OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

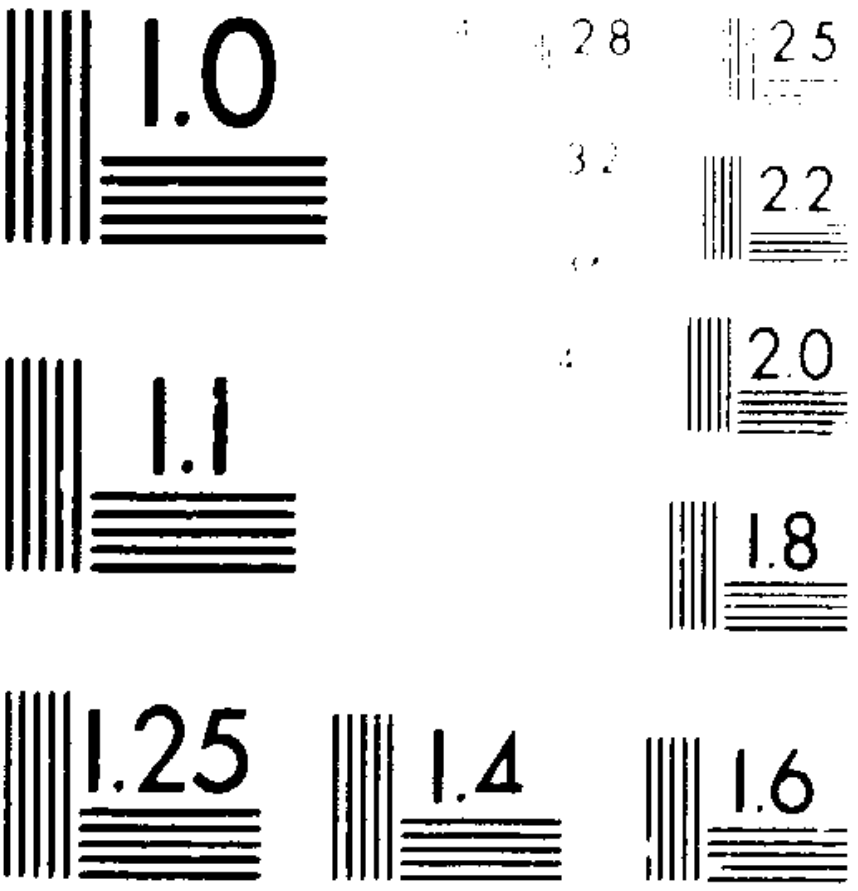
FAIR USE POLICY

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org



14564

1985

MICROBIAL MODIFICATIONS OF CASSAVA: TRADITIONAL AND EXPERIMENTAL PROCESSES

by

K.F. Gregory

Department of Microbiology
University of Guelph
Guelph, Ontario, Canada

Cassava (Manihot esculenta Crantz) is a major root crop in the low-land tropics. It is commonly called manioc in French-speaking countries, mandioca in Brazil, yuca in Spanish-speaking regions of South America, tapioca in much of Southeast Asia, and cassava in most English-speaking areas. Various species names have been applied to diverse cultivars and the epithet M. utilissima Pohl is still used by some authors to designate cultivars with high concentrations of HCN and cyanogenic glucosides (usually the 'bitter' cultivars). Since there is a continuous gradient of cultivars with varying cyanide concentrations, however, and there are no morphological features which correlate with cyanogenic glucoside concentrations (Rogers, 1965), most authorities prefer the use of the single species name, M. esculenta. The plant is believed to have originated in Central America or nothern South America (Renvoize, 1972). It was introduced to West Africa by Portuguese traders in the 16th century, to India in the 17th century, and to East Africa in the 18th century (reviewed by Lancaster et al., 1982). Subsequently it spread throughout the tropics.

Annual world production of cassava in 1982 was estimated at 129 million metric tons (FAO, 1983) with the greatest production occurring in Brazil, Thailand, Indonesia, Zaire and Nigeria. Most of the cassava grown in Thailand and Indonesia is exported as an animal feed, while most of that grown in Africa is consumed directly by humans. Cassava is an important dietary staple for 450 to 500 million people in 26 tropical countries and an estimated 50 million people in Africa derive

from E. Wilm

more than 500 kcal per day from this crop (Cock, 1982). About 65% of the total cassava production from 1975 to 1977 was used for direct human consumption (FAO, 1980). Of this total, about one half was processed in various ways, including microbiological fermentations. Many of the processing techniques used were developed by the Amerindians of South America and subsequently modified in various ways in various regions. Some of these techniques did not immediately follow the geographical spread of cassava, however, and it was not until settlers from Brazil came to West Africa, after 1800, that the preparation of gari became common and contributed to the popularity of cassava in Africa (Lancaster et al., 1982). The traditional techniques for processing cassava into foods for human consumption have been comprehensively reviewed by Lancaster et al., (1982), and Lancaster and Coursey (1984).

Cassava processing techniques are designed both to preserve cassava and to detoxify it. Cassava begins to become stale and undergo discolouration within about three days after harvesting, if it is not processed in some way (Oke, 1983). The cause and nature of cassava toxicity are described in an excellent review by Bourdoux et al. (1980). Both the leaves and the roots contain glucosides which liberate hydrogen cyanide in two enzymatic steps. The principal cyanogenic glucoside is linamarin (about 95% of the total), while the remainder is lotaustralin. The tissues also contain the enzymes which catalyze these reactions. Linimarase, which causes the initial hydrolysis, is presumed to be rate limiting, and any process which ruptures the cell wall brings this enzyme in contact with its substrate, thereby initiating the liberation of HCN. After ingestion, HCN is detoxified by reactions with thiosulphate or L-cysteine which yield thiocyanate. Hence a combination of high cyanide intake with a low protein intake (especially if the proteins are low in the sulfur-containing amino acids) can lead to cyanide toxicity, sometimes manifested as a syndrome called tropical ataxic neuropathy. The

thiocyanate ion, resulting from the detoxification of cyanide, behaves similarly to iodide and competes with iodide for uptake by the thyroid gland. At higher concentrations it also inhibits the incorporation of iodine into thyroglobulin. For these reasons, people on a high cassava, low iodide diet are prone to endemic goitre unless the cassava has been adequately processed.

The HCN released from cassava, after the root has been shredded or grated, probably exerts a selective effect on the microflora responsible for the various fermentations of cassava. It was observed that cassava roots rapidly developed toxicity, after grinding, for filamentous fungi being used for single-cell protein production, unless the pulp was heated immediately after grinding (Reade and Gregory, 1975; Mikami et al., 1982). The heating was presumed to inactivate linimarase and prevent the release of cyanide. Linimarase decomposes at 72 C. (Joachim and Panditteseker e, 1944).

The cassava root is extremely low in protein. An average cassava variety contains only 3.5% crude protein (total N x 6.25) (Grace, 1971) of which only 40-60% is true protein (Pond and Maner, 1974). Many common cultivars contain about 1% protein. Clearly, any diet based heavily upon cassava must contain enough protein-rich foods or protein deficiency diseases could develop. Any process which would increase the protein content of cassava-based foods could be of great importance.

Traditional Fermented Foods from Cassava

Gari

The most commonly used cassava product in West Africa is a fermentation product, of grated cassava, called gari. It is particularly popular in Nigeria but it is also of considerable importance in Ghana, Cameroon, Sierre Leone, Guinea, Benin and Togo (Lancaster et al., 1982). Methods for preparing gari vary considerably from place to place. The following description is adapted from those given by Lancaster et al. (1982) and Oke (1983).

To make gari, cassava roots are washed, peeled (with a loss of about 13-20% of their substance), and shredded. The pulp is then placed in cloth bags or sacks made from jute. It is then left to ferment for about 4 days (the time may vary from 3 to 10 days). During the fermentation, pressure is applied to squeeze excess liquid out of the fermenting pulp. This is commonly done by placing heavy stones or logs on top of the sacks or by tying the necks of the sacks around strong sticks and twisting the sticks so as to tighten the sacks. In another procedure the pulp is pressed between two boards. Ngaba and Lee (1979) described a procedure used in Cameroon in which 3 or 4 poles are bound tightly in parallel around the sack. The extruded juice, which contains much of the cyanogenic glucoside, is discarded. The fermented pulp is removed from the sacks, sieved to remove any fibrous material, dried, and heated or 'garified' in shallow iron pans, at about 80-85 C to make white gari, while being stirred continuously until it becomes light and crisp. Yellow gari is made by frying in a pan with palm oil. The product is a free-flowing, granular meal with a faint sour odour. A finer gari is produced by sieving the product to produce very fine particles. Good quality gari should swell to over three times its own dry volume in hot water. For safe storage, gari must contain less than 12% moisture.

Contradictory reports have been published concerning the microorganisms

involved in the gari fermentation. Collard and Levi (1959) concluded that the fermentation proceeded in two stages. They isolated a Corynebacterium species, from the first stage, that they named Corynebacterium manihot. It was able to ferment starch with the production of organic acids. After the first two days the bacterium was replaced by Geotrichum candidum which they believed produced a variety of aldehydes and esters responsible for the characteristic taste and aroma of the gari. They were able to shorten the fermentation period to 24 h by using a starter culture of these two organisms. Akinrele (1964) noted a biphasic rise in temperature during the fermentation of cassava pulp, which he attributed to the two-stage fermentation described by Collard and Levi (1959) but he did not culture the microorganisms involved. Several later investigators were unable to isolate Corynebacterium sp. in appreciable quantitites from fermenting cassava (Okafor, 1977; Abe and Lindsay, 1978; Ngaba and Lee, 1979; and Vinck, 1982). It is interesting to note, however, that Whitby (1968) found an amylolytic Corynebacterium sp. present in the initial stages of maize fermentations leading to the production of banku, kenkey, and akpler in Africa. Okafor (1977) considered Leuconostoc spp. to be important in the gari fermentation, while Abe and Lindsay (1978) and Vinck (1982) concluded that Streptococcus spp., especially S. faecium, played a dominant role. Ngaba and Lee (1979) studied the microflora in traditionally fermented cassava, as well as in cassava fermented in the laboratory, and found that several isolates of Lactobacillus plantarum, L. buchneri, and cocci resembling Leuconostoc spp., were able to produce the gari flavour. At the 48 h time period of the laboratory fermentation, only Lactobacillus spp. and Streptococcus spp. were found and the authors concluded that these species were primarily responsible for the acid production and gari flavour development.

