amount of DDT a rat would consume in this study in the 28-day period, one finds that 18.48 μg of DDT "would" have been the amount consumed by a rat eating 15g of food per day containing 200g brine shrimp cyst per kilogram diet. This would be equivalent to 0.16% of the LD_{50} (113 mg/kg body weight) of a 100 gram rat. Since the value for DDT is several years old and it has been established that levels have decreased since, (26), one may, with a reasonable amount of security, assume that brine shrimp cysts are safe for consumption from the point of view of pesticide contamination.

CHAPTER III

EXPERIMENTAL PROCEDURES

Preliminary Chemical Analyses

Basic Composition

Samples of San Francisco Bay brine shrimp cysts were vacuum dried to a constant weight (110°C) and then ashed in a muffle furnace for 24 hours by gradually increasing the temperature to 450°C. The dried weight was used to calculate percent moisture, and the ashed weight was used to calculate total mineral content. Total calories per gram of cyst were determined by adiabatic bomb calorimetry.

The total extractable lipid in another sample of the same batch of dried brine shrimp cyst was determined by 8 hour extraction with petroleum ether (Malinkroidt ASC) on a Goldfisch apparatus, according to the AOAC procedure (30).

Protein was estimated from total Kjeldahl nitrogen x 6.25 (48).

The percent of the cyst consisting of chitin was measured by an adaptation of a method previously used in this department (6). Vacuum dried samples were combined with 2% KOH for 24 hours in a steamer at 100°C. A washing procedure from Giles et al. (49) was used for further purification of the residue (2 times each with hot water, cold 5% HCl, cold water, acetone, and ether). The washed residue was allowed to sit until the ether evaporated and was then vacuum dried to a constant weight. At this point the nitrogen content was determined by the Kjeldahl method to compare with pure chitin (6.89%N) (49).

Protein Utilization Studies

Conventional Rats

Male weanling Sprague-Dawley rats were used in all animal feeding experiments. Groups of 5 rats each were fed diets containing varying levels and sources of protein as indicated in Table 1. All rats were housed individually in a temperature and light-controlled room.

During a 28-day period, food intake, weight gain, and fecal output were recorded. Food and water were available *ad libitum*. Five reference animals (with the same starting age and weight as test rats) were sacrificed at the onset of the study to represent 0-day nitrogen content of animals fed the test diets.

At the end of 28 days, the animals were sacrificed, the intestinal tract contents removed, and carcass nitrogen was determined by the method of Mickelsen and Anderson (50). NPU was calculated from nitrogen intake and retained carcass nitrogen.

TABLE 1. Composition of rat rations

I.	Non-Protein Basal Mixture	% by Weight
	Corn oil	5%
	Vitamin mix (1)	2%
	Mineral mix (3)	5%
	Sucrose (to equal)	100%
II.	Protein Sources	% by Weight*
	Casein (2)	5%
		10%
		15%
		20%
	Casein Plus Brine Shrimp	5% + 5%
	Cyst Mixture	10% + 10%
	Soy Protein (5)	5%
		10%
		20%
	Soy Protein Plus Brine	5% + 5%
	Shrimp Cyst Mixture	10% + 10%
	Brine Shrimp Cyst (4) Alone	10%

* As protein

(1) Purchased from Nutritional Biochemicals, Cleveland, Ohio. See appendix for composition.

(2) Purchased from General Biochemicals, Chagrin Falls, Ohio.

(3) Wesson Modified Osborne Mendel Salt Mix, General Biochemicals, Chagrin Falls, Ohio. See appendix for composition.

(4) Purchased from San Francisco Bay Brand, Inc., Division of Metaframe Corp., Menlo Park, California.

(5) Assay Protein Cl, Skidmore Enterprises, 3217 Beull Avenue, Cincinnati, Ohio 54211.

$$\text{NPU} = 100 \times \text{Carcass N gain} \ \frac{(\text{experimental} - 0 \text{ controls})}{\text{N intake}}$$

Ground Brine Shrimp Cysts

Ground brine shrimp cysts were combined with casein for a total protein level of 20% with 10% of the protein from brine shrimp cysts and 10% from casein. This was done to determine whether or not an increase in utilization would result from grinding the cysts, above that, when the whole brine shrimp cysts (10%) plus casein (10%) diet was fed. In preliminary studies, the 20% protein level, produced maximum growth.

Cyst Breakage

The best estimate of the degree of availability of the brine shrimp protein to the rat was the percent of the hard chitinous shells which were ruptured during passage through the intestinal tract. If the cysts were ruptured in the intestinal tract, a nucleotide peculiar to brine shrimp cysts should be liberated and readily absorbed. For those cysts that were not broken in the intestinal tract, the nucleotide remained in the intact cyst and should appear in the feces. By determining the total amount of this nucleotide in the feed and feces, an indication of cyst breakage was secured.

The quantities of p^1 p^4 diguanosino 5' tetraphosphate ($G_{p4}G$) remaining in the feces were used as a measure of cysts which were not ruptured during passage through the intestinal tract. The difference between the percent intact cysts in the feces and 100% represented broken shells and protein made available to the rat.

Germfree Rats

Germfree studies were planned with the assistance and guidance of Dr. C. K. Whitehair, Department of Pathology. In order to get an indication as to the importance of the intestinal microorganisms on cyst breakage, germfree rats were fed diets similar to those fed conventional rats (Table 2). The only differences were the addition of nutrients expected to be damaged by 2.5 megarads irradiation from a cobalt^{60} source, (thiamin, Vitamin K and α-tocopherol) (33). The diets were vacuum sealed in airtight bags before sterilization, and the bags were not opened until the rats were fed.

The germfree rats (two males and two females per diet) were housed in individual cages contained within a polyvinyl isolator. All equipment was steam sterilized and then transferred into the isolators with 2% peracetic acid by sterile transfer techniques (33).

Weight gain and food intake were recorded and feces collected for the entire 28-day period. Littermates of the germfree rats (two males and 2 females per diet) were conventionalized (i.e., removed from sterile conditions) and used as x-germfree controls. They were caged individually on a standard rack in the same room as their

TABLE 2. Composition of germfree rat rations

I.	Non-Protein Basal Mixture	% by Weight
	Corn oil	5%
	Vitamin fortification mix (1)	2%
	DL-a-Tocopheryl Acetate (2)	.4%
	Thiamin-HCl (2)	.02%
	Menadione (2)	.05%
	Mineral mix (3)	5%
	Sucrose (to equal)	100%
II.	Protein Source	
	Casein (2)	10%
		20%
	Brine Cyst Plus Casein (2)	10% + 10%
	Soy Protein (2)	10%
		20%
	Brine Cyst Plus Soy Protein	10% + 10%
	All diets received 2.5 megarads irridiation before use.	

(1) Purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. See appendix for composition.

(2) Purchased from General Biochemicals Corp., Chagrin Falls, Ohio.

(3) Wesson Modified Osborne Mendel Salt Mix, General Biochemicals, Chagrin Falls, Ohio. See appendix for composition.

(4) Purchased from San Francisco Bay Brand, Inc., Division of Metaframe Corp., Menlo Park, California.

germfree counterparts with identical diets and comparable measurements.

The group of four weanling germfree and x-germfree rats were fed each diet specified in Table 2, and four germfree rats were sacrificed at the onset to provide data for NPU.

Statistical Analysis

This study incorporated several treatments in the experimental design. Hence, the necessity of using multiple comparison statistical analyses was inherent. Making all possible comparisons in this case was neither necessary nor practical. For that reason, the most critical comparisons were selected and calculated by the Bonferroni t-Statistic (52) also known as Dunn's multiple comparisons procedure. In this statistic $t_B = \frac{\bar{Y}_1 - \bar{Y}_2}{2MS_e/x}$ where $\bar{y}_1$ and $\bar{y}_2$ are means to be compared, MS_e is the Error Mean Square, and x is the number of rats per group. Bonferroni t-statistic is based on Student's t-distribution and is suggested for planned comparisons. Making fewer comparisons allows one to split up the level of significance between any of those comparisons which he wishes to make rather than only those which are orthogonal. This test also does not require a prior significant overall F-ratio and it has the advantage of being more sensitive where specific non-independent comparisons are being made.

Specific Nutrient Analysis

Amino Acids

Acid hydrolysis of brine shrimp cysts was performed in our laboratory and the clear hydrolysate was then analyzed by the method of Bergen (53) with the Technicon Auto Analyzer. The hydrolysate was prepared as follows:

11 mg vacuum dried cyst, 1 ml 1mM Norleucine, 1 ml 12N HCL, and 75 ml 6N HCL were placed in a 125 ml erlenmyer flask. The flask was flushed with N_2, capped tightly and hydrolyzed at 14 lb. pressure for 16 hrs. The solution was then filtered through Whatman #1 filter paper, flash evaporated and resuspended in 2 ml pH 1.9 buffer (.3N Lithium, 0.05M Citrate). This procedure gave an estimate of : methionine, cystine, phenylalanine, tryosine, histidine, threonine, valine, leucine, isoleucine, arginine, glutamine, asparagine, serine, proline, glycine and alanine.

Vitamins

All vitamin determinations were performed on brine shrimp cysts obtained from San Francisco Bay Brand, Inc, which were ground to a fine powder in a ball mill before analysis.

Thiamin

The thiamin content of brine shrimp cysts were determined by the combination of two adaptations of the thiochrome procedure. The extraction procedure from Freed (54) was used to obtain a clear filtrate. Aliquots of the filtrate were then examined with the assistance of Eleanor Schlenker by the procedure of Leveille (55).

Niacin

The niacin content of brine shrimp cysts was determined microbiologically according to the procedure in Methods of Vitamin Assay, by Freed (54). *Lactobacillus plantarum*, and Difco stock, basal, and niacin assay media were used. The method measures nicotinic acid, nicotinamide and nicotinuric acid.

Riboflavin

The riboflavin content of brine shrimp cysts was determined chemically by the Autoanalyzer Industrial Method No. 140-71P (56). This is a fluorometric procedure designed for the Technicon Autoanalyzer.

Pantothenic Acid

The pantothenic acid content of brine shrimp cysts was determined microbiologically according to Freed (54). *Lactobacillus plantarum* and Difco pantothenate assay media were used. Clarase (54) was used as the hydrolytic enzyme in place of mylase P.

Retinol

The retinol content of brine shrimp cysts was determined by the method of Thompson (57). The technique described for butter and cheese was used, and the fluorescence was measured in the petroleum ether eluate of the unsaponifiable fraction on a Turner fluorimeter (330 nM, excitation and 480 nM emission).

Carotenoid pigments were not analyzed as they have been previously identified and characterized by Fisher, Kon and Thompson (45), Krinsky (43) and Petracek and Zechmeister (58).

Minerals

All mineral determinations were performed on brine shrimp cysts from San Francisco Bay Brand, Inc. which were ground in a ball mill before analysis. The following minerals were determined on a Jarrel Ash atomic absorption spectrophotometer by the method of Ullrey (59): calcium, magnesium, iron, zinc, manganese, copper. The method consisted of wet digestion of dry ground cysts with nitric and perchloric acids followed by atomic absorption spectrophotometry (30). Atomic emission spectrophotometry was used for sodium and potassium.

Phosphorus was determined by the Gomorri modification of the Fisk and Subarrow method (60).

CHAPTER IV

RESULTS

Preliminary Chemical Analyses

Data from preliminary analyses are presented in Table 4. The high value for dry matter was expected due to the dehydration of the cysts which represents the dormant stage. Dehydration is an important factor in preserving the viability of the cysts. The value for ash includes minerals adhering to the outside of the shell as well as those within. Pre-washing was not considered advisable in order to keep results as near those which would be found in actual use as possible. The value for fat represents ether extractable substances (i.e., lipid material except for the canthaxanthin pigment, which is tightly bound to chitin).

Fifty percent was the estimate used for the protein content of BSC. The actual value is closer to 55% (calculated: total Kjeldahl N X 6:25), but the lower value was used in planning diets to compensate for the non-utilizable nitrogen in chitin. Chitin nitrogen was unavoidably included in the total Kjeldahl nitrogen analysis. Borgstrom (27) also mentions the importance of correcting for chitin nitrogen in shelfish meals used as protein sources. Much speculation has been made as to the dietary value of chitin, but whether or not it can be used as a nutrient source still remains unknown. The caloric value of the total cyst is high but does not represent true metabolizable energy.