Although most of the above studies did not reveal the presence of large numbers of yeasts and molds in freshly prepared gari, Adenkule and Ayeni (1974),

while studying the occurrence of mycotoxic flora in some Nigerian foods, found that gari was among the most highly contaminated foods. Many of the isolated species, in the genera Aspergillus, Cladosporium and Penicillium, were shown to have toxigenic potential in Wistar rats. Lewis and Aderoji (1978) found that peppers and cassava preparations in the form of 'eba' and 'gari' are potent factors in the etiology of duodenal ulcers in Ibadan, Nigeria. It is not clear whether the cassava itself, the fermentation process, or the presence of contaminating microorganisms is responsible for this effect.

The conclusion to be drawn from the above reports appears to be that the gari fermentation is quite variable. This is not surprising in view of the variations occurring in the cassava roots and in the physical conditions of the fermentation (temperature, moisture content, and degree of anaerobiosis). It seems likely that a higher quality, safer and more uniform product could be produced more quickly if carefully selected starter cultures were used as an inoculum and the fermentation conditions and subsequent roasting conditions were accurately controlled. Meuser and Smolnek (1980) developed a process for mechanizing the prodution of gari under controlled conditions but the process utilized the indigenous microflora and no microbiological studies were reported.

One of the purposes of fermenting cassava, prior to its consumption, is to detoxify it by eliminating hydrogen cyanide and the cyanogenic glucosides found in the roots. Considerable cyanide may, nevertheless, remain in the gari. Olarewaju and Boszormenyi (1975) determined the cyanide content of 12 commerical gari samples collected at the Ife town market. Eleven of the 12 samples contained cyanide. The white samples had HCN contents ranging from 19.7 to 27.9 mg/kg dry weight; the yellow samples had from 0.00 to 1.98 mg/kg; while the slightly coloured samples had intermediate values. The authors attributed the low cyanide content of yellow gari to its different roasting in palm oil.

It has often been assumed that the linamarin in cassava hydrolyzed spontaneously as gari became acid during fermentation (Collard and Levi, 1959). Ikediobi and Onyike (1982a), however, showed that the release of HCN decreased as the pH dropped below 6.0 (the optimum for linimarase). The addition of exogenous linimarase greatly accelerated the rate of detoxification and consistently yielded gari with 'innocuous' levels of cyanide. These authors identified two fungi capable of producing the enzyme in commercial quantities (Ikediobi and Onyike (1982b). Ejiofor and Okafor (1984) identified several bacteria and yeasts capable of breaking down linamarin and tested four of these as inocula in fresh cassava pulp. Over twice as much HCN was released, within 72 h, from the inoculated pulp than from the noninoculated pulp, leading the authors to suggest that these organisms could be used in comercial detoxification of pulp during the production of gari. These data indicate, as suggested previously by Olarewaju and Boszormenyi (1975), that 'the fermentation step in the gari processing step is not so important from the point of view of detoxification as it is generally supposed.' It appears that in traditional gari production, the plant's autogenous enzymes are responsible for most of the conversion of cyanogenic glucosides to HCN, some of the HCN is lost by volatilization during the fermentation (which is favoured by a pH low enough to keep the cyanide present in the volatile HCN form), and most of the remaining HCN is driven off during the roasting process.

Linamarin was found to be toxic when fed to rats but the effects appeared to be due to cyanide released from the glucoside (Hill, 1977). The site of hydrolysis has not been established but Fomunyam et al. (1984) showed that common intestinal bacteria are able to hydrolyze linamarin.

The fermentation process used to produce gari does not appear to improve the nutritional value of cassava. The protein content of cassava, already extremely low, was found to be decreased by 36% (Favier et al., 1971) to 45% (Ezeala, 1984)

during the production of gari. The chemical nutritional score of the protein was decreased by about 3% and the biological value by about 2% (Ezeala, 1984). Okezie and Kosikowski (1981a), recognizing the potential protein deficiency resulting from diets containing large amounts of cassava, produced a food similar to gari but containing 11-12% protein, by fermenting a mixture of cassava, acid whey powder, and soybean concentrate. Several vitamins and minerals were also found to be present in lower concentrations in gari than in the raw peeled root (Favier et al., 1971). The fermentation adds acids and flavouring components, however, which preserve the cassava and increase its palatability. Lactic acid was the principal acid found by several authors in gari (Akinrele, 1964; Dougan et al., 1983). Dougan et al. (1983) found small amounts of oxalic acid (0.04%) in gari and detected alkylpyrazines which had aromas typical of gari.

Other fermented foods from cassava

There is a wide variety of other traditional foods produced from cassava by fermentation. Many of these are produced by variations on the gari process but have been less well studied. These will only be reviewed briefly. Further details are given by Djien (1982), Grace (1971), Lancaster et al., (1982) and Lancaster and Coursey (1984).

Farinha de mandioca (also called farinha de mesa, farinha seca, and farinha suruhy) is produced in Brazil by a process similar to that used with gari except that the fermentation time is shorter -- just overnight (Grace, 1971; Lancaster et al., 1982). Alternatively, the pulp may be mixed with a small amount of pulp which has been left to ferment for three days. The pulp is sometimes squeezed with a screw-press but traditionally this is done by a basket-work cylinder known as a tipiti. The pulp is sieved to remove coarse fibers, then cooked on a hot clay or stone griddle.

Attieke, popular in the Ivory Coast, is a fermented paste prepared from

cassava (Lancaster et al., 1982). The process again resembles that used with gari but the pulp is more finely ground and the fermentation is limited to two days. The fermented paste is crumbled by hand, steamed, and consumed with milk or with meat and vegetables.

In Zambia, cassava roots (soaked or unsoaked according to taste) are sometimes mixed with a fermentation starter called kapapa, consisting of partially dried cassava slices that have developed a coating of mould. The mixture is ground together, fermented 1-6 days, sun-dried, and pounded again into a flour.

The term fufu, also foofoo, fuifui, foutou and vou-vou, is used to refer to a wide variety of pastes prepared from cassava and other starchy foods. These foods may or may not have undegone a fermentation process. For example, one type of fufu is prepared from gari.

Other variations on cassava fermentations occur in various localities. For example, the author visited several small starch factories in Colombia in which some of the starch, intended for human consumption, was fermented. Following extraction from the rasped roots and purification by settling in large vats, the starch was transferred to a smaller vat and allowed to ferment for a day or two before being pulverized and air dried. The fermentation was said to impart a desirable flavour.

Other fermented starchy foods

Maize products. At least 20 different fermented maize products are found in Africa (Djien, 1982). These are self-inoculated, mostly bacterial, fermentations not unlike that of gari. A wide variety of fermented foods is produced in Oriental countries where soybeans and filamentous fungi predominate (Wang and Hesseltine, 1979; Hesseltine and Wang, 1980). This starting material is not equivalent to the high starch -- low protein substrate provided by cassava but these foods are mentioned here since they illustrate the acceptability of many filamentous fungi for

human nutrition. Species in the genera Aspergillus, Mucor, Rhizopus and Neurospora are common in foods such as miso (Japan), sufu (China), tempeh (Indonesia) and ontjom (Indonesia), respectively. This is in marked contrast to Occidental countries where Penicillum species are the only filamentous fungi used to modify foods (roquefort and camembert types of cheese). The moulds are used in conjunction with yeasts and bacteria in some of these foods. In fact, yeasts are widely used around the world in fermented foods and as a leavening agent in bakery products. On a global basis, there is probably less consumer resistance to yeasts in food than to any other type of microorganism.

Experimental Fermented Foods and Single-cell Protein from Cassava

Many studies have been published on the growth of microorganisms on cassava plus inorganic forms of nitrogen for the synthesis of protein. Most of these studies were designed to produce a low cost, protein-enriched feed for domestic livestock and only a few were designed to produce human food. The reasons for this are the much more extensive safety evaluations required for a novel human food, the problems resulting in human nutrition from the high nucleic acid levels found in microbial cells, and the perceived problem in gaining public acceptance of novel foods. Each of these reasons is discussed briefly below.

The Protein-Calorie Advisory Group (PAG), now disbanded, of the United Nations system, issued guidelines for preclinical testing of novel sources of protein, human testing of supplementary food mixtures, and the production of single-cell protein for human consumption (Protein Advisory Group, 1976). No attempt will be made to review these here. Since these guidelines involve quite extensive testing procedures (for example, they suggest multigeneration animal feeding trials), targeting a product for domestic animals, in the first instance, has been favoured.