Sparre (28) presents the following limits for properties of fish meal: moisture 6-10%; fat 5-12%; protein 60-75%; and ash 10-20%.

This varies considerably from one product to another, but it is apparent that brine shrimp eggs compare favorably with these limits (Table 3). Thus, one sees that brine shrimp cysts which require no processing are indeed a likely prospect for improving the quality of animal feeds.

The low moisture content (6%) of brine shrimp eggs would assist in preventing both mold growth and fat oxidation. These two would be the major source of spoilage expected with this type of product.

TABLE 3. Proximate analysis of San Francisco Bay brine shrimp cysts

Dry Matter	No. of Samples	%	Sd
		94%*	
ash	6	6%	± .1
fat	5	13%	± 1.29
protein	3	58%	(N X 6.25)
chitin	6	10%	± .494
kcal/g	3	5.115	± .032

*From the work of Eleanore Hague Smith, Fall, 1969, Department of Human Nutrition and Foods, Michigan State University.

Protein Utilization Studies

Conventional Rats

A number of comparisons are of major importance in evaluating the protein in the brine shrimp cyst. The weight gain and food intake of the rats fed 10% brine shrimp cyst diet were equal to that of the rats fed the 5% casein ration. Growing rats fed the ration containing the 10% protein from casein significantly ($P < .005$) exceeded the growth produced and food consumed by the rats fed the 10% protein in the brine shrimp cyst ration. This suggests that the protein in brine shrimp cysts alone, when incorporated in a diet, was approximately 1/2 as good as casein in growth promoting activity. This theory was further supported by the results secured when brine shrimp cysts were incorporated with casein in the same diet. A 10% protein diet in which 5% was from brine shrimp cysts and 5% was from casein produced a weight gain significantly different ($P < .005$) from that produced by either 5% casein or 10% casein alone (Figures 4 & 5). Rats fed a 5% casein diet, a 10% casein diet and a mixed diet of 5% brine shrimp cysts plus 5% casein gained an average of 0.57g, 4.07g and 2.03g per day respectively. This trend was repeated again at the level of 20% protein, 10% from brine shrimp cysts plus 10% from casein. The rats fed the mixed diet grew significantly better than those fed the 10% casein ration ($P < .005$). These rats equaled the 15% casein average daily gain of 6.43g, but gained less weight (6.71g/d) than the 20% casein (7.34g/d) fed rats (Figures 4 & 5), even though the difference was not supported statistically.

Figure 6 summarizes the growth patterns of the rats fed diets containing protein from soy or brine shrimp cyst. The group fed the 5% soy protein showed no increase in body weight throughout the 28-day assay period. The addition of 5% protein from brine shrimp cyst to that diet improved the growth significantly ($P < .005$) (.11g per day increased to 1.85g per day), but it did not equal the 10% soy control (4.06g per day). The second mixed diet, 10% soy and 10% brine shrimp cyst, likewise exceeded the 10% soy protein ration in growth promotion but was

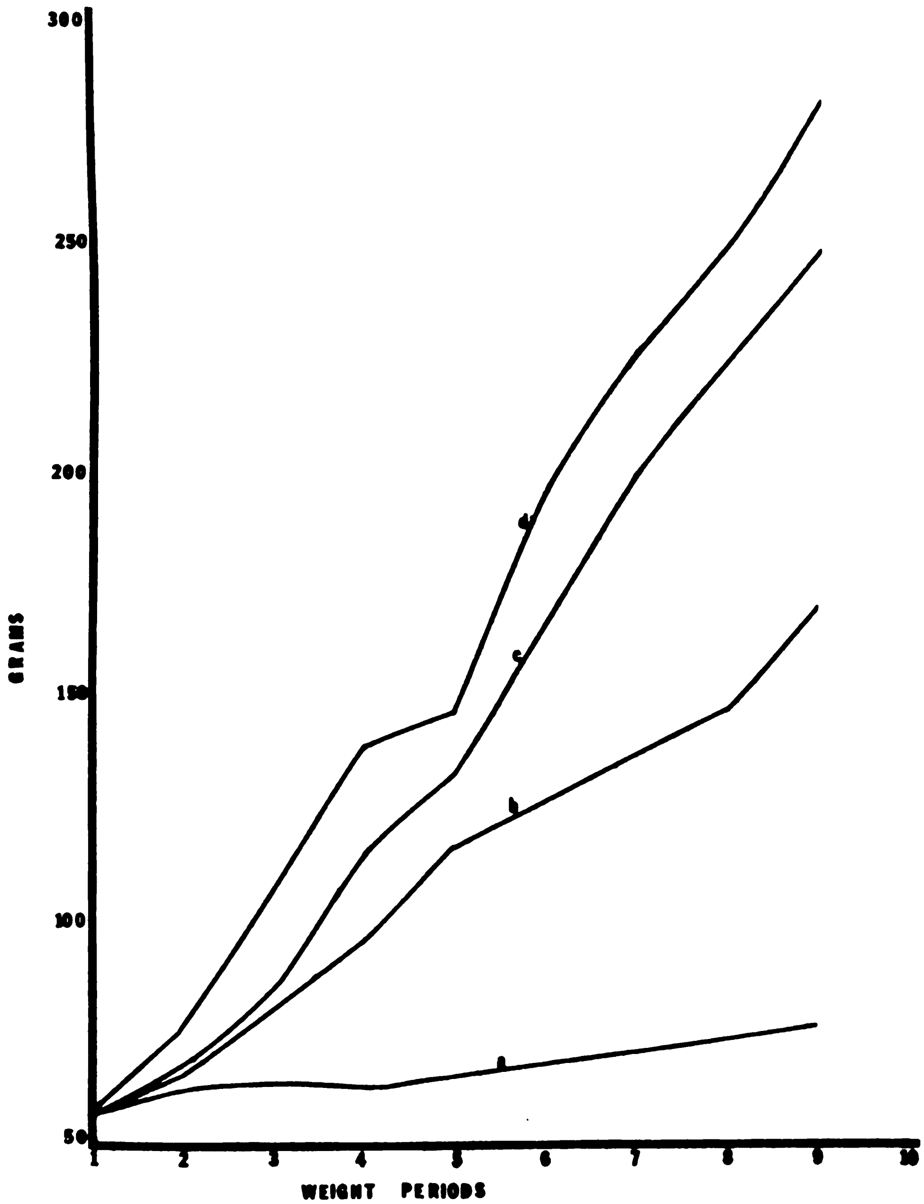

Figure 4. Absolute weights of rats fed diets containing

a) 5% protein from casein
b) 10% protein from casein
c) 15% protein from casein
d) 20% protein from casein

Weight periods were three days.

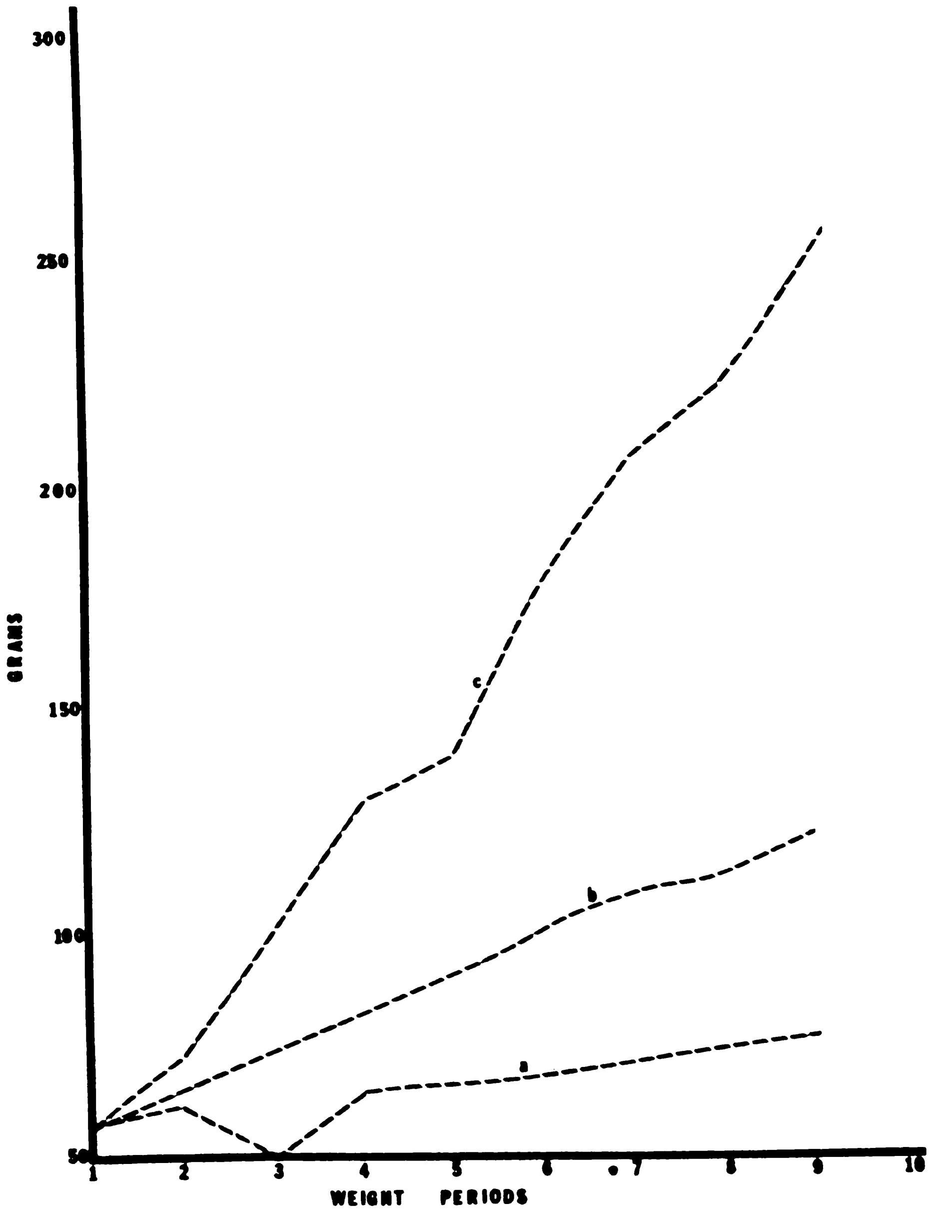

Figure 5. Absolute weight of rats fed diets containing

a) 10% protein from brine shrimp cyst
b) 5% protein from brine shrimp + 5% protein from casein
c) 10% protein from brine shrimp cyst + 10% protein from casein

Weight periods were three days. (5 rats per diet)