The high nucleic acid levels in microbial cells are an inescapable consequence

of the rapid protein-synthesizing ability of these organisms since ribosomes, the cell's protein-synthesizing machinery, contain about 50-60% ribonucleic acid. During the metabolism of nucleic acids in the body, the purines become converted to uric acid. This does not pose a problem for most animals since they possess the enzyme uricase. This enzyme converts uric acid in the blood plasma to the more soluble allantoin which is readily excreted by the kidneys (see review by Kihlberg, 1972). Human beings, and some other primates, however, lack uricase so that the level of uric acid in the blood may rise until uric acid and its salts (chiefly calcium urate) can form crystals in the kidney (kidney stones or renal calculi) or in the joints, where they cause a painful inflammatory condition called gout. The Protein Advisory Group (1976) proposed a maximum consumption of 2 g of nucleic acid per day, in the form of single-cell protein, as a safe practical limit for most adult populations. The total nucleic acid ingestion from all sources should not exceed 4 g per day. Whereas this restriction severely limits the amount of untreated microbial cells which can be incorporated into diets, several procedures have been devised for reducing the levels of RNA (see review by Solomons, 1983). The cheapest, most desirable procedure for lowering the RNA content of cells is a heat-shock process first described by Maul et al. (1970) for yeast. The technique has since been adapted to other microorganisms including the filamentous fungus Fusarium graminearum, which has been given U.K. Government clearance to be sold as a human food (Solomons, 1983).

There have been several excellent general reviews published on single-cell protein (Kihlberg, 1972; Litchfield, 1983; Solomons, 1983; Steinkraus et al., 1980; Tannenbaun and Wang, 1975). Some studies which have specifically concerned cassava will be considered here. The semi-solid processes will be considered first since they most closely resemble the traditional fermentation processes. The low moisture, so-called 'solid-fermentation' processes, will then be discussed and finally

the various liquid culture, aerated fermentor systems will be considered. The protein contents of the products are progressively higher as we move through this sequence.

Semi-solid Fermentations

If the protein in gari is assumed to be totally microbial protein (which is unlikely to be the case) and the cells are assumed to be 50% protein (probably an underestimate for bacterial cells), the maximum mass of microbial cells in the gari studies by Favier et al. (1971) (1.25% protein) was 2.5% on a dry weight basis. An increase in the level of microbial protein in fermented cassava could only be possible if additional nitrogen were available, either from a nitrogenous compound or by biological nitrogen fixation. The effect of adding supplemental nitrogenous compounds to an anaerobic cassava fermentation was investigated by W. Sprung and R.E. Smith (see Gregory, 1977) who found that the addition of mineral salts and ammonium nitrate actually decreased the protein content of a 'cassava silage' to 0.76% from 1.08%. If the cassava pulp were first heat sterilized and inoculated with a Rhizopus oligosporus culture, however, the protein content increased to 3.65%. When the ground cassava was spread in thin layers, inoculated with R. oligosporus and discontinuously perfused with recycled nutrient solution, the protein content was raised to 6.44%. Under these conditions, however, a low pH, such as found in gari, was not produced. It was concluded that the nearer the conditions approached a liquid, aerated system, the higher the protein content.

A process modelled on the Indonesian processes for tempeh and ontjom, was developed at the Tropical Products Institute in London (Brook et al., 1969; Stanton and Wallbridge, 1969 and 1972). In this process the roots were peeled, dried, ground to a flour, and pasteurized for 18 h at 70 C. Spores of several fungal species (Rhizopus, Mucor, Actinomucor or Monilia) were added with ammonium nitrate, monopotassium phosphate, and water. The paste-like mixture was extruded

into spaghetti-like strands, placed in shallow trays, and allowed to incubate for three days at 30 C. Rhizopus stolonifera appeared to give the best results. Increases in protein concentrations from as low as 0.2% to as high as 4% were reported. Although this represented a several fold increase in protein, the product was still a low protein food. No nutritional, toxicological or palatability tests appear to have been published.

Trevelyan (1974) also studied the use of Rhizopus oryzae in a tempeh-like system for producing protein-enriched cassava. He achieved a maximum protein content of 4.3% but found the process susceptible to contamination by fungi and bacteria, and he criticized the potential of the process for use in a village-level technology, on several grounds. These included the fact that the protein level remained low, that nonassimilated ammonium salts, etc. could not be left to remain in the product, and that the distribution of inoculum and nitrogenous compounds to dispersed villages would be difficult. Vanneste (1982) recently rep[illegible]chieving protein contents from 12.8% to 17.5% from cassava inoculated with [illegible]es of fungi and grown in fifty-liter rotating vessels.

Low-moisture fermentations

A major factor limiting the growth of fungi, in the semi-solid processes described above, is that the penetration of oxygen into the substrate is insufficient to support maximum growth of these aerobic organisms. This problem was partly overcome by the development of 'solid fermentations' in which the moisture content of the cassava substrate is kept so low that air can readily permeate through the granulated substrate (Senez et al., 1980; Raimbault and Alazard, 1980; Raimbault, 1981). The process developed at the Office de la Recherche Scientifique et Technique Outre-mer (ORSTOM), utilizes a fungus called Aspergillus hennebergii (A. niger group). The procedure requires a very heavy inoculation of spores, homogeneously distributed with mineral salts, throughout the starchy substrate

which has been prepared in a porous, granulated form. Briefly the coarsely ground raw material, containing 30-35% moisture, is heated to 70-80 C to gelatinize the starch. After cooling, the preparation is mixed with an aqueous spore suspension and the nitrogenous and phosphate salts, then brought to a moisture content of 55%. Mechanical stirring results in uniform granules. The mixture can be prepared and incubated (at 35-40 C, pH 3.5-4.5, about 30 h) in a modified commercial bread-making blender. Air is introduced through perforations at the bottom of the tank. The pH, moisture and temperature are automatically controlled by probes that activate a spray with water or mineral solutions. The fungus invades the cassava granules, hydrolyzing the starch and largely replacing the starch granules with fungal biomass granules. The mixture is then dried to yield a final product which contains 18-20% true protein (Lowry method) and 25-30% residual carbohydrate.

The ORSTOM process has been studied in our laboratory with thermotolerant fungi (R.E. Smith, C. Eknamkul and P. Bicho, unpublished data). Cephalosporium eichhorniae 152, a fungus found to give good results in SCP production from cassava in liquid cultures, was unable to grow at the high salt concentrations required for this process. Another thermotolerant fungus, Sporotrichum pulverulentum 141, was found to grow well and produce a product containing up to 36.7% protein after incubation at 45 C for 50 h. The protein was found to contain 3.3% methionine, which is unusually high since most sources of SCP are deficient in this amino acid. The process with S. pulverulentum 141 has not been scaled up to a pilot plant scale nor have nutritional and toxicological evaluations been performed. The fungus was not observed to cause any harmful effects when inoculated intranasally into immune-impaired (gamma-irradiated) rats and mice.

The ORSTOM type of process has several noteworthy advantages over other procedures for producing protein-enriched cassava. The fermentation is not readily

contaminated because of the low pH and the high osmotic pressure. A dewatering procedure is not required prior to final drying. Finally, there is no effluent from the process, such as encountered with liquid culture systems, which represents both a waste of nutrients and a source of environmental pollution. A major disadvantage of this procedure for the production of human food, however, is that there is no obvious way by which the nucleic acid content of the product could be reduced. Any nucleotides released from the cells, after a heat-shock process, for example, would remain in the final product.

Liquid Culture Systems

Yeasts have been more widely used for the production of single-cell protein in aerated fermentors than any other type of microorganism, so it is not surprising that several processes have been described for their production from cassava. Most yeasts are not able to hydrolyze starch, however, so that yeast-based processes usually include a procedure for hydrolyzing the starch prior to growth of the yeast. The hydrolysis can be effected by acid, added enzymes, or enzymes produced by a second microorganism. Strasser et al. (1970) used both acid and enzymatic hydrolysis to hydrolyze cassava starch before the growth of three species of yeast. The best culture, Candida utilis, yielded a product with 35% crude protein. They described a process in which soluble and insoluble portions of cassava were separated; the soluble portion hydrolyzed and used for yeast production, while the insoluble portion was allowed to ferment to form gari. Subsequently the two product streams were combined and dried. The enzymatic hydrolysis of starch followed by the growth of Candida utilis was the subject of a patent by Scott and Houghton (1976) and a process described by Musenge et al. (1980), which yielded biomass with 62.25% protein. Okezie and Kosikowski (1981b) carried these processes further by partially purifying the protein from mechanically and chemically disrupted cells of Candida tropicalis and Candida utilis grown on

hydrolyzed cassava. Their products contained 68-71% protein, and the purification process achieved a 75-80% reduction in the nucleic acid level.

The Adour Speichim process utilizes a strain of Candida tropicalis which has some amylase activity so that protein enrichment of cassava can be achieved without a separate hydrolysis step (Azoulay et al., 1980a; Revuz and Voisin, 1980). The commercial names Yucaprina (for Spanish and Portuguese speaking countries) and Yucapreen (English and French speaking countries) have been assigned to the product which contains about 21.3% crude protein (Azoulay et al., 1980b). Since the product is a yeast, the product has been recommended for human nutrition. Although the process has been claimed to be a 'revolution for the third world' (Azoulay et al., 1980b), there appear to be some problems associated with it. The amylase in the yeast is intracellular so that only 'soluble' starch can be utilized. This implies that an extensive treatment of the cassava starch would be required before a high yield of cells could be obtained. Azoulay et al. (1980a) noted that about 25% of the initial substrate remained unutilized and the yield was only 36 to 46% of the dry weight of the substrate. These values for substrate utilization, yield and protein content are all markedly inferior to those for many other processes which have been described.

Noparatnaraporn et al. (1983) studied the production of SCP from cassava starch by the photosynthetic bacterium Rhodopseudomonas gelatinosa. The bacterium was amylolytic. The author's recommended medium contained 2 g starch + 2 g soybean meal per liter. Since the proposed substrate concentration is an order of magnitude lower than that used in most SCP processes and the input of protein, as soybean meal, was equivalent in amount to a large proportion of the protein in the product, this approach does not seem to offer much promise.

Therkildsen (1980) studied the symbiotic growth of a bacterium (Bacillus subtilis NCIB 8646) and a yeast (Candida utilis ATCC 9256). The bacterium was an

active producer of alpha amylase and hydrolyzed cassava starch into glucose which was utilized by the yeast. The product consisted of 2-5% B. subtilis and contained 52% prot in.

A two-stage SCP process from cassava was studied by Opoku and Adoga (1980). A filamentous fungus, Trichoderma reesei, was grown on whole cassava medium to give 0.74 g dry cells/g cassava. The solid biomass was separated to yield a product with 42% protein, while the culture filtrate, which still contained an appreciable amount of glucose, was inoculated with a yeast and growth of the mixed culture permitted. The fermentation took ca. 48 h for completion but ultimately gave a very high yield (0.85 g/g cassava) and up to 51% protein.

A comparison of mixed culture growth (C. utilis and Endomycopsis fibuligera) with the growth of C. utilis following starch hydrolysis with commercial amyloglucosidase was carried out by Sawada et al. (1982). Their results indicated that the later process was economically more advantageous.

The use of filamentous fungi (moulds) for producing microbial protein from cassava has the advantages that: (1) these organisms are usually amylolytic; (2) they will grow at a low pH which inhibits most bacteria; (3) they can be cheaply harvested by filtration, and (4) they confer a texture to the product not found with unicellular organisms. They tend to have lower protein contents than yeast and bacteria, however, so that strains must be carefully selected for high protein content. Some moulds produce mycotoxins so that safety evaluations must be very carefully performed. Gray and Abou-El-Seoud (1966) first reported the aerated, liquid cultivation of filamentous fungi on cassava but their products only contained 13-14% crude protein. De Lamo and De Menezes (1979) found that Gliocladium deliquescens could reduce the organic matter poilution in the waste water from cassava processing plants by 80%, while producing a protein-rich product. Muindi and Hanssen (1981a, 1981b) produced microbial biomass from cassava with

Trichoderma harzianum. The product (grown on 4% cassava root meal + inorganic nitrogen at 23 C, pH 4.0-4.2 for 60 h) contained 38% crude protein on a dry weight basis.

Thermotolerant fungi have also been studied for the production of SCP from cassava (reviewed by Gregory, 1982). In addition to the advantages already listed for filamentous fungi, thermotolerant species have the additional advantages that: (1) they tend to grow faster than mesophilic species; (2) their high growth temperature reduces the cooling costs involved when a large mass of cells is grown in an aerated fermentor -- a particularly important consideration in tropical countries, and (3) the high growth temperature, in combination with the low pH, presents conditions which strongly resist the growth of contaminants. There are the additional disadvantages, however, that (1) the solubility of oxygen is less at high temperatures so that the rate of oxygen transfer to the growing cells tends to be rate limiting, and (2) some fungi capable of growing at body temperature (37 C), which includes most thermotolerant species, are capable of being 'opportunistic pathogens', so that cultures must be selected with this is mind.

Gregory et al. (1977b) screened 147 cultures of thermotolerant fungi for cultures which would grow well at pH 3.5 at temperatures of 45 C or higher, were amylolytic, and had over 44% crude protein in their mycelium. Rat-feeding trials were used to select those cultures which gave the highest protein efficiency ratios. The culture which scored highest, according to these criteria, was designated Cephalosporium eichhorniae 152 (ATCC 32722). The crude protein content of the mycelium was 49% but the true protein was 38%; because of the high content of nonprotein nitrogen (Mikami et al., 1982). The product harvested from cultures grown on whole cassave contained 41% crude protein and 31% true protein.

An intensive nutritional evaluation of C. eichhorniae 152, in comparison to the two next best cultures, confirmed that it was nutritionally best (Alexander et al.,

1979). Although the methionine content (at 1.9%) was higher than that of the other cultures examined, it was suboptimal for nutritional purposes. When methionine supplementation was used with all diets, however, the feeding tests showed that the weight gain, feed efficiency, and protein efficiency ratio were at least as good with C. eichhorniae 152 mycelium as the values obtained with a control diet based on casein. Muindi et al. (1981) conducted detailed nutritional evaluations of the biomass produced by C. eichhorniae 152 on cassava in comparison with cotton-seed cake and soybean meal. The results were not as good as those reported for the pure mycelium but methionine supplementation was not used. Biological Value and Net Protein Utilization estimates for the SCP-enriched cassava meal were similar to those obtained with cotton-seed cake but were lower than those obtained with soybean meal.

Kuo et al. (1979) did sub-chronic toxicity tests of C. eichhorniae 152 and another thermotolerant fungus. Analytical tests for six common mycotoxins were all negative. No differences were found between rats fed C. eichhorniae mycelium (at levels up to 40% of the diet) from rats fed a standard soybean-based diet except for slightly higher kidney weights and serum alkaline phosphatase levels in rats fed the microbial diets. Since these changes have previously been observed in rats fed high levels of protein in the form of yeast, bacterial or fungal cells, and negative findings were obtained in the other clinical, hematological and histological examinations, it was concluded that this fungus was not toxic to the rats in the 90 day feeding trials.

Mikami et al. (1982) showed that C. eichhorniae 152 is obligately acidophilic and is unable to grow at the pH values found in the tissues where rare opportunistic infections have been reported by species of the same genus. (There have been no reports infections by C. eichhorniae). Inoculations by various routes, of resting and germinated spores of this fungus into immune-impaired adult

mice (gamma-irradiated), new-born mice and one-week old chickens, failed to incite an infection. It was concluded that C. eichhorniae 152 did not pose a biohazard. (In earlier studies an asporogenous mutant of Aspergillus fumigatus I-21 had been used as a prototype culture for the use of thermotolerant fungi for producing microbial protein from cassava (Reade and Gregory, 1975). Since it was not possible to prove that this culture could not serve as an opportunistic pathogen, it was recommended (Gregory, 1971) that that culture not be used for practical biomass conversion processes.)

Using a least-cost formulation for the production of biomass from cassava by C. eichhorniae 152, Charonesiri and Gregory (unpublished data), found the cost of supplements per kg product to be approximately $US 0.05/kg. Considering the present market value of sobyean meal (about $US 0.21/kg), and bearing in mind that it takes about 2 kg of cassava meal to produce 1 kg of microbial biomass, it follows that the cost of cassava would have to be less than $U.S. 0.08/kg, even if all production costs were zero, in order to

produce biomass cheaper than soybean meal. Itappears unlikely that any process can produce microbial biomass from cassava at a cost that could compete economically with soybean meal, at the present time, as a protein-rich animal feed. Microbially-enriched cassava products could, however, rank among the cheapest forms of protein so that if the products were shown to be safe and attractive for human consumption, such products could be valuable human food sources. The necessity for an attractive taste cannot be overemphasized. A large number of protein-rich foods have been tested for the relief of malnutrition in developing countries (a comprehensive review was prepared by Orr, 1972). It is evident that the success of a food is at least partly dependent upon its being highly palatable.

LITERATURE CITED

Abe, M.G., and R.C. Lindsay. 1978. J. Food Prod. 41: 781. Cited by: K.S. Djien, Indigenous fermented foods. Economic Microbiol. 7: 15-38, 1982.

Adenkule, A.A., and O. Ayeni. 1974. Occurrence and distribution of mycotoxic flora in some Nigerian foods. Egyptian J. Microbiol. 9: 85-95.

Akinrele, I.A. 1964. Fermentation of cassava. J. Sci. Food Agric. 15: 589-594.

Alexander, J.C., C.Y. Kuo, and K.F. Gregory. 1979. Biological evaluations of two thermotolerant filamentous fungi as dietary protein sources for rats. Nutr. Rep. Internat. 20: 343-351.

Azoulay, E., F. Jouanneau, J. Bertrand, A. Raphael, J. Janssens, and J.M. Lebeault. 1980a. Fermentation methods for protein enrichment of cassava and corn with Candida tropicalis. Appl. Environ. Microbiol. 39: 41-47.

Azoulay, E., et al. 1980b. Le manioc protéiné: une révolution pour le tiers-monde. Adour Expansion 15: 13-23. (Cited by: Abstracts on Cassava. Vol VII, p. 183 Centro Internacional de Agricultura Tropical, Cali, Colombia, 1981).

Bourdoux, P., M. Mafuta, A. Hanson, and A.M. Ermans. 1980. Cassava toxicity: the role of linamarin. in: A.M. Ermans, N.M. Mbulamoko, F. Delange and R. Ahluwaba, Role of Cassava in 'The Etiology of Endemic Goitre and Cretinism', International Development Research Centre, Ottawa, Canada. pp. 15-27.

Brook, E.J., W.R. Stanton, and A. Wallbridge. 1969. Fermentation methods for protein enrichment of cassava. Biotechnol. Bioeng. 11: 1271-1284.

Cock, J.H. 1982. Cassava: a basic energy source in the tropics. Science 218: 755-762.

Collard, P., and S. Levi. 1959. A two stage fermentation of cassava. Nature 183: 620-621.

Dougan, J., J.M. Robinson, S. Sumar, G.E. Howard, and D.G. Coursey. 1983. Some flavoring constituents of cassava and of processed cassava products. J. Sci. Food Agric. 34: 874-884.

Djien, K.S. 1982. Indigenous fermented foods. in A.H. Rose (ed.) Economic Microbiology, Vol. 7. Fermented Foods. Academic Press, London, New York, pp. 15-38.