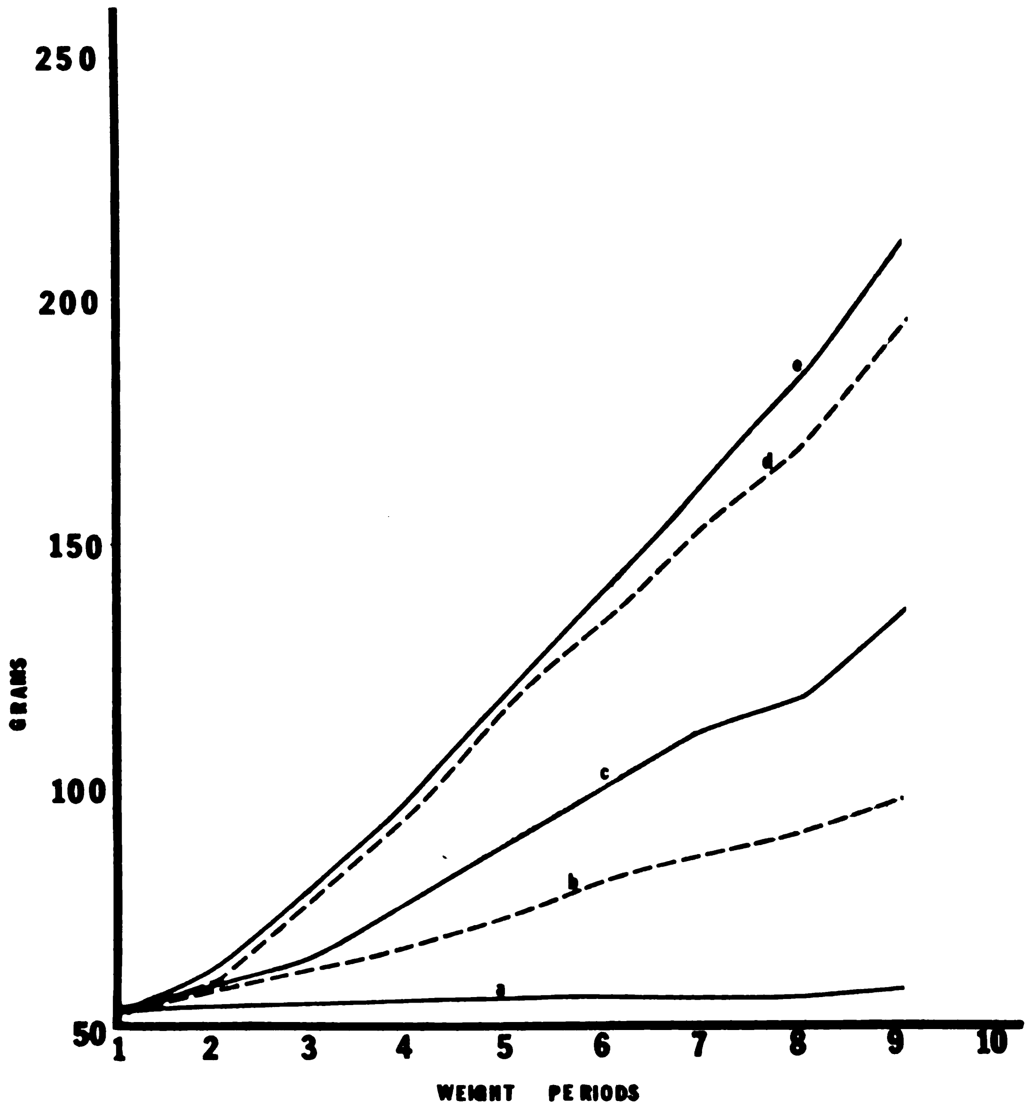

Figure 6. Absolute weight gain of rats fed diets containing

a) 5% protein from soy
b) 5% protein from soy + 5% protein from BSC
c) 10% protein from soy
d) 10% protein from soy + 10% protein from BSC
e) 20% protein from soy

Weight periods were three days. (5 rats per diet) significantly less efficient than the 20% soy ration ($P < .005$).

Food intake was not mentioned in all cases above, but it followed a trend identical to the growth patterns so it was clearly the feed efficiency which was responsible for differences in weight gain. The exception to this was the 12.05g, 12.43g and 12.22g per day intakes of the rats fed the 10% soy ration, 5% soy-5% brine shrimp cyst, and 10% soy, 10% brine shrimp cyst respectively. However, weight gain was consistent with the quality and quantity of the dietary protein available to the rats at these levels (Figure 6).

In the one study in which brine shrimp cysts were ground in a ball mill before being incorporated into the ration (Figure 7) no difference between the utilization of that diet (10% brine shrimp cyst and 10% casein) and the 20% casein diet was observed ($P < .001$). Average daily gains were 7.17g and 6.80g respectively; average daily feed consumptions were 16.81 and 16.95, which were not significantly different ($P > .001$).

In terms of PER at the 20% protein level, the mixed diet containing 10% protein from casein and 10% protein from brine shrimp cyst was as efficiently used ($P > .005$) as the 20% casein ration (Table 4). This was also true when soy was mixed with brine shrimp cyst in the diet. The ration containing 10% protein from soy plus 10% from

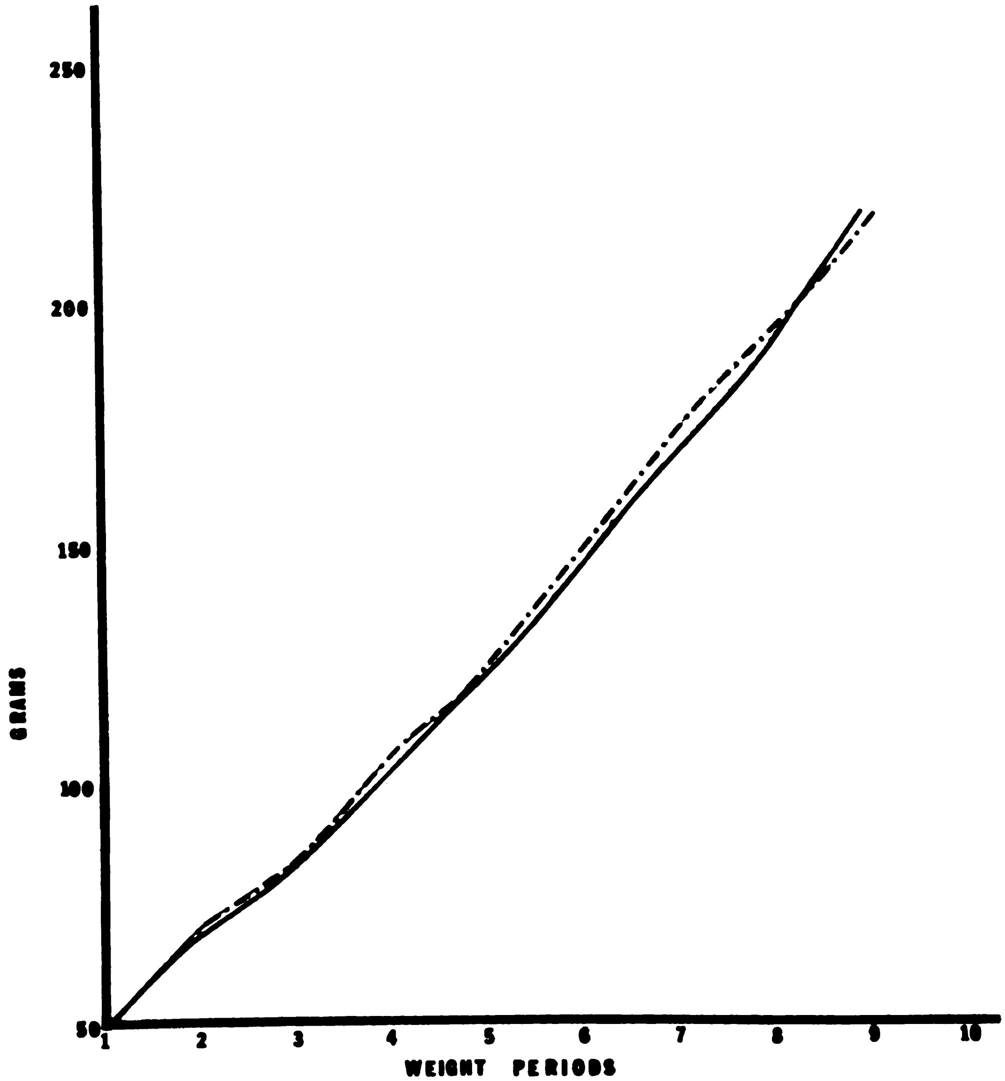

Figure 7. Absolute weight gain of rats fed a 20% casein diet (————) and of rats fed a ration containing 10% casein plus 10% protein from ground brine shrimp cyst (-·-·-·-·-·-). Weight periods were three days. (5 rats per diet)

brine shrimp cyst resulted in a PER which was not statistically different ($P > .005$) from the PER resulting from the 20% casein ration (Tables 4 and 5).

From the weight gain data in Table 4, it is apparent that there is no significant difference between the weight gain of rats fed the 15% protein from casein diet and rats fed the 10% protein from casein plus 10% from brine shrimp cyst. This similarity did not hold true for PER. The 15% casein ration when consumed by growing rats was more efficiently used per gram consumed ($P < .005$) than was the mixed protein ration containing 10% from casein plus 10% from brine shrimp cyst.

When brine shrimp cyst was used as the sole source of protein at the 10% protein level, PER was quite low. When casein was mixed with brine shrimp cyst in the diet, there was a marked increase in the PER over that of the 10% brine shrimp cyst diet (Table 4). This was seen in both mixed rations containing brine shrimp cyst plus casein (5% + 5% and 10% + 10%). The combination of soy protein with brine shrimp cyst in rations, (5% protein from soy + 5% from brine shrimp cyst), when fed to rats, did not significantly increase the PER in comparison to the PER of the 10% brine shrimp cyst diet (Table 5). There was, however, a marked increase of the PER produced by the 10% soy plus 10% brine shrimp cyst ration in comparison to the PER of the 10% protein from brine shrimp cyst diet.

TABLE 4. Weight gain and feed consumption of weanling Sprague-Dawley rats fed rations containing brine shrimp cysts and/or casein and PER and NPU values of the proteins. (1)

Dietary Protein Level and Source	28-day Weight Gain g.	Feed Consumption g/da/rat	PER	NPU
5% Casein	16 ± 5	5.87	2.11 ± .47	41% ± 23
10% Casein	114 ± 20	12.50	3.63 ± .60	72% ± 9
15% Casein	180 ± 13	16.16	2.84 ± .32	58% ± 4
20% Casein	204 ± 6	17.25	2.29 ± .10	50% ± 2
10% Brine Shrimp Cyst	16 ± 9	5.48	1.07 ± .44	18% ± 7
5% + 5% Casein + Brine Shrimp Cyst	57 ± 11	8.47	2.48 ± .72	58% ± 9
10% + 10% Casein + Brine Shrimp Cyst	188 ± 8	17.25	1.94 ± .08	42% ± 5
10% + 10% Casein + Ground Brine Shrimp Cyst	229 ± 9	16.84	2.13 ± .06	--------
20% Casein	217 ± 35	16.95	1.99 ± .20	--------

(1) 5 rats per diet.

TABLE 5. Weight gains, feed consumption, of weanling Sprague-Dawley rats fed rations containing brine shrimp cysts and/or soy protein and PER and NPU values of the proteins. (1)

Dietary Protein Source	28-Day Weight Gain g.	Feed Consumption g/da/rat	PER	NPU
10% Brine Shrimp Cyst	16 ± 9	5.48	1.07 ± .44	18% ± 7
10% Soy	113 ± 7	12.05	2.77 ± .1	52% ± 10
20% Soy	184 ± 14	16.47	2.09 ± .1	38% ± 9
5% + 5% Soy + Brine Shrimp Cyst	52 ± 14	12.43	1.54 ± .3	20% ± 8
10% + 10% Soy + Brine Shrimp Cyst	155 ± 17	12.22	2.49 ± .1	45% ± 6

(1) 5 rats per diet.

In this study the values for Net Protein Utilization (NPU) were low for the casein fed rats. However, the data (Table 4 & 5) did confirm better Net Protein Utilization in the diets containing casein than those containing soy. When brine shrimp cysts were added to the ration containing protein from 10% soy plus 10% protein from brine shrimp cyst there was no difference between the utilization of that mixture and the casein and brine shrimp cyst mixture (10% and 10%) ($P > .005$). Likewise, the addition of brine shrimp cyst to the ration containing 5% protein from casein plus 5% protein from BSC resulted in a higher NPU value than that for the ration containing twenty percent protein from casein and was equal to that of the ration providing 15% protein from casein alone ($P > .05$) (Table 4).

<u>Germfree Rats</u>

The germfree rats exhibited a positive increase in weight gain with increasing protein content of the diet (Figure 8). Few statistically significant differences in growth parameters were seen between the germfree rats and their littermates, which had been removed from germfree conditions (x-germfree).

The absence of microorganisms in the gastrointestinal tract of the rats did not significantly change the utilization of brine shrimp cyst protein when combined with casein. Figures 9 & 10 demonstrate that the same pattern of growth occurs in both germfree rats and conventionalized

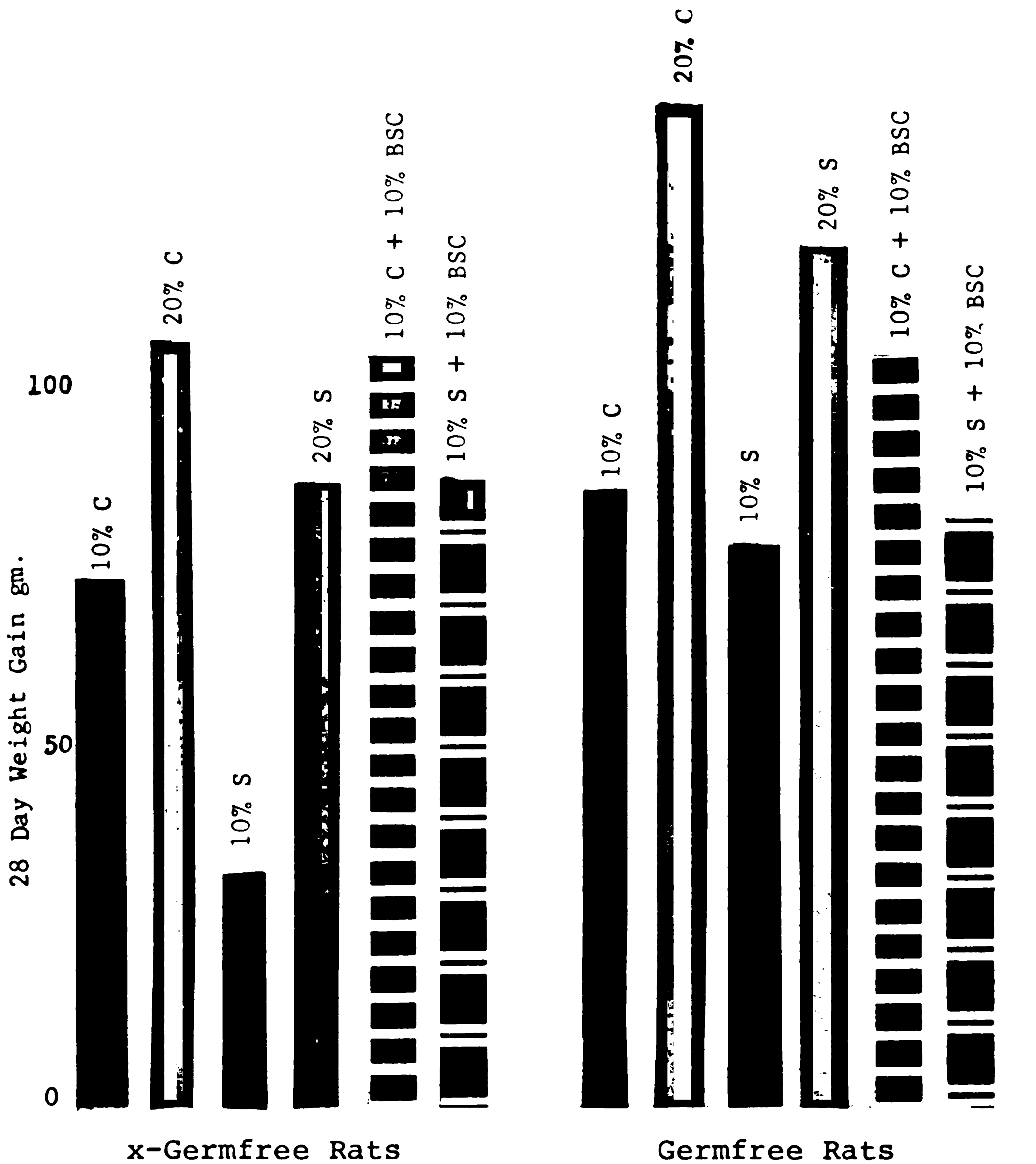

Figure 8. Twenty-eight-day weight gain of germfree and x-germfree rats fed diets containing casein, soy and brine shrimp cyst rations.

Legend

C = casein as protein source
S = soy as protein source
BSC = brine shrimp cyst as protein source
C + BSC = mixed protein source
S + BSC = mixed protein source

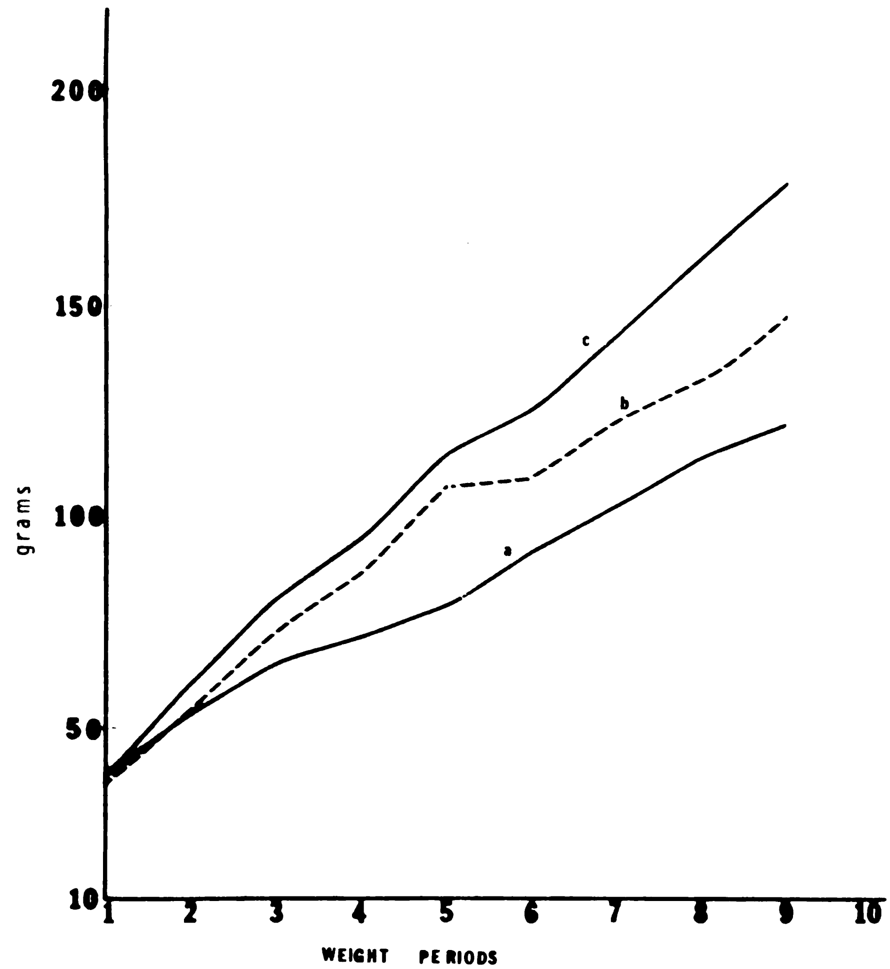

Figure 9. Absolute weight of germfree rats fed diets containing

a) 10% protein from casein
b) 10% protein from casein + 10% protein from BSC
c) 20% protein from casein.

Four rats per group. Weight periods were every three days.

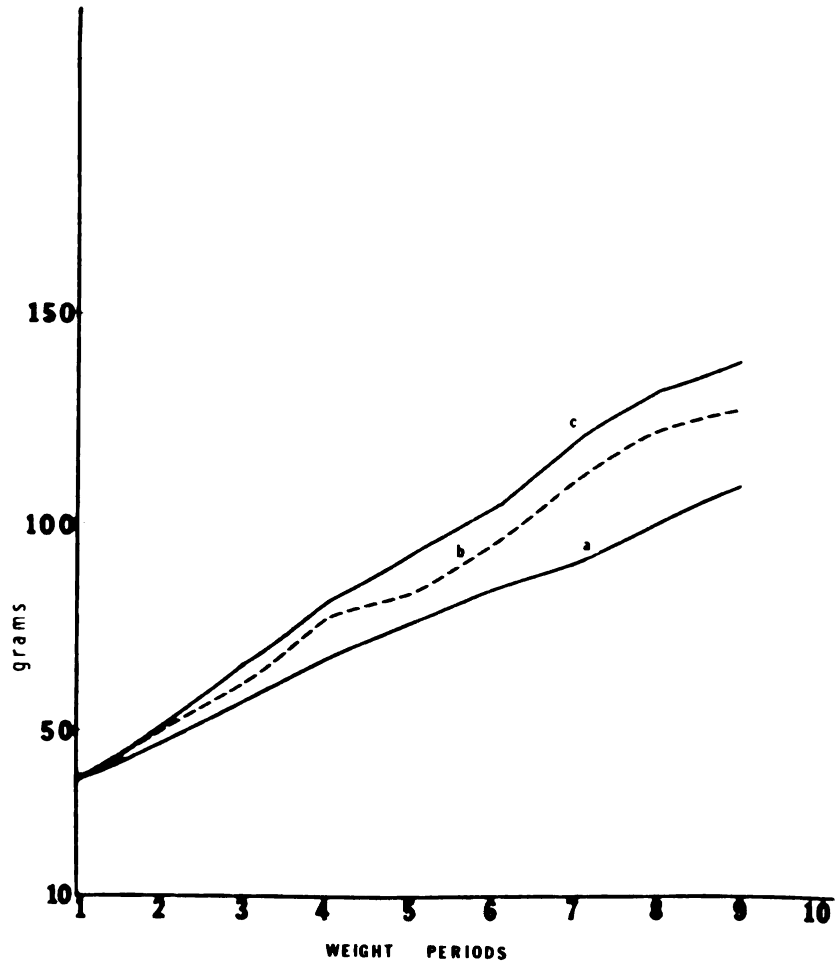

Figure 10. Absolute weight of x-germfree rats fed diets containing

a) 10% protein from casein
b) 10% protein from casein + 10% protein from BSC
c) 20% protein from casein.

Four rats per group. Weight periods were every three days.

controls, when fed diets in which casein and/or casein plus brine shrimp cysts are the protein sources. No statistically significant differences were found between the germfree rats fed mixed diets and their x-germfree controls in any parameter measured ($P > .005$) (weight gain, PER, NPU) Tables 6 & 7). The germfree rats fed the casein and brine shrimp cyst mixture grew at a rate equal to that of the x-germfree rats fed the same mixture (Figures 9 and 10). The germfree rats fed the soy and brine shrimp cyst mixture also grew at rates which equalled that of their x-germfree controls.

When germfree rats were fed diets containing either casein or soy as the sole source of protein, they gained more weight than their x-germfree littermates at both the 10% and 20% protein levels (Figure 9). Better PER values were also obtained by the germfree rats regardless of the source of dietary protein. There were no differences observable between any comparisons made of NPU, whether within germfree treatments or between germfree and x-germfree littermates.

Cyst Breakage

The diguanosine nucleotide analyses left little doubt that brine shrimp cyst were ruptured during passage through the intestinal tract of rats.

The amount of p^1,p^4 diguanosine-5^1-tetraphosphate (G_p4G) available to the rats per day was calculated by

TABLE 6. Weight gain and feed consumption of germfree rats fed rations containing brine shrimp cysts, soy protein and casein as the protein sources and PER and NPU values of the proteins. (1)

Dietary Protein Level and Source	28-Day Weight Gain	Feed Consumption g/da/rat	PER	NPU
10% Casein	85 ± 5	10.82	2.80 ± .25	45% ± 11
20% Casein	138 ± 30	13.23	1.83 ± .45	39% ± 11
10% Soy	78 ± 14	9.42	3.00 ± .65	33% ± 2
20% Soy	119 ± 23	13.57	1.57 ± .36	31% ± 1
10% + 10% BSC + Casein	104 ± 27	11.94	1.70 ± .23	26% ± 3
10% + 10% BSC + Soy	81 ± 23	9.81	1.35 ± .26	30% ± 7

(1) 4 rats per diet.

TABLE 7. Weight gain, feed consumption of x-germfree rats fed rations containing brine shrimp cysts, soy protein and casein as the protein sources and PER and NPU values of the proteins. (1)

Dietary Protein Level and Source	28-Day Weight Gain	Feed Consumption g/day/rat	PER	NPU
10% Casein	73 ± 13	10.89	2.44 ± .6	56% ± 2
20% Casein	106 ± 18	11.99	1.60 ± .21	32% ± 5
10% Soy	32 ± 4	10.02	1.23 ± .48	27% ± 18
20% Soy	86 ± 13	12.07	1.30 ± .15	31% ± 3
10% + 10% BSC + Casein	104 ± 3	13.59	1.26 ± .22	22% ± 1
10% + 10% BSC + Soy	87 ± 20	11.34	1.34 ± .15	29% ± 1

(1) 4 rats per diet.

multiplying the average daily weight of food intake and average daily feces weight times their respective G_p4G content as determined by analyses. The amount of G_p4G remaining in the feces daily was then subtracted from the amount of G_p4G consumed daily to give a value for the amount which was theoretically absorbed. The mg G_p4G absorbed was divided by the mg G_p4G in the diet and multiplied by 100 to give the percent absorbed. This value also represents the percent of brine shrimp cyst which were ruptured during passage through the intestinal tract.

The results (Table 8) revealed that at lower protein levels almost all the eggs are ruptured (to the extent of 91-95%), but at the higher level of protein intake only 68% of the cysts protein became available to the rats.