De Lamo, P.R., and T.J.B. De Menezes. 1979. Bioconversão das águas residuais do processamento de mandioca para produção de biomassa. Coletânea do Instituto de Tecnologia de Alimentos. 10: 1-14. (Cited by: Abstracts on Cassava, vol. VIII (2), Centro Internacional de Agricultura Tropical, Cali, Colombia, p. 57.)

Ejiofor, M.A.N., and N. Okafor. 1984. Microbial breakdown of linamarin in fermenting cassava pulp, in E.R. Terry, E.V. Doku, O.B. Arene, and N.M. Manhungu (ed.). Tropical Root Crops: Production and Uses in Africa. Proc. 2nd Triennial Symposium Internat. Soc. Trop. Root Crops. International Development Research Centre, Ottawa, Canada, pp. 105-107.

Ezeala, D.D. 1984. Changes in the nutritional quality of fermented cassava tuber meal. J. Agric. Food Chem. 32: 467-469.

Food and Agriculture Organization. 1980. Food Balance Sheets Averate. 1975-1977. FAO Rome.

Food and Agriculture Organization. 1983. FAO Production Yearbook, 1982. FAO Rome, pp. 128-129.

Favier, J.C., S. Chevassus-Agnes, and G. Gallon. 1971. La technologie traditionelle du manioc au Cameroun: influence sur la valeur nutritive. Ann. Nutr. Alim. 25: 1-59.

Fomunyam, R.T., A.A. Adegbola, and O.L. Oke. 1984. Hydrolysis of linamarin by intestinal bacteria. Can. J. Microbiol. 30: 1530-1531.

Grace, M. 1971. Processing of Cassava Agricultural Services. Bull. No. 8, FAO, Rome.

Gray, W.D., and M.O. Abou-El-Seoud. 1966. Fungal protein for food and feeds III. Manioc as a potential crude raw material for tropical areas. Econ. Bot. 20: 251-255.

Gregory, K.F. 1977. Cassava as a substrate for single-cell protein production: microbiological aspects, in B. Nestel and M. Graham (ed.). Cassava as Animal Feed. International Development Research Centre, Ottawa, Canada, pp. 72-78.

Gregory, K.F., A.E. Reade, J. Santos-Nunez, J.C. Alexander, R.E. Smith, and S.J. MacLean. 1977. Further thermotolerant fungi for the conversion of cassava starch to protein. Anim. Feed Sci. Technol. 2: 7-19.

Gregory, K.F. 1982. Feed enrichment with microbial protein. Conservation Recycl. 5: 33-40.

Hesseltine, C.W., and H.L. Wang. 1980. The importance of traditional fermented foods. Bioscience 3: 402-404.

Hill, D.C. 1977. Physiological and biochemical responses of rats given potassium cyanide or linamarin, in B. Nestel and M. Graham, Cassava as Animal Feed, International Development Research Centre, Ottawa, Canada, pp. 33-42.

Ikediobi, C.O., and E. Onyiko. 1982a. Linamarase activity and detoxification of cassava (Manihot esculenta) during fermentation for gari production. Agric. Biol. Chem. 48: 1667-1669.

Ikediobi, C.O., and E. Onyiko. 1982b. Use of linamarase EC-2.4.1.63 in gari production. Process Biochem. 17(4): 2-5.

Joachim, A.W.R., and D.G. Panditteseker e. 1944. Investigation of the hydrocyanic acid content of manioc (Manihot utilissima). Trop. Agric. 100: 150-163.

Kihlberg, R. 1972. The microbe as a source of food. Ann. Rev. Microbiol. 26: 427-466.

Kuo, C.Y., J.C. Alexander, J.H. Lumsden, and R.G. Thomson. 1979. Subchronic toxicity test for two thermotolerant filamentous fungi used for single cell protein production. Can. J. Comp. Med. 43: 50-58.

Lancaster, P.A., J.S. Ingram, M.Y. Lim, and D.G. Coursey. 1982. Traditional cassava-based foods: survey of processing techniques. Econ. Bot. 3: 12-45.

Lancaster, P.R., and D.G. Coursey. 1984. Traditional post-harvest technology of perishable tropical staples. FAO Agric. Services Bull. No. 59, Food and Agriculture Organization of the United Nations, Rome, pp. 25-34.

Lewis, E.A., and E.A. Aderoju. 1978. Factors in the aetiology of chronic duodenal ulcer in Ibadan. Trop. Geograph. Med. 30: 75-79. Cited in Abstracts on Cassava, Centre Internacional de Agricultura Tropical, Cali, Colombia, 1981, p. 140.

Litchfield, J.H. 1980. Microbial protein production. Bioscience 30: 387-396.

Maul, S.B., A.J. Sinsky, and S.R. Tannenbaum. 1970. New process for reducing the nucleic acid content of yeast. Nature (London) 228: 181.

Meuser, F., and H.D. Smolnik. 1980. Processing of cassava to gari and other foodstuffs. Starke 32(4): 116-122. Cited in Abstracts on Cassava, Vol. VI, 1980, Centro Internacional de Agricultura Tropical, Cali, Colombia, p. 170.

Mikami, Y., K.F. Gregory, W.L. Levadoux, C. Balagopalan, and S.J. Whitwill. 1982. Factors affecting yield and safety of protein production from cassava by Cephalosporium eichhorniae. Appl. Environ. Microbiol. 43: 403-411.

Muindi, P.J., and J.F. Hanssen. 1981a. Nutritive value of cassava root meal enriched by Trichoderma harzianum for chickens. J. Sci. Food Agric. 32: 647-654.

Muindi, P.J., and J.F. Hanssen. 1981b. Protein enrichment of cassava root meal enriched by Trichoderma harzianum for animal feed. J. Sci. Food Agric. 32: 655-661.

Muindi, P.J., S. Thomke, and J.F. Hanssen. 1981. Protein quality studies on rats fed on cassava-root meal enriched with Cephalosporium eichhorniae 152 or with conventional plant-protein supplements. Anim. Feed Sci. Technol. 6: 197-208.

Musenge, H.M., J.G. Anderson, and R.S. Holdom. 1980. Growth of Candida utilis on enzymatically hydrolysed cassava. Biotechnol. Letters 2(1): 35-40.

Ngaba, P.R., and J.S. Lee. 1979. Fermentation of cassava (Manihot esculenta Crantz). J. Food Sci. 44: 1570-1571.

Noparatnaraporn, N., Y. Nishigawa, M. Hayashi, and S. Nagai. 1983. Single cell protein production from cassava starch by Rhodopseudomonas gelatinosa. J. Ferment. Technol. 61: 515-520.

Okafor, N. 1977. Microorganisms associated with cassava fermentation for gari production. J. Appl. Bacteriol. 42: 279.

Oke, O.L. 1983. Processing and detoxification of cassava, in F. Delange and R. Ahluwabia, Cassava Toxicity and Thyroid: Research and Public Health Issues. Internat. Dev. Res. Centre, Ottawa, Canada. pp. 129-133.

Okezie, B.D., and F.V. Kosikowski. 1981a. Acid whey powder modification of gari from cassava. J. Dairy Sci. 64: 416-421.

Okezie, B.O., and F.V. Kosikowski. 1981b. Extractability and functionality of protein from yeast cells grown on cassava hydrolysate. Food Chem. 6(11): 7-18.

Olarewaju, O.C., and Z. Boszormenyi. 1975. The process of detoxification and residual cyanide content of commercial gari samples. W.A.J. Biol. Appl. Chem. 18: 7-14.

Opoku, A.R., and P.A. Adoga. 1980. Two-stage fermentation method for production of protein-enriched feed from cassava. Enzyme Microbial Technol. 2: 241-243.

Orr, E. 1972. The use of protein-rich foods for the relief of malnutrition in developing countries: an analysis of experience. Tropical Products Institute, London, 71 pp.

Protein-Calorie Advisory Group. 1976. Documents on Single Cell Protein issued by PAG. PAG Guideline No. 6 for preclinical testing of novel sources of protein (1972 revision); PAG Guideline No. 7 for human testing of supplementary food mixtures (1972 revision); PAG Guideline No. 12 on the production of single-cell protein for human consumption (1972). Protein Advisory Group of the United Nations System. United Nations, New York.

Pond, W.G., and J.H. Maner. 1974. Swine production in temperate and tropical environments. W.H. Freeman & Co., San Francisco.

Raimbault, M. 1981. Ferrmentation en milieu solide. Croissance de champignons filamenteux sur substrat amylace. ORSTOM, Paris.

Raimbault, M., and D. Alazard. 1980. Culture method to study fungal growth in solid fermentation. European J. Appl. Microbiol. Biotechnol. 9: 199-209.

Reade, A.E., and K.F. Gregory. 1975. High temperature production of protein-enriched feed from cassava by fungi. Appl. Microbiol. 30: 897-904.

Renvoize, B.S. 1972. The area of origin of Manihot esculenta as a crop plant -- a review of the evidence. Econ. Bot. 26: 352-360.

Revuz, B., and D. Voisin. 1980. La manioc protéiné, procédé Adour Speichim. Industries Alimentaires et Agricole, 1980: 1079-1084.

Rogers, D. 1965. Some botanical and ethnological considerations of Manihot esculenta. Econ. Bot. 19: 369-377.

Sawada, H., T. Yoshida, H. Toguchi, M. Sundhogue, and S. Chaitiumvong. 1982. Process design and economic studies of single cell protein production from cassava. Conservation and Recycling 5: 13-29.

Scott, H.L., and E. Houghton. 1976. Biochemical process for the synthesis of protein from cellulose and starch-containing plants. U.S. Patent 3,979,522.

Senez, J.C., M. Raimbault, and F. Deschamps. 1980. Enrichissement en protéines de substrats amylacés par fermentation en milieu solide. Rev. Mond. Zootech. (FAO) 35: 36-39.

Solomons, G.L. 1983. Single cell protein. CRC Critical Rev. Biotechnol. 1: 21-58.

Stanton, W.R., and A. Wallbridge. 1969. Fermented food processes. Process Biochem. April, pp. 45-51.

Stanton, W.R., and A.J. Wallbridge. 1972. Improvements relating to the fermentation of cassava and other vegetable substances British Patent 1,277,002, 4 pp.

Steinkraus, K.H. 1980. Special analysis: food from microbes. Bioscience 30: 383-407.

Strasser, J., J.A. Abbott, and R.F. Battey. 1970. Prccess enriches cassava with protein. Food Engineering, May, pp. 112-116.

Tannenbaum, S.R., and D.I.C. Wang (eds.). 1975. Single-cell Protein II. MIT Press, Cambridge, Mass. 707 pp.

Therkildsen, H. 1980. Symbiotic growth of a yeast and a bacteria in batch fermentors. Biotechnol. Letters 2: 117-122.

Trevelyan, W.E. 1974. The enrichment of cassava with protein by moist-solids fermentation. Trop Sci. 16(4): 179-194.

Vanneste, G. 1982. Enrichissement protéinique du manioc par fermentation fongique. Revue Fermental. Ind. Aliment. 37: 19-24.

Vinck, D. 1982. La transformation du manioc en gari. These Ing. Chim. Ind. Agric., Gembloux, Belgique, Faculte des Sciences Agronomiques de l'Etat.

Wang, H.L., and C.W. Hesseltine. 1979. Mold-modified foods, in H.J. Peppler and D. Perlman (eds.). Microbial Technology, Academic Press, N.Y., pp. 95-129.

Whitby, P. 1968. Foods of Ghana. Food Research Institute, Accra. Cited by: K.S. Djien, Indigenous fermented foods, Econ. Microbiology 7: 15-38, 1982.

Appendix A

Answers to Questions Posed by
K. Venkataraman, Officer-in-Charge
UNIDO Technology Programme, 25 January, 1985

(Note: These responses are the personal opinions of the author)

1. **What is the present state of the art?**

   Up to the present time only processes in which yeast cells are grown on hydrolyzed cassava starch appear to be immediately available for use in producing human food. (The Adour Speichim process uses a yeast which will hydrolyze starch by itself but, as described, it appears to be an inefficient process.) The only process which has been used for producing a yeast biomass, from cassava, with a low nucleic acid content is expensive. Without this step, only a small amount of the biomass could be safely consumed. Processes which utilize filamentous fungi would yield lower cost products. These products have not, thus far, been adapted for human consumption.

2. **What technical possibilities are available for improving cassava fermentation?**

   Most conventional fermentors are stirred by impellor blades. Air lift fermentors might produce less shearing action on filamentous fungi and could produce higher yields than those processes studied to date.

3. **Can the work on improved animal fodder based on cassava be extended to food?**

   Yes. Several processes for producing protein-rich microbial products from cassava have been described. The products are not able to compete in cost with some other low cost protein-rich supplements. In order to provide variety to human diets, however, some of these products could be valuable and sufficiently cheap. More extensive, longer term, multi-generation animal feeding trials would first need to be undertaken, however, and low-cost methods for the elimination of nucleic acids would have to be adapted to these products. An approach which appeals to me is the one proposed by Strasser *et al*. (1970) in which part of the cassava is fermented to produce gari, and part of it used to produce a protein-rich microbial biomass. I believe the microorganism used should be a filamentous fungus (possibly a thermotolerant species). Special attention should be given to selecting a fungus with a desirable taste. Some fungi, for examle, have a mushroom flavour and could produce a very tasty product. The combination of such an SCP product with gari (a high quality gari produced with selected starter cultures) could produce a food which would be very attractive as well as being highly nutritious.

4. **Have the microorganisms found in traditional fermented foods been clearly identified? How can they be modified to improve their efficiency?**

There are marked differences in reports on the microorganisms involved in the gari fermentation. The traditional gari fermentation could probably be improved if a detailed microbiological study were performed on many gari samples produced in various places. A variety of starter cultures could be tested until a formulation was developed that reproducibly gave a high quality gari in a short period of time. The fermentation and roasting processes should be carefully studied and accurately controlled so that the gari produced is free of both cyanide and cyanogenic glucosides, and stored under conditions where mycotoxin-producing fungi could not grow on it.

5. **What is the cost of adding amino acids and vitamins to the gari after production rather than improving them during production?**

The addition of amino acids would be very impractical. Sometimes the addition of an amino acid to a protein, which is deficient in that amino acid, could be valuable. The protein content of gari is so low, however, that there is little point in trying to balance its amino acid content.

The formulation of foods in which cassava, either fermented or nonfermented, is mixed with a high protein food component such as proposed by Okezie and Kasikowski (1981a), could be useful. In general, any leguminous plant, such as soybeans or peanuts (groundnuts), would markedly raise the protein level. Formulations of this nature could also raise the vitamin content.

6. **What type of technology, equipments and skills are required for producing the improved microorganisms and the other elements of gari production?**

Microbiologists, nutritionists, pathologists and engineers would all be required for an integrated, co-ordinated attack on this problem. The equipment required would include fermentors, with associated instrumentation, and grinding devices. It is tempting to suggest that the new techniques in biotechnology, such as genetic engineering, be applied to the problem. It is not immediately obvious, however, how these could be profitably employed. Genetic engineering should be looked upon as one of many techniques available to solve a particular problem. If a problem arises where it can provide the best solution, it should be applied. It would be a mistake to design a problem so as to utilize a particular technique rather than using whatever techniques are required to solve a particular problem.

7. **What programmes need to be developed to test for safety and public acceptability, and what are their costs?**

Specific recommendations have been prepared by the Protein/Calorie Advisory Group of the U.N. (see Appendix B). I have no experience with programmes on public acceptability and I am unable to estimate costs. The extensive review on protein-rich food plans by Orr (1972) indicates the problems to be expected.

8. **Can the microorganisms be packaged for use and storage in tropical climates, and at what cost?**

Any microorganism, which would likely to involved, could be packaged for storage. The cost would vary so greatly according to the local conditions and the longevity of different organisms that no cost estimate at this time would be meaningful. An improved gari inoculum possibly could be used for individual family production of gari but protein enrichment processes would probably require some degree of centralization in an industry.

9. **What are the appropriate marketing, dissemination and extension techniques, and the role of voluntary agencies?**

I don't know. It may be premature to consider this subject.

10. **Which are the institutions which could carry out the different aspects of the program?**

I would prefer to defer making specific suggestions at the present time except to note that the scientists affiliated with the Guelph/Waterloo Biotechnology Institute, which was recently created jointly by the University of Guelph and the University of Waterloo, have considerable expertise in many of the aspects involved. African universities and research institutes should be involved as much as possible.

APPENDIX B

PAG GUIDELINE (NO. 6) FOR PRECLINICAL TESTING OF NOVEL SOURCES OF PROTEIN*

Although prior history of safe use may be taken into account in the evaluation of a protein source proposed for general consumption, this alone is insufficient to preclude adequate preclinical testing by currently-available, more objective animal feeding studies. Careful attention must be given to the development of new varieties of conventional foods of improved protein quality developed through application of technological processes. Before they may be used as human food sources, new foods must be evaluated with respect to the quality of their protein content and their safety for use. This requirement may apply to new varieties of conventional foods where the composition has been genetically changed, but it applies especially to new foods developed by isolation from conventional sources by unusual techniques and to yeasts, bacteria, molds or

*Originally issued on 13 March 1970; revised version issued 13 March 1972.

algae, i.e. the so-called single cell proteins. Processes involving the use of solvent extraction or unusual heating conditions or the utilization of food additives in a variety of combinations may result in changes in digestibility, absorption and nutritive or toxicological safety of the food in question.

The development of a protocol for a specific food material will depend upon its similarity to a conventional food, the kind of process applied in its preparation and the conditions of its intended use as prepared for consumption.

The guideline for preclinical testing of novel sources of protein which follows has been prepared in general terms to describe the categories of information which must be developed in some cases but not necessarily in all. It is intended that this guideline serve as a general recommendation rather than as a series of mandatory procedures. The extent of animal testing considered necessary prior to undertaking trials in human subjects will depend on the protein product. In the event that the observations and results of a preclinical appraisal of a novel protein are to be submitted to a regulatory or institutional group as a basis for clinical trials, it is advisable to review the proposed protocol in advance with such an agency in the interest of saving time and effort.

Products intended for use in animal feeds may not require as extensive testing as is suggested here for human foods, but foods derived from such animal sources (meat, milk or eggs) must be considered from the viewpoint of the possible presence of residues transmitted from animal feeds. Controlled tests in farm animals may contribute useful information concerning safety or nutritional value for man.

With respect to single cell proteins (SCP), particular attention must be directed to the composition of the media from the viewpoint of the possible presence of chemical components regarded as hazardous to health. The source materials which form the substrates for the growth of potentially nutritive microorganisms are a) food and agricultural products such as molasses, whey and starch; b) industrial by-products such as cellulosic wastes and sulfite liquor; c) hydrocarbons such as petroleum fractions and natural gas; and d) alcohols. The microorganisms grown on these media include various strains of yeast, bacteria and fungi.