Specific Nutrient Analysis

Amino Acids

Compared to casein and soy, brine shrimp eggs are a good source of amino acids. In each case they are from 50-100% of the casein values and equal or exceed soy protein in 5 essential amino acids and 3 non-essential amino acids (Table 9). Tryptophan has been omitted from the tables because it was not measured.

In this study different protein levels were used, thereby resulting in different levels of intake and different requirement limits. However, it was necessary to establish a relative standard for comparison between

TABLE 8. The percent of brine shrimp cysts ruptured during passage through the intestinal tract of rats as calculated by the amount of diguanosine nucleotides excreted by the rat.

Diets Containing	G_p4G Absorbed From Diet (mg) (1)	G_p4G In Feces (mg) (2)	% Cyst Rupture
10% protein from bine shrimp cyst	11.67	1.04	91%
5% protein from casein + 5% protein from brine shrimp cyst	8.04	.36	95%
10% protein from casein + 10% protein from brine shrimp cyst	42.60	13.32	68%

(1) Based on average daily food intake of rats.

(2) Based on average daily feces.

TABLE 9. Amino acid content of brine shrimp cyst, casein and soy protein g/16 g N.

AA	BSC	Cas (1)	Soy (2)
Met	2.00	2.95	1.0*
Phe	3.74	5.25	5.0
His	1.80	3.10	2.6
Thre	3.97	4.70	3.9*
Lys	7.16	8.20	6.8*
Val	5.50	7.20	5.5*
Leu	4.87	9.75	7.5
Ile	4.88	6.85	6.5
Cys	.14	.34	.6
Arg	5.46	4.00*	8.3
Glu	13.56	22.30	19.5
Tyr	5.12	6.10	3.4*
Asp	10.25	7.20*	6.2*
Ser	5.95	5.90*	6.9
Pro	3.10	11.60	2.5*
Gly	3.85	2.00*	4.1
Ala	5.94	3.50*	3.6*

*BSC exceeds soy protein or casein.

(1) Advances in Pro. Chem. Anson & Edsall V5, 1949, p. 218, Acad. Press (29) (30) (31).

(2) Skidmore Enterprises Assay Protein Cl, 3217 Beull Ave., Cincinnati, Ohio 54211.

diets. For this reason all dietary amino acid levels have been compared to the requirement of a normal growing rat so defined by the National Research Council (61). That norm is a male rat consuming 15 g per day and gaining approximately 5 grams of body weight per day (61).

Table 10 compares the percent of the rats amino acid requirement which is met by three of the 10% protein diets (casein, soy and brine shrimp cyst). Notice that the most limiting amino acid in each case is histidine for casein, methionine for soy protein, and histidine for brine shrimp cysts.

Since the requirement is not met by these diets, we would expect the lower daily weight gain which occurred; casein (4 g/da), Soy (4 g/da) and brine shrimp cyst (0.57 g/da). Brine shrimp cysts as the sole source of protein did not permit consumption of adequate amounts of diet to promote growth. In fact, the ration containing 10% protein from brine shrimp cysts permitted growth of the rats equal to the ration containing 5% protein from the casein diet (0.62 g/da wt. gain) (Table 11). These two diets also promoted equal feed consumption (10% BSC protein: 5.9 g/da; 7.5% casein: 5.5 g/da). In fact, Table 11 shows that both 5% casein and 10% protein from brine shrimp cyst; provide similar percentages of the rat's requirement for the essential amino acids. For all practical purposes brine shrimp cysts have not proved desirable as the sole source of protein in a ration but make a valuable contribution when

TABLE 10. Amino acids essential to rats, percent of the requirement available from diets containing 10% protein from casein, 10% protein from soy and 10% protein from brine shrimp cyst based on 28-day intake. (1)

Amino acids needed for growth (2)		% requirement supplied by		
Name	g/da	Casein %	Soy %	BSC %
Met	.090	41	13	16
Phe	.12	40	37	12
His	.045	27	21	6
Thre	.075	68	54	25
Lys	.135	72	57	33
Val	.09	61	44	17
Leu	.112	71	52	17
Ile	.082	86	79	35

(1) Five rats per group.

(2) Nutrient Requirement of Laboratory Animals #10, Sec. Rev. 1072 National Academy of Science, p. 64.

TABLE 11. Percentages of essential amino acids required by the rat as provided by casein or brine shrimp cysts. These values are based on the average daily feed intake of the rats fed these rations for 28 days. (1)

Amino Acid	Required g/day	Dietary Protein Source % from 5% CAS	% from 10% BSC	% from 5% Soy
Met	.090	9*	16	4*
Phe	.120	9	12	11
His	.045	6	6*	6
Thre	.075	15	25	16
Lys	.135	16	33	17
Val	.090	14	17	18
Leu	.112	16	17	16
Ile	.082	22	40	27

*Limiting AA

(1) 5 rats per group.

combined with casein or soy. The value of combining two protein sources is represented in Table 12. Because of the increased intake (8.47 g/da) of the mixed diet (5% casein + 5% BSC) the rats received almost as great a percent of the requirement of essential amino acids from the added 5% BSC as they did from the 10% brine shrimp cyst diet alone (Table 11). This 5% casein and 5% brine shrimp cyst protein combination improved intake and growth because of an increase in the essential amino acid consumed from the brine shrimp cysts.

The increase in the essential amino acids available from brine shrimp cyst is even greater at higher protein levels (Table 13). A mixed diet containing 10% protein from casein and 10% protein from brine shrimp cyst provided essential amino acids in amounts approaching that percentage of the requirement provided by a ration containing 20% protein from casein. Brine shrimp cyst and soy protein underwent the same beneficial supplementation from being combined in a ration. The deficiency in essential amino acid illustrated in Table 10 (10% BSC) was markedly improved when 5% soy protein and 5% brine shrimp cyst protein were combined (Table 14). Both feed intake (12.43 g/da) and weight gain (1.85 g/da) were improved over the diet containing 10% protein from brine shrimp cyst. At the 20% protein level (10% from brine shrimp cyst and 10% from soy) the dietary essential amino acids approached that provided by soy protein (Table 15).

TABLE 12. Percentages of essential amino acids required by the rat as supplied by casein and brine shrimp cysts when both contribute 5% protein to the ration. These values are based on the average daily feed intake of the rats for 28 days. (1)

		Dietary Protein Source			
Amino Acid	Required g/day	% from 5% CAS	+	% from 5% BSC	Total %
Met	.090	13		12	25
Phe	.120	13		9.5	22
His	.045	9		5	14
Thre	.075	22		19	41
Lys	.135	24		25	49
Val	.090	20		13	33
Leu	.112	23		13	36
Ile	.082	29		27	56

(1) 5 rats per group.

TABLE 13. Percentage of essential amino acids required by the rat as supplied by casein and brine shrimp cysts when both contribute 5% protein to the ration. These values are based on the average daily feed intake of the rats for 28 days. (1)

		Dietary Protein Source			
Amino Acid	Required g/day	% from 20% CAS	% from 10% CAS +	% from 10% BSC*	Total %
Met	.090	113	56	51	107
Phe	.120	113	56	40	96
His	.045	74	37	21	58
Thre	.075	189	94	79	173
Lys	.135	200	99	104	203
Val	.090	169	84	55	139
Leu	.112	197	98	54	152
Ile	.082	238	119	112	231

*Based on 68% cyst breakage.

(1) 5 rats per group.

TABLE 14. The percentage of essential amino acids required by the rat as supplied by soy protein and brine shrimp cysts. These values are based on the average daily feed intake of the rats for 28 days. The amino acid values for BSC are based on the rupture of 68% of the BSC during digestion.

		Dietary Protein Source			
Amino Acid	Required g/day	% from 5% Soy	+	% from 5% BSC	Total %
Met	.090	7		18	25
Phe	.120	19		14	33
His	.045	11		7	18
Thre	.075	28		28	56
Lys	.135	30		38	68
Val	.090	23		20	43
Leu	.112	27		19	46
Ile	.082	48		48	96

TABLE 15. The percentage of essential amino acids required by the rat as supplied by soy protein and brine shrimp cysts. These values are based on the average daily feed intake of the rats fed for 28 days. The amino acid values for BSC are based on the rupture of 68% of the BSC during digestion.

		Dietary Protein Source			
Amino Acid	Required g/day	% from 20% Soy	% from 10% Soy +	% from 10% BSC	Total from Soy + BSC %
Met	.090	37	15	27	42
Phe	.120	105	42	21	63
His	.045	60	24	11	35
Thre	.075	151	61	42	103
Lys	.135	160	65	56	121
Val	.090	124	50	29	79
Leu	.112	146	60	29	89
Ile	.082	219	82	61	143

Vitamin and Mineral Determinations

Fish are known to have high concentrations of B-Vitamins; and this is also true of brine shrimp cysts as can be seen in Table 16. Sparre (28) reported values for the amount in whole fish meal of riboflavin (7.3 mg/kg), niacin (126 mg/kg) and pantothenic acid (30.6 mg/kg). Brine shrimp eggs are better sources of riboflavin (23 mg/kg) and pantothenic acid (72 mg/kg) and contain almost as much niacin (108 mg/kg). He also mentions considerable loss (50%) of pantothenic acid during storage but with brine shrimp cysts as long as the cysts remained whole and viable storage losses should be zero.

The mineral levels found in brine shrimp cysts were good indications that the eggs would be a good supplementary source. Highest were those common to salt water and probably involved in exterior contamination (Na, K). Wickins (26) reports zinc (SFB - 59 ppm and Utah - 66 ppm) and copper (SFB - 5 ppm and Utah - 27.5 ppm) in brine shrimp cysts, which varies somewhat from the data in Table 17, but, as has been discussed previously, exterior contamination has not been accounted for, and it is not known whether or not his values are for prewashed eggs.

Values for the mineral content of fish meal (28) are: zinc (70 ppm), iron (250 ppm), copper (7 ppm), manganese (4 ppm). When these concentrations are compared to the values in Table 17, they support the fact that brine shrimp

TABLE 16. Vitamin content of San Francisco Bay brine shrimp cyst moisture content 6%.

Vitamin	μg/g	SD	IU	No. Replicates
Thiamin	7.13	± 0.44	--	7
Niacin	108.68	± 5.42	--	6
Riboflavin	23.15	± 3.03	--	8
Pantothenic Acid	72.56	± 1.28	--	5
Retinol	10.48	± 0.54	35	5

TABLE 17. Mineral analysis of San Francisco Bay brine shrimp cysts, moisture content 6%.

Mineral	ppm	SE	No. Replicates
Phosphorus	8765	± 433	16
Potassium	8133	± 295	16
Sodium	5344	± 286	16
Calcium	4781	± 381	20
Magnesium	2735	± 177	20
Iron	681	± 176	20
Zinc	75	± 3.5	16
Manganese	32	± 40	20
Copper	2	± 0.05	20
Selenium	0.834	--------	2*

*Performed by John Hitchcock, Department of Animal Husbandry, Michigan State University.

eggs are a valuable source of nutrients and should be further developed.

CHAPTER V

DISCUSSION

On the basis of the reports in the literature, it appears that the major anticipated difficulty in the utilization of BSC by animals was going to be the apparent indigestibility of the shell protecting the brine shrimp cysts. This assumption did not materialize. The protein was not only available to the rat, but when combined with casein or soy it contributed significantly to the growth of weanling rats. On the basis of weight gain alone, the protein from the whole brine shrimp cyst combined with casein or soy proved to be half as good as casein at the levels tested.