It is important, therefore, to recognize the possibility of the presence of contaminants derived from the source materials (e.g., polycyclic aromatic hydrocarbons from petroleum, mercury from sulfite liquor), from the media in which microorganisms are growing and from extraction or refining, as well as reaction products resulting from heat processing. Substances used as lubricants or binders (e.g., in texturization) should also be considered in this connection.

The physical and chemical identity of the industrial product should be established to be essentially the same as that of the material tested experimentally. To be truly significant, studies should be conducted on the SCP product as made on a commercial scale rather than on laboratory batches. Minor variations in processing conditions need not necessitate repetition of the entire series of preclinical or clinical studies.

1. Introduction

1.1 Categories of information needed

1.1.1 Toxicological safety as predicted from information concerning methods of production, chemical and physical properties, content of microorganisms and their metabolites, toxicological effects on laboratory animals and the responses of normal human subjects to limited feeding studies.

1.1.2 Nutritional value as predicted first from chemical composition with particular

emphasis on amino acid content and availability, then by means of short-term rat feeding studies designed to estimate the efficiency of absorption and utilization of nitrogen content.

1.1.3 Sanitation with respect to the source of the raw material and the conditions under which it is processed as well as potential pathogenicity should be taken into account.

1.1.4 Acceptability from the standpoint of taste and other organoleptic properties, including its tendency to induce "taste fatigue". Esthetic considerations, cultural and religious patterns of food acceptance should also be considered in this connection.

1.1.5 Technological properties from the point of view of incorporation of the product into currently acceptable foods and fabrication into new food items.

1.2 Tests and procedures to be used

1.2.1 Chemical analyses for proximate composition of the basic product, for the amino acid composition of its protein component, for its content of nonprotein nitrogenous components, for the presence of contaminants, residues of pesticides or solvents (depending upon the source of the raw material), for naturally-occurring or adventitious toxins and for food additives subject to tolerance limits.

1.2.2 Microbiological examinations for viable microorganisms, both pathogenic and nonpathogenic, aerobic and anaerobic, vegetative and spore-forming. In the case of proteins of microbial origin, consideration should be given to the composition of the medium or substrate on which the organism is grown.

1.2.3 Safety evaluations based on feeding studies in rodents and other experimental mammals. The initial short-term studies should be followed by long-term (at least 2-year) tests in rats.

1.2.4 Protein quality studies in young rats and other laboratory mammals to indicate the value of the protein product for promoting growth and nitrogen retention when fed as the sole source of protein and as a supplement to other foods.

1.2.5 Studies of acceptability in preliminary feeding studies of the protein in normal human adults and children. The extent to which these tests should be carried out will depend upon the novelty of the protein product as well as on the results of the preceding studies.

1.2.6 Extensiveness of the preclinical testing program should be decided for each new protein proposed for human food use based on a consideration of its source, composition and nature of the process employed in its production. As examples, the species of fish used for the production of fish protein concentrate, the microorganisms used as single cell proteins and the extraction systems employed in processing may determine how much preclinical evaluation is required.

1.2.7 Choice of procedures should be exercised with judgment based on experience. A product intended for use by infants will demand more exhuastive preclinical evaluation than products intended for use by children above the age of one year. No advantage is to be gained in employing a multiplicity of preclinical evaluations directed toward the same end in terms of the information furnished.

1.2.8 Limitation of the tests and procedures should be kept in mind. Whereas animal testing procedures are capable of establishing safety with reasonable certainty, no methods are available by which safety can be assured in an absolute sense. Furthermore, chemical or other nonbiological methods for predicting nutritive value have certain limitations despite the high degree of correlation between the results of such tests and those based on animal feeding studies.

2. Evaluation procedures

2.1 Chemical

A novel source of protein for human food should be subjected to the following analyses:

2.1.1 Proximate composition, i.e., moisture, total solids, total nitrogen, fat (ether extract), ash, crude fiber and available carbohydrate (by difference).

2.1.2 Protein. a) The nitrogenous components should be hydrolyzed and the amino acid spectrum determined by chromatography. The essential amino acid composition should be expressed on a N x 6.25 (N = 16 per cent) basis and the ratio of the total essential amino acids to N x 6.25 should also be calculated. b) The available lysine content should be determined by the method of Carpenter et al. (1-3). Since lysine is the principal, though not the only, essential amino acid likely to become bound and thus unavailable as a result of heat processing, the slightly modified Carpenter method is especially useful as a quality control procedure. c) The presence and amount of non-protein nitrogenous components such as glucosamines, amides and amines should be determined, particularly in the case of products derived from animal sources. d) The content of nucleic acid should be determined in single cell proteins.

2.1.3 Fat. The solvent extract should be analyzed for the presence and content of triglycerides, steroids and phospholipids. If the ether extract is greater than a fraction of a per cent, the fatty acid profile should be determined by gas chromatography, with special reference to fatty acids of unusual structure. If fat is present in calorically significant amounts, the ratio of polyunsaturated to saturated fatty acids should also be calculated from the determination of each group. Single cell proteins derived from petroleum hydrocarbons should be analyzed for total and polycyclic aromatic hydrocarbons by a suitable quantitative method.

2.1.4 Ash. Ash should be analyzed for its content of calcium, phosphorus, iron, iodine, alkali and alkaline earth elements and heavy metals. Products of marine and sulfite liquor origin should also be analyzed for mercury, arsenic and fluorine. In the light of current concern over mercurial contamination of fish from lakes, streams and marine waters, attention should be directed to the possible presence of inorganic and particularly alkyl mercury in protein concentrates derived from fish or algae.

2.1.5 Vitamins. Analyses should be conducted for all of the major vitamins except those for which a low lipid content or instability under processing conditions indicate little likelihood of their presence in significant amounts.

2.1.6. Food additives. Analyses should be conducted for those food additives permitted in the product under investigation for which tolerance limitations have been established.

2.1.7 Processing damage. Useful information concerning the effect of heat on the product may be obtained not only by determinations of available lysine, as mentioned above, but also by spectrophotometric examination for products of the Maillard reaction, or, in the case of leguminous proteins, for the heat-labile antitryptic factor or the concomitant enzyme urease. Useful information concerning the effect of alkali treatment of the product may be obtained by the determination of lysinoalanine (4).

2.1.8 Physical properties. Though not relevant to the preclinical evaluation of the nutritional quality or safety of novel proteins, it would be expected that studies of their physical properties, e.g. solubility, wettability, viscosity, etc., would be conducted to establish their technological utility as foods or food supplements.

2.1.9 Miscellaneous. Depending upon the nature of the raw material and the conditions employed in its production, special analyses of the protein product should be conducted for:

a) Solvent residues, such as polycyclic or chlorinated hydrocarbons;
b) Pesticide residues;
c) Naturally-occurring toxic substances, e.g. gossypol, hemagglutinins and marine toxins. It should be noted that there are no satisfactory nonbiological tests for the latter category of substances.

2.2 Biochemical

As an indication of the digestibility of the protein product, in vitro enzyme studies may be conducted to determine the rate and degree of hydrolysis by pepsin and pepsin plus trypsin under conditions simulating those in the human gastrointestinal tract (5-7). As discussed below, calculations based on the essential amino acid content of enzymatic hydrolyzates have been adapted for estimating the biological value (utilization) of proteins.

2.3 Microbiological

While this is discussed more fully elsewhere, mention may be made of the need for microbiological examination of new sources of protein to determine the number or types of microorganisms indicative of unsanitary conditions of production or processing and to establish their freedom from toxigenic organisms. In the case of single cell proteins, taxonomic and toxicologic studies should be conducted on the organisms from which they are derived to define their identity as non-pathogenic species. The end products should also be examined to rule out the presence of viable organisms.

2.4 Protein quality

2.4.1 Predictive tests. Though the above-mentioned chemical examinations yield valuable information for predicting safety and nutritional value, they cannot be considered substitutes for biological appraisals of protein quality in the intact animal. For examp amino acid profiles as determined either microbiologically or chromatographically on protein hydrolyzates fail to differentiate between free, bound and nonbound forms, or between differences in rate and degree of digestibility. Moreover, interpretations of amino acid profiles are based on comparis with some more or less arbitrarily-chosen "ideal" protein, such as that of whole egg o human milk, or on comparison with the FA reference pattern or the amino acid require ments as observed in young adult males.

The utility as well as limitations of estimat the biological value of proteins from their e sential amino acid content (8-11) is discuss in several monographs (12, 13). Their ma use is for screening products prior to anin testing.

The "chemical score" of Mitchell and Bloc (8, 9) is based on the ratio of the essential amino acid in greatest deficit compared wi its content in a reference protein, e.g. wh egg. The "essential amino acid index" (E/ is predicated on the hypothesis that the bio logical value of a protein is a function of t levels of all of these amino acids in relati to their content in the reference protein (10, 11). Experience has shown that the chemical score, based as it is on a single limiting amino acid, tends to underestima the biological value, whereas the modifie EAA index gives values which correlate m closely with biological values. However, these chemical ratings are based on analy of complete hydrolyzates of proteins, the fail to take into account differences in dig ibility of the proteins or availability of in vidual amino acids.

2.4.2 Bioassay procedures. Ideally, the nutritional evaluation of protein food sho be made in relation to their potential role the diet of the population for whom they a

intended. Whereas this would preclude assigning a single numerical rating to a given protein which is applicable under all conditions of use, it would avoid the fallacy of assuming that such a rating, based on a single level of feeding as the sole source of nitrogen for the test animal, provides a true measure of its nutritional value as a supplement to the diet or to specific foods for man.

Experience over the years has shown that the most useful preliminary tests for the appraisal of the nutritional value of proteins are based on the short-term growth responses of rats to the ingestion of suboptimal levels of the test protein. Variations of this basic procedure have been described in which the responses are expressed in terms of weight gain per unit weight of protein (N x 6.25) or nitrogen ingested. These methods do not distinguish between the utilization of protein for maintenance and for growth.

2.4.2.1 Protein Efficiency Ratios

The most commonly used of these procedures involves the determination of the Protein Efficiency Ratio (PER) in which the average net gain in body weight per unit weight of protein (N x 6.25) is compared with that observed for the reference protein, casein. Because of differences in response to the same standard casein, in the same or different laboratories, PER values are customarily adjusted to an assumed value of 2.5 for casein.

This procedure has been refined (14, 15) to balance the fat, i.e. caloric density, and mineral content of the test and reference protein diets as far as possible. The diets are fed ad libitum to groups of ten weanling male rats (weighing 40-50g at 20-23 days) for a period of 28 days. In the case of low-protein sources, e.g. rice, cassava, etc., it is not possible to achieve the usual 9 or 10 per cent levels of dietary protein specified for the PER test. Hence, the tests are run in comparison with standard casein at correspondingly lower levels. Conventional PER determinations are deficient as bioassays in that they are performed at single protein levels, thus ignoring differences in slope of the dose-response curve at the suboptimal level. Improved design of the PER test may be achieved by either a multilevel assay in which the growth responses at two or three levels of the test protein are compared with two or three suboptimal levels of the reference protein (16), or by means of a slope-ratio assay employing multiple levels in which the slope of response to the test protein is compared with that of the reference protein (17). In the conventional PER test, the observed value of grams gain in body weight per 100 grams protein consumed is adjusted to an assumed value of 2.5 for casein to compensate for interlaboratory variations due to age, strain and pretest dietary history of the rat and differences in the actual casein used.

Because of its relative simplicity and low cost, the PER method is the most widely-favored method for the evaluation of protein quality. However, it has certain limitations. For example, like any procedure based solely on gain in body weight, it takes no account of composition of the weight increment. A more significant criticism, however, resides in the fact that it is a measure of the combined effects of digestibility of the dietary components and their utilization for tissue synthesis. More informative procedures permit differentiation between the proportion of dietary nitrogen absorbed, which is a function of the susceptibility of the protein source to the action of the gastrointestinal proteolytic enzymes, and the proportion retained in the body, which is a function not only of the essential amino acid composition and content, but of the total protein content and the caloric density of the diet.

2.4.2.2 Net Protein Ratio and Net Protein Utilization

Less frequently employed are the methods based on estimation of Net Protein Ratio (NPR),

in which the weight loss of a comparable group receiving a protein-free diet is added to the weight gain of the protein-fed group, or the Net Protein Utilization (NPU), in which the total body nitrogen of both the protein-fed and protein-free groups are determined. These tests are generally performed on groups of four to six 28- or 30-day-old rats fed the test or control diets for a ten-day period. The differences in terms of weight gain (NPR) or nitrogen retention (NPU) are expressed in relation to the weight of protein or nitrogen consumed.

The NPU procedure is more laborious than those based simply on growth response since it involves tedious and time-consuming analyses of carcasses. In theory at least, it is the product of the coefficients of digestibility of the nitrogenous component of the diet and its retention or biological value. When conducted under standardized conditions with respect to the age, initial weight, etc., of the test animals, the NPU method gives values which correlate quite closely with biological values, since within classes of foods digestibility will vary only within small limits. However, NPU is subject to the same criticisms mentioned previously for PER. It must be recognized that nutritional assays employing suboptimal levels of dietary protein reflect not only the qualitative adequacy of the essential amino acid content but the proportion of the total essential amino acid content in the protein. Thus a protein containing even a "balanced" essential amino acid combination may yield a poor response if the ratio of essentials to nonessentials is too low.

2.4.2.3 Nitrogen balance procedures

For a more definitive and detailed appraisal of the nutritive value of a protein source, nitrogen balance procedures must be used. These were applied originally to studies in man (18), but in the laboratory dogs (19, 20) and especially rats (21, 22) have been widely used. By this technique, one can differentiate between the degree of digestibility of the nitrogen source and the proportion of nitrogen retained for storage or anabolism. Digestibility is measured in terms of the ratio of the absorbed N (i.e. the difference between the ingested and intestinally excreted nitrogen, the latter corrected for "metabolic N") to the total N intake. Nitrogen retention is calculated from the ratio of the retained N (i.e. the difference between absorbed N and that eliminated in the urine, corrected for so-called endogenous N) to the absorbed N. These values are based on analyses of food, fecal and urinary N over the 3- to 5-day collection period, which follows a similar adjustment period. Several proteins, including the reference protein, may be tested in successive weeks, the metabolic and endogenous N corrections being derived from a basal collection period during which a protein-free diet is fed. If the rats are in an actively-growing stage over a series of tests, it is a further refinement to repeat the basal period at the beginning and end of the series so as to interpolate correction values for metabolic and endogenous loss appropriate for each week. The errors involved in the interpolations are discussed in a critical review by Njaa (23).

In testing a series of proteins sequentially, any residual effect of the previous week's test diet not fully offset by the adjustment period can be balanced by assigning the rats to test and reference proteins according to a Latin square design.

In addition to yielding more detailed information than growth methods, the nitrogen balance procedure is not significantly affected by differences in maintenance requirements since the test collection periods are very short.

In comparative tests of biological value by this procedure, greater precision can be achieved by standardizing the food consumption at a constant level, e.g. 8-10g per day, because the degree of nitrogen retention is

influenced more by food intake than by the protein level of the diet (23). It is recommended that instead of a protein-free diet, one containing a low level of protein, e.g., 3 to 4 per cent lactalbumin or whole egg protein, be fed during the basal period to avoid confusing catabolic with maintenance losses. The diets are fed ad libitum, which introduces a source of error inasmuch as the ratio of weight gain to food consumption may be influenced by the latter, which in turn is influenced by factors of palatability and toxicity of the protein source.

2.4.2.4 Limitations of bioassay results

It should be pointed out that none of the animal assays for protein quality can be assumed to provide an absolute measure of nutritive value for man. Aside from possible species differences, these procedures have certain limitations with respect to i) differentiation between maintenance and growth requirements, ii) the assumptions in estimating metabolic and endogenous losses, iii) the effects of ad libitum vs. controlled food intake and iv) dietary levels of proteins, calories and other nutrients not completely balanced between test and control diets. Furthermore, any test of protein efficiency based on feeding a single source must be interpreted with reservation since, except for milk or milk substitutes, proteins are consumed as components of, or as supplements to, mixed diets. Nevertheless, reasonably good correlation is observed between the growth and nitrogen retention procedures and the relative ranking of individual protein sources is quite similar in each of the methods described. Especially in chemically-treated proteins the determination of the limiting amino acid by rat assay may give valuable information in relation to the chemically-determined composition of the protein. Special reference is made to the review entitled "Evaluation of Protein Quality" of the Committee on Protein Malnutrition of the Food and Nutrition Board (12) and that of Njaa (23). Irwin and Hegsted (24) have discussed the limitations not only of the widely-used short tests for rating proteins, e.g. PER and NPU, but of the more informative nitrogen balance procedures. The errors involved in estimating corrections for endogenous and metabolic nitrogen excretion are relatively small compared to the basic error implicit in the evaluation of an individual protein as the sole source of this nutrient in the diet.

2.5 Safety (25)

2.5.1 Related factors.

2.5.1.1 Nutritional adequacy of test diet

Growth depression or any other adverse effect observed in the course of short-term nutritional assays should be viewed in the light of possible toxicity of the protein source. It is necessary to differentiate between low weight gain due to nutritional inadequacy and toxicity of either the protein per se or any adventitious contaminants. In order to avoid confusing nutritional insufficiency with toxicity in safety evaluation studies, the basal diet to which the test protein is added as a supplement should itself be nutritionally adequate for normal growth and development of the animal species employed. The protein content of the basal diet rather than that of the test material should be relied upon to satisfy the amino acid requirements.

2.5.1.2 Identity of test materials

In applying the results of safety evaluation studies to products made, or to be made, commercially, the identity and reproducibility of the test material with that produced in practice must be established by chemical and other relevant procedures.

2.5.1.3 Natural toxicants

Naturally-occurring toxic substances found in plants include carcinogens (e.g. cycad nuts, oil of sassafras), goiterogens (Brassica species), hemagglutinins (e.g. ricin, phaseo-

lotoxin in legumes), lathyrogens (e.g. vetch, sweet peas), cyanogenic glycosides (certain beans and nuts) and estrogens (in seeds and leafy vegetables) (26). Marine sources of protein, such as fish or shellfish found in tropical waters and the algae or plankton on which they feed, may contain highly toxic substances. Naturally-occurring toxic agents may be avoided either by care in the selection of the raw materials or by appropriate methods of storage, heat processing or extraction.

2.5.1.4 Microbiological toxins

Raw materials subject to microbial contamination and spoilage must be examined for the presence of pathogenic organisms, e.g. Salmonellae, Shigella, Staphylococci and Clostridia, and for the endotoxic and exotoxic substances they produce. Raw materials exposed to warm, humid conditions which induce fungal growth must be examined for the possible presence of mycotoxins such as the aflatoxins.

2.5.1.5 Extraction residues

Protein concentrates which have been isolated or refined by means of solvent extraction should be analyzed for the possible presence of solvent residues and any products which may be formed, particularly by the use of reactive chlorinated hydrocarbons. In the event that any such residues are present, toxicological data should be available to establish safe limits.

Single cell proteins produced by growing microorganisms on sulfite liquor, carbohydrates (e.g. molasses) or hydrocarbon media must be evaluated to establish the nonpathogenicity