In Figure 11 the solid line represents the standard curve obtained with 28-day weight gain was plotted against protein level. The grams gained were 16g, 114g, 180g and 204g, respectively. For the purpose of comparison, the dotted lines in Figure 11 represent the weight gains of the rats fed brine shrimp cyst plus casein plotted over the casein standard curve. Notice that the 5% and 5% mixture (b) produced a weight gain approximately equivalent to what one would expect from 7-1/2% casein (57g); the 10% and 10% mixture (c) produced a weight gain equal to 15%

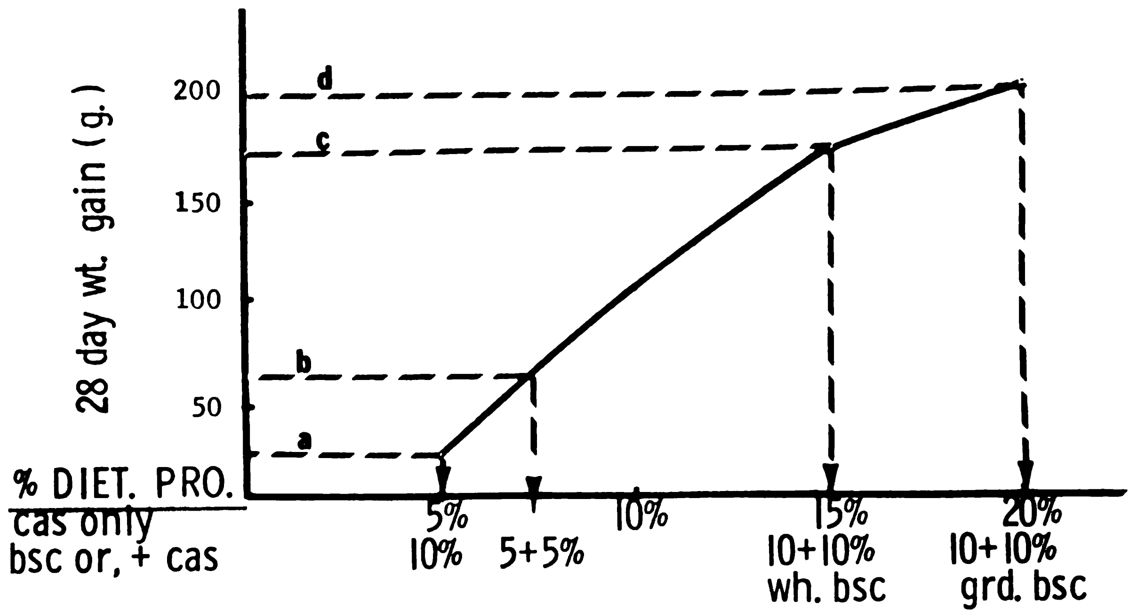

Figure 11. Growth curve: 28-day weight gain vs. % protein from casein at different concentrations in the ration. Dotted lines indicate extrapolation of weight gained by rats fed mixed diets containing different protein levels.

a) BSC diet containing protein at 10% from BSC
b) 5% protein from whole BSC + 5% from casein
c) 10% protein from whole BSC + 10% from casein
d) 10% protein from ground BSC + 10% from casein

(wh = whole, grd = ground)

casein (188g); and the 10% and 10% mixture (d) containing ground brine shrimp cyst produced a weight gain equal to 20% casein (204g). Line a) represents the weight gain resulting when weanling rats were fed the ration containing 10% protein from brine shrimp cysts.

The difference between the two 10% plus 10% diets can be explained by the difference in cyst breakage: 68% when whole cysts were in the diet, 100% when ground cysts were used.

Figure 12 reveals that a soy and BSC mixture is also better than BSC alone. The two mixed diets (lines a and b in Figure 12) fall approximately half way between the value produced by the soy control diets.

The effect of the BSC on the PER values was greater with each when the BSC was added to the casein ration than when added to the soy ration. A higher PER score resulted when rats were fed the casein ration containing 5% protein from BSC and 5% from casein than when the protein levels from both sources was at a level of 10% (Table 18). This, it to be expected since the PER value decreases with increasing protein level in the ration. The opposite occurred with the soy protein rations. There the higher PER value occurred when the ration contained 10% protein from BSC and 10% from soy.

Table 19 lists PER from highest to lowest value. Casein was a more efficiently used source of protein than soy. It was, therefore, not surprising that more efficient

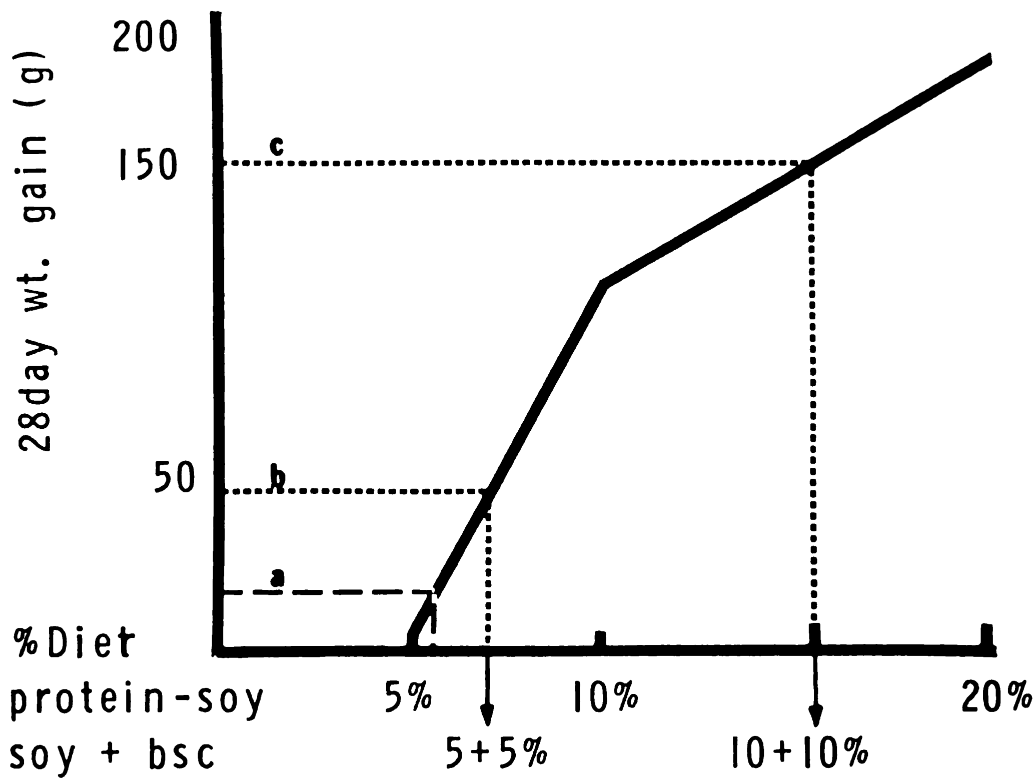

Figure 12. Growth curve: 28-day weight gain vs. % protein from different levels of soy protein. Dotted lines indicate extrapolation of weight gained by rats to protein concentration.

a) Diet containing 10% protein from BSC
b) Diet containing 5% protein from BSC + 5% protein from soy
c) Diet containing 10% protein from BSC + 10% protein from soy

TABLE 18. PER of rats fed diets containing casein, soy, brine shrimp cyst or mixtures thereof as the protein sources. The percentages listed represent the level of protein contributed to the ration by the listed products.

PER	Protein Source	PER	Protein Source
3.63	10% casein	2.77	10% soy
2.84	15% casein	*2.49	10% + 10% soy
*2.48	5% + 5% BSC protein + casein	2.09	20% soy
2.29	20% casein	*1.54	5% + 5% BSC protein + soy
2.11	5% casein		
*1.94	10% + 10% BSC protein + casein	.47	5% soy
1.07	10% BSC protein		

*Mixed diets

use of casein was observed at lower protein levels (i.e., 10%) and less efficient use at higher protein levels (i.e., 20%). Soy protein was simply not as efficient at supplementing the brine shrimp cyst as was casein, so it took a higher level of total protein to promote growth.

When used alone in the diet, brine shrimp cyst was not as efficiently used as casein or soy; but when it was combined with either of these, the resulting PER was enhanced greatly. This is shown by the fact that the PER of the 5% casein plus 5% brine shrimp cyst diet surpasses that secured with the 20% casein ration and approached the PER of the 15% casein ration.

In both cases of supplementation of brine shrimp cysts (with casein or with soy), NPU was in agreement with PER; and in the case of the 5% casein plus 5% brine shrimp cyst diet, the NPU equaled that of the 15% casein (58%).

The inability to show a statistically significant difference between the growth parameters of germfree rats fed mixed diets (10% casein or soy plus 10% brine shrimp cyst) and their conventionalized littermates (x-germfree rats) helped to answer the question of microbial influence upon intestinal cyst breakage (13). The actual mechanism for cyst breakage in the intestinal tract remains to be found. Horne (13) tested digestive enzymes (*in vitro*) against cyst viability and found none of the enzymes tested resulted in a decrease in the viability of brine shrimp cyst. The weight gain of the germfree rats fed 10% casein

(85 ± 5), although not statistically different from those germfree rats fed a mixed diet (104 ± 27), was great enough to suggest that brine shrimp cysts were used as a protein source by the germfree rats. Therefore cyst breakage which cannot be shown to occur <u>in vitro</u> with digestive enzymes, does occur <u>in vivo</u>.

The San Francisco Bay brand brine shrimp cyst used in this study contained approximately 12 mg G_p4G per gram of cyst as determined in our laboratory. The fact that this nucleotide disappears from the ingested brine shrimp cysts as they through the intestinal tract of the rat was strong evidence to support potential protein availability and cyst breakage during that time. This quantitative evidence outweighed the fact that viable cysts do appear in the feces (15, 16) and that cysts were not liable to digestive enzymes <u>in vitro</u> (13). Also, it supported the idea that brine shrimp cysts were used as a source of nutrients by growing mammalians. The diet containing the 10% casein plus 10% brine shrimp cyst mixture released less G_p4G to the animal than the 5% casein plus 5% brine shrimp mixture or the diet of 10% brine shrimp cyst alone. This occurrence could be related to two factors: (1) the need of the animal (as more protein was supplied more was wasted), (2) the bulk of the diet itself. It does not appear as though chitin was used by the rats because feeding BSC in the diet resulted in considerable bulk in the feces. The rats fed the ration containing 20% protein from casein excreted 3.07 g/da total fecal material. The rats fed the ration containing 10% protein from casein plus 10% protein from BSC (a total of 20% protein) excreted 6.63 g/da total fecal matter. Average daily food intakes for these two groups were the same (17 g/da).

By combining protein sources one can achieve a combination of essential amino acids superior to that in one single source. Assuming availability it was this type of supplementation which occurred in the mixed diets tested in this study. In the casein-brine shrimp cyst mixture, the brine shrimp cyst contributed additional methionine and histidine which are two essential amino acids in low quantities in casein. Methionine, histidine supplementation occurred with soy protein and brine shrimp cyst, but the level of methionine was so much lower in the soy than in casein that it was still limiting in the mixed diet.

It was also interesting at this point to compare the previously mentioned amino acids in whole fish meal to those in brine shrimp cyst (Table 19).

TABLE 19. A comparison of the amino acids required by the rat for growth which are found in whole fish meal versus BSC. Expressed in g/16 g N.

Amino Acid	Whole Fish Meal (28)	BSC
Met	2.49	2.0
Phe	3.55	3.74
His	1.84	1.80
Thre	4.0	3.97
Lys	8.18	7.16
Val	5.33	5.50
Leu	6.85	4.87
Ile	6.12	4.88

Brine shrimp cysts compares very well with whole fish meal. Just as fish meal has been used as a protein supplement, brine shrimp cysts should be seriously tested and considered.

Cole and Brown (25) compared habitats of Artemia throughout the world and illustrated the variety of mineral waters in which brine shrimp can survive. The large standard errors for the mineral data were considered inherent in the nature of the product; (i.e., due to the tremendous environmental variability possible). It is, therefore, imperative that any use of brine shrimp cyst be based on analyses specific to the locale from which they were taken.

The determination of the water soluble vitamins (thiamin, niacin, riboflavin and pantothenic acid) performed on the San Francisco Bay Brand brine shrimp cyst revealed that they are excellent sources (Table 20). The thiamin content (.7 mg/100 g) was equivalent to that of lean pork (.7 mg/100 g) (62), the riboflavin (2.5 mg/100 g) and niacin (0.6 mg/100 g) contents approached that of beef liver (3.26 mg/100 g) niacin (9.6 mg/100 g) (62) and the pantothenic acid level (7.2 mg/100 g) exceeded that of pork liver (6.4 mg/100 g) (63).

Brine shrimp exceeded the requirement of the rat for growth in every vitamin measured. Table 21 compares the requirement of the rat per gram of diet and the content of brine shrimp cyst per gram. In a mixed diet in which 10% of the protein was from brine shrimp cyst and 10% was from casein, there would be 200g brine shrimp cyst/kg diet (BSC = 50% protein) or 20% of the diet by weight would be from brine shrimp cysts. If the rat consumed 15 grams of diet per day, he would be getting 3g brine shrimp cyst per day.

By calculating the amount of vitamins in three grams of brine shrimp cyst it was found that, for the vitamins determined, brine shrimp cyst met the requirement of the rat by values greater than 100% in every case. Thus, if we assume 100% availability then theoretically no addition of these vitamins would be required if brine shrimp cyst were

TABLE 20. Vitamin content of San Francisco Bay brine shrimp cyst as compared to the requirement of the rat for growth.

Vitamin	Requirement µg/g Diet (1) 90% dry wt.	BSC content µg/g Cyst 90% dry wt.
Thiamin	1.25	6.7
Niacin	15	103
Riboflavin	2.5	22
Pantothenate	8	68.9
Retinol	.06	9.57

(1) Nutrient Requirements of Laboratory Animals #10, Sec. Rev. 1972. National Academy of Sciences, p. 64.

TABLE 21. Estimated amounts of vitamins the rat would require from a diet consumed at the rate of 15/g per day compared to those which would be provided by that diet if it contained 200 g brine shrimp cyst per Kg (i.e., the rat consumed 3 g BSC/day).

Vitamin	Requirement per Day (1) in µg/15 g diet 90% Dry Wt.	Amount Provided by 3 g BSC/day in µg
Thiamin	18.75	21
Niacin	225	324
Riboflavin	37.5	69
Pantothenate	120	217
Retinol	.9	30

(1) Nutrient Requirements of Laboratory Animals #10, Sec. Rev. 1972. National Academy of Sciences, p. 64.

used to supplement the protein content of a diet at a level to provide 10% of the protein.

A similar approach was used to examine the results of the mineral determinations (Table 22) on a per gram basis. If the rat were to consume brine shrimp cyst alone the mineral requirements of the rat in all cases but two (Mn., Cu.) would be exceeded. On the basis of the diet which contained 20% brine shrimp cyst by weight (10% protein from brine shrimp cyst and 10% from casein), brine shrimp cysts provided amounts in excess of the requirement for 6 of the 10 minerals; falling short only in phosphorus, calcium, manganese and copper. Although no correction can be made for variability of minerals from one source to another, these data suggest that brine shrimp cyst would very likely be a potential source of minerals.

The fact that brine shrimp cyst is not only a good source of protein but contain generous amounts of essential vitamins and minerals should add even more impetus to their development and use as a worldwide food source for domestic animal production. Depending upon the requirement of the particular animal and the nutrient content of the particular cysts used, fewer additions would be needed to formulate a ration upon which animals used for human consumption could be raised.

Much work remains to be done before the actual use of BSC could be commerically utilized. Following is a list of

TABLE 22. Mineral content of San Francisco Bay brine shrimp cyst compared to the requirement of the rat for growth expressed in ppm in the diet.

Mineral	Requirement (1) of the Rat ppm (90% Dry Wt.)	Content of BSC ppm (90% Dry Wt.)	Contribution to the diet Containing 20% BSC ppm (90% Dry Wt.)
Phosphorus	4000	8392	1753
Potassium	1800	7786	1626
Sodium	500	5116	1068
Calcium	5000	4577	956
Magnesium	400	2618	547
Iron	35	652	136
Zinc	12	71	15
Manganese	55	30	6.4
Copper	5	1.9	.4
Selenium	.04	.802	.16

(1) Calculated from values in Nutrient Requirements of Laboratory Animals #10, Sec. Rev. 1972. National Academy of Sciences, p. 64.

suggestions which the author believes should be examined in any future work performed on brine shrimp cysts:

1. Long term studies to determine and insure the safety of feeding BSC.
2. Further pesticide analyses.
3. Feeding BSC to domestic animals which might benefit from the protein, vitamins, and minerals supplementary effects of BSC.
4. Studies with larger numbers of animals to improve statistical relationships and verify the protein use and value.
5. Analysis of minerals on washed cysts.
6. Analyses of the vitamins relevant to the requirement of other test animals.
7. Further identifications of lipids.
8. The extent to which chitin is used by the test animal, if at all.
9. The mechanism by which the cysts are ruptured during intestinal passage.
10. Tryptophan analysis of protein from BSC.
11. Amino acids by microbial methods.

The above are primarily of nutritional interest, but production potential, identification of populations which are likely to use BSC as a source of protein, and a program to motivate the use of BSC protein must all be seriously considered if BSC are to be a contributing factor to the world's need for food.

CHAPTER VI

SUMMARY

Brine shrimp cysts (BSC) were analyzed and found to contain dry matter (94%), ash (6%), total extractable lipid (13%), protein (58%), chitin (10%), and total calories (5100 per gram). Protein quality was evaluated by PER, NPU and weight gain of male Sprague-Dawley rats. The BSC were fed to rats in diets containing various levels of proteins from BSC, casein, or soy protein. Mixtures of BSC plus casein or soy were also well consumed by the rats, and the animals did not appear to utilize the available protein. The mixed diets containing either soy or casein in combination with BSC were well consumed by rats, and produced good weight gains.

Diets containing 5% protein from casein and 5% from BSC produced weight gains which were half way between 5% casein diets and 10% casein diets. When the protein level was doubled (10% protein from casein and 10% protein from BSC), the growth produced in rats fed this mixture was equal to the weight gain of rats fed 15% casein diets. Similar data were obtained from soy:BSC combination diets. It appeared that BSC protein could be used by the rats if combined with another good quality protein source.

Germfree studies, using the same mixtures and protein levels revealed that BSC was well used in the absence of intestinal microorganisms when combined with casein or soy. In these studies the chitin shell was broken, and the protein was made available to the rat.

Percent cyst breakage was estimated by measuring the p^1,p^4 diguanosine-5^1-tetraphosphate ($G_{p4}G$) content of food and fecal samples. This nucleotide peculiar to the cysts acted as a marker. As eggs were broken open during passage through the rat's intestinal tract, the $G_{p4}G$ was made available and absorbed by the rat. The $G_{p4}G$ remaining in the feces represented that in the eggs which had remained intact through the tract. Those cysts which were ruptured presumably provided the protein for the growth produced by BSC. At the 20% protein level (10% protein from BSC and 10% protein from casein) 68% cyst breakage occurred. At the 10% protein level, 90% cyst breakage occurred whether the diet contained BSC as the sole source of protein or a mixture of half BSC protein and half protein from casein.

Amino acid analyses of BSC revealed them to be limiting in histidine, phenylalanine, and methionine in relation to the requirement of the growing rat. All other essential amino acids appeared to be in adequate supply. On a gram per gram basis, BSC represented a good supplementary source of essential amino acids.

These small crustacean eggs are an exceptionally good source of B Vitamins: thiamin (7.13 μg/g) niacin (108 μg/g), riboflavin (23 μg/g), and pantothenic acid (72 μg/g). They are not a realistic source of retinol (10 μg/g and do not contain a biologically active carotenoid pigment for mammalian systems.

The mineral levels in BSC gave additional evidence for their high nutritional quality. Potassium, sodium, magnesium, iron, zinc, and selenium were found to be in excess of the rats requirement in the mixed diet providing 10% protein from BSC and 10% protein from casein. Variability in the mineral content must be expected depending upon the source of the eggs. This must be examined separately for different geographical locations.

In conclusion it is the opinion of the author that this study has revealed a useable and potentially valuable new food source which should be seriously considered as one means of increasing the worlds food supply.

LITERATURE CITED

LITERATURE CITED

1. BORGSTROM, G. World Food Resources. Intext Educational Publishers, New York. 1973.

2. TANNENBAUM, S. R. Potential for New Protein Sources. pp. 139-154. In Scrimshaw, N. and A. M. Altschul. Amino Acid Fortification of Protein Foods. Vail-Ballou Press, 1971.

3. WHITAKER, D. The Brine Shrimp Artemia and Its Environment. Science Monthly, 51:192, 1940.

4. ZAHL, P. A. Life in a Dead Sea - Great Salt Lake. National Geographic, 132:252, 1967.

5. MORRIS, J. E. and AFZELIUS, B. A. The Structure of the Shell and Outer Membranes in Encysted Artemia salina Embryos During Cryptobiosis and Development. Journal of Ultrastructure Research, 20:244, 1967.

6. RUDALL, K. M. Chitin Protein Complexes in Insect Cuticle. Advances in Insect Physiology, 6:257, 1963.

7. TOSHER, A. B. and HACKMAN, R. H. Application of Ethylenediaminetetra-acetic Acid in the Isolation of Crustacean Chitin. Nature, 180:40, 1957.

8. HEMPEL-ZAWITKOWSKA, J. Resistance of Eggs of Artemia salina L. to Low Temperature as Related to Several Chosen Environmental Factors. Polish Archives of Hydrobiology, 18(3):287, 1971.

9. BOWEN, S. T. The Genetics of Artemia salina III. Effects of X-Irradiation and of Freezing Upon Cysts. Biological Bulletin 125(3):431, 1963.

10. IWANAMI, Y. Retaining the Viability of the Resting Egg of Brine Shrimp (Artemia salina) in Organic Solvents. Experimental Cell Research, 78:470, 1973.

11. SNIPES, W. C. and GORDY, W. Radiation Damage to Artemia Cysts: Effects of Water Vapor. Science, 142:503, 1963.

12. STOCCO, D. M., BURS, P. C. and WARNER, A. H. Effect of Annoxia on Nucleotide Metabolism in Encysted Embryos of the Brine Shrimp. Developmental Biology, 27:497, 1972.

13. HORNE, F. Effects of Digestive Enzymes on the Hatchability of Artemia salina. Trans American Microscopic Society, 85(2):271, 1966.

14. DUTREIU, J. and CHRESTIA-BLANCHINE, D. Resistance des Oeufs Durables Hydrates d'Artemia salina a' l' anorie. C. R. Academie of Science Paris. t. 263. (10 October 1966) Serie D.

15. PROCTOR, V. Viability of Crustacean Eggs Recovered from Ducks. Ecology, 45:656, 1964.

16. PROCTOR, V. and MALONE, C. Further Evidence of the Passive Dispersal of Small Aquatic Organisms Via the Intestinal Tract of Birds. Ecology, 46:728, 1965.

17. LOFFLER, H. Vogelzug Und Crustaceenverbreitung. Zoologischer Anzeiger. Supplement. 27:311, 1964.

18. EWING, R. D. and CLEGG, J. S. Evidence for a Single Macromolecular Form of Lactate Dehydrogenase in Artemia salina. Archives of Biochemistry and Biophysics, 150:566, 1972.

19. EMMÈRSON, D. N. Some Aspects of Free Amino Acid Metabolism in Developing Encysted Embryos of Artemia salina the Brine Shrimp. Comparative Biochemistry and Physiology, 20:245, 1967.

20. ZASLOFF, M. Non-enzymatic Binding of Formylmethionyl-transfer RNA_f to Artemia salina Ribosomes. Journal of Molecular Biology, 76:455, 1973.

21. ZASLOFF, M. and OCHOA, S. Polypeptide Chain Initiation in Eukaryotes. Journal of Molecular Biology, 73: 65, 1973.

22. PROCTOR, V. and MALONE, C. Dispersal of Acquatic Organisms: Viability of Dissemenules Recovered from the Intestinal Tract of Captive Killdeer. Ecology, 48:672, 1967.

23. AL-UTHMAN, H. S. Ecological Observations on Artemia salina (L) (Anostraca:Artemiide) in Iraq. Bulletin of the Biological Research Center, Baghdad, 5:49. 1971.

24. KRISNA, P. and BAID, I. C. Experimental Studies in the Population of Artemia salina L. Proceedings of the National Academy of Science, U.S. 28:416, 1958.

25. COLE, G. A. and BROWN, R. J. Chemistry of Artemia Habitat. Ecology, 48(5):858, 1967.

26. WICKINS, J. F. Food Value of Artemia to Palaemon serratus Larvae. Journal of Experimental Biology and Ecology, 10:151, 1972.

27. BORGSTROM, G. ed. Fish as Food II, Academic Press, New York. 1962.

28. SPARRE, T. Chapter 13 from Fish as Food III. Borgstrom. Academic Press, New York, 1962.

29. National Academy of Science. Evaluation of Protein Quality, Publication 1100. U.S. Government Printing, Washington, D. C. 1963.

30. Association of Official Analytical Chemists. Official Methods of Analysis of the Association of Official Analytical Chemists. ed. Wm. Horwitz. Association of Official Analytical Chemists, Washington, D.C., 1970.

31. BENDER, A. E., KIHLBERG, R., LOFQUIST, B. and MUNCK, L. Evaluation of Novel Protein Products. Pergamon Press, New York, 1970.

32. LUCKEY, T. P. Germfree Life and Gnotobiology. Academic Press, New York, 1963.

33. COATES, M. ed. The Germfree Animal in Research. Academic Press, New York, 1968.

34. MIAKAWA, M. and LUCKEY, T. D. ed. Advances in Germfree Research and Gnotobiology. C. R. C. Press, Cleveland, Ohio. 1968.

35. GORDON, H. A. and PESTI, L. The Gnotobiotic Animal as a Tool in the Study of Host and Microbial Relationships. Bacteriological Reviews, 35:390, 1971.

36. MIRAND, E. A. and BACK, N. ed. Germfree Biology Experimental and Clinical Aspects. Plenum Press, New York, 1969.

37. National Academy of Science. Nutrient Requirement of Laboratory Animals, #10. U. S. Government Printing Office, Washington, D. C., 1972.

38. OACE, S. A Purified Soy Protein Diet for Nutrition Studies with Germfree Rats. Laboratory Animal Science, 22(4):528, 1972.

39. WOSTMANN, B. S. and KELLOG, T. F. Purified Starch-Casein Diet for Nutrition Research with Germfree Rats. Laboratory Animal Care, 17(6), 1967.

40. THOMPSON, G. R. and TREXLER, P. C. Gastro Intestinal Structure and Function in Germfree or Gnotobiotic Animals. Gut, 12:230, 1971.

41. COMBE, E. Nitrogen Metabolism in Germfree Rats. International Conference on Gnotobiology, 1972.

42. TESHIMA, S. and KANAZAWA, A. Utilization and Biosynthesis of Sterols in Artemia salina. Bulletin of the Japanese Society of Scientific Fisheries, 37(8):720, 1971.

43. KRINSKY, N. I. The Carotinoids of the Brine Shrimp Artemia salina. Comparative Biochemistry and Physiology, 16:181, 1965.

44. PETRACEK, F. J. and ZECHMEISTER, L. Reaction of β-Carotene with N-Bromosuccinimide: The Formation and Conversion of Some Polyene Ketones. Journal of the American Chemical Society, 78:1472, 1956.

45. FISHER, R. L., KON, S. K. and THOMPSON, S. Y. Vitamin A and Carotinoids in Certain Invertebrates I. Marine Crustacea. Journal of Marine Biological Association, 31:229, 1952.

46. MEAD, C. G. and FINEMORE, F. J. The Occurrence of Ascorbic Acid Sulfate in the Brine Shrimp. Biochemistry, 8(6), 2653, 1969.

47. Pesticide and Toxic Substance Effects Laboratory. Pesticide Reference Standards and Supplemental Data, Environmental Protection Agency, North Carolina, 1973.

48. BRADSTREET, R. B. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York, 1965.

49. GILES, C. H., HASSAN, A. S. A., LAEDLOW, M. and SUBRAMANIAN, R. B. R. Adsorption at Organic Surfaces. III. Some Observations on the Constitution of Chitin and on Its Absorption of Inorganic and Organic Acids from Aqueous Solutions. Society of Dyers and Colorists Journal, 74:674, 1968.

50. MICKELSEN, O. and ANDERSON, A. A. A Method for Preparing Intact Animals for Carcass Analysis. Journal of Laboratory Clinical Medicine, 53:282, 1959.

51. WARNER, A. H. and MCCLEAN, D. K. Studies on the Biosynthesis and Role of Diguanosine tetraphosphate During Growth and Development of Artemia salina. Developmental Biology, 18:278, 1968.

52. KIRK, R. E. Experimental Design: Procedures for the Behavioral Sciences. Wadsworth Publishing Company, Inc., Belmont, California, 1968, p. 79.

53. MAKDANI, D. D., HUBER, J. T. and BERGEN, W. G. Effect of histidine and methionine supplementation on the nutritional quality of commercially prepared fish protein concentrate in rat diets. Journal of Nutrition 101:367, 1971.

54. FREED, M. ed. Methods of Vitamin Assay. 3rd ed. Interscience Publishers, New York, 1966.

55. LEVEILLE, G. A. Modified Thiochrome Procedure for the Determination of Urinary Thiamin. American Journal of Clinical Nutrition, 25:273, 1972.

56. Technicon Instruments Corporation. Riboflavin Vitamin B. in Food Products, Autoanalyzer II Industrial Method #140, p. 71. Continuous Flow Analyses Reference Library, Tarrytown, New York, p. 2684, 1971.

57. THOMPSON, J. N., ERDODY, P., MAXWELL, W. B. and MURRAY, T. K. Fluorometric Determination of Vitamin A in Dairy Products. Journal of Dairy Science, 55(8):1077, 1972.

58. PETRACEK, F. J. and ZECHMEISTER, L. Structure of Canthazanthin. Archives of Biochemistry and Biophysics, 61:137, 1956.

59. Association of Official Analytical Chemists. Official Methods of Analysis of the Association of Official Analytical Chemists. ed. Wm. Horwitz. 7.073(6) Association of Official Analytical Chemists, Washington, D.C., 1970.

60. GOMORRI, G. A Modification of the Colorimetric Phosphorus Determination for use with the Photoelectric Coloremeter. Journal of Laboratory Clinical Medicine, 27:955, 1942.

61. National Research Council. Nutrient Requirement of Laboratory Animals, #10, 2nd ed., 1972.

62. WATT, B. K. and MERRILL, A. L. Composition of Foods. Agricultural Handbook No. 8. United States Government Printing Office, Washington, D.C., 1963.

63. WOHL, M. G. and GOODHART, R. S. Modern Nutrition in Health and Disease. Lea Febiger, Philadelphia, PA., 1968.

TABLE 1A. Twenty-eight day weight gain data for male Sprague-Dawley rats.

Dietary Protein Source	Average Daily Gain		
	Conventional*	Germfree**	X-Germfree**
Casein			
5	0.57	----	----
10	4.07	3.03	2.63
15	6.43	----	----
20	7.31	4.92	3.80
Soy			
5	0.11	----	----
10	4.06	2.80	1.16
20	6.57	4.25	3.10
Brine Cyst + Casein			
5 + 5	2.03	----	----
10 + 10	6.71	3.72	3.71
Brine Cyst + Soy			
5 + 5	1.85	----	----
10 + 10	5.52	2.90	3.12
Brine Cyst Alone			
10	.57	----	----
Ground BSC + Casein			
10 + 10%	7.17		
20% C	6.80		

* Five rats per group.

** Four rats per group.

TABLE 2A. Twenty-eight day average daily feed intake for male Sprague-Dawley rats.

Dietary Protein Source	ADI Conventional*	ADI Germfree**	ADI X-Germfree**
Casein			
5	5.87	-----	-----
10	12.50	10.82	10.89
15	16.16	-----	-----
20	17.25	13.23	11.99
Soy			
5	4.63	-----	-----
10	12.05	9.42	10.02
20	16.47	13.57	12.07
Brine Cyst + Casein			
5 + 5	8.47	-----	-----
10 + 10	17.25	11.94	13.59
Brine Cyst + Soy			
5 + 5	12.43	-----	-----
10 + 10	12.22	9.81	11.34
Brine Cyst Alone			
10	5.48	Died	Died
Ground BSC + Casein			
10 + 10	16.84		
20% Casein	16.95		

* Five rats per group.

** Four rats per group.

TABLE 3A. Average daily feces for twenty-eight day period for male Sprague-Dawley rats.

Dietary Protein Source	Conventional*	Germfree**	X-Germfree**
Casein			
5	0.6	----	----
10	1.70	1.00	1.04
15	3.28	----	----
20	4.31	2.19	2.01
Soy			
5	0.29	----	----
10	1.63	.93	.873
20	3.07	2.36	1.88
Brine Cyst + Casein			
5 + 5	1.27	----	----
10 + 10	6.63	2.21	2.61
Brine Cyst + Soy			
5 + 5	0.76	----	----
10 + 10	2.71	1.87	2.27
Brine Cyst Alone			
10	.813	----	----
Ground BSC + Casein			
10 + 10%	2.91		
20% Casein	2.97		

* Five rats per group.

** Four rats per group.

TABLE 4A. Statistical comparisons of rat growth data using Bonferoni t-statistic. α level: + = .05
++ = .01
+++ = .005

I. Conventional Rats

Number	Comparison	Wt. Gain	PER df 40	NPU df 40
1	5% C vs. 10% BSC	-	+++	+++
2	5% C vs. 5% C + 5% BSC	+++	-	+
3	10% C vs. 5% C + 5% BSC	+++	+++	-
4	10% C vs. 10% C + 10% BSC	+++	+++	+++
5	10% C vs. 10% BSC	+++	+++	+++
6	15% C vs. 10% C + 10% BSC	-	+	-
7	20% C vs. 10% C + 10% BSC	-	-	-
8	5% C + 5% BSC vs. 10 BSC	+++	+++	+++
9	10% S vs. 10% BSC	+++	+++	+++
10	10% S vs. 5% S + 5% BSC	+++	+++	+++
11	10% S vs. 10% S + 10% BSC	+++	-	-
12	20% S vs. 10% S + 10% BSC	+++	-	-
13	5% S + 5% BSC vs. 10% BSC	+++	-	-
14	10% S + 10% BSC vs. 10% C + 10% BSC	+++	++	-
15	5% S + 5% BSC vs. 5% C + 5% BSC	-	-	+++

TABLE 4A. (Cont'd)

II. Germfree Rats

Number	Comparison	Wt. Gain df 44	PER df 40	NPU df 40
1	10% C vs. 10% + 10% C	-	+++	-
2	20% C vs. 10% + 10% C	-*	-	-
3	X10% C vs. 10% C	-	-	-
4	X20% C vs. 20% C	-	-	-
5	X10% + 10% C vs. 10% + 10% C	-	-	-
6	X10% C vs. 10% + 10% C	-	+++	-
7	X20% C vs. 10% + 10% C	-	-	-
8	X10% S vs. 10% S	++	+++	-
9	10% S vs. 10% + 10% S	-	+++	-
10	X20% S vs. 20% S	-*	-	-
11	20% S vs. 10% + 10% S	+	-	-
12	X10% + 10% S vs. 10% + 10% S	-	-	-
13	X10% S vs. X10% + 10% S	+++	-	-
14	X20% S vs. X10% + 10% S	-	-	-
15	10% C vs. 10% S	-	-	-
16	X10% C vs. X10% S	+	+++	
17	20% S vs. 20% C	-	-	
18	X20% S vs. X20% C	-	-	
19	10% C vs. 20% C	+++	++	
20	10% S vs. 20% S	+	+++	
21	X10% C vs. X20% C	-*	+	
22	X10% S vs. X20% S	+++	-	

* Approached significance

TABLE 5A. Equipment used in germfree isolators.

1. Individual stainless steel cages
2. Fiberglass cafeteria trays (two per cage; one to catch feces, and one as a lid to retain the rat)
3. Food cups and retainers to decrease spillage (one per rat)
4. Water bottles (one per rat)
5. Large storage bottles of sterile water
6. Cloth gloves
7. Triple-beam balance and containers to restrain rat
8. Tongs
9. Paper bags
10. Rubber bands
11. Paper towels
12. Cloth towel
13. Pencil (irradiated)
14. Food packets (irradiated), (one per rat)
15. Culture tubes with sterile swabs

TABLE 6A. Content of vitamin diet fortification mix.*

Vitamin	g/K Fortification Mix
A (200,000 IU/g)	4.5
D (400,000 IU/g)	0.25
E (a-tocopherol)	5.0
C	45.0
Inositol	5.0
Choline Chloride	75.0
Menadione	2.25
P-aminobenzoic acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridonine HCl	1.0
Thiamin HCl	1.0
Calcium pantothenate	3.0
Biotin	0.020
Folic acid	0.090
B-12	0.00135

* Wesson Modified Osborne Mendel Salt Mix #170900
General Biochemicals, Chagrin Falls

TABLE 7A. Content of Wesson Osborne Mendel Salt mix.

Mineral	Present Composition
Calcium carbonate	21
Calcium phosphate tribasic	14
Cupric sulfate	0.039
Ferric phosphate	1.47
Magnesium sulfate	9
Manganese sulfate	0.020
Potassium aluminum sulfate	0.009
Potassium chloride	12
Potassium iodide	0.005
Potassium phosphate monobasic	31
Sodium Chloride	10.5
Sodium fluoride	0.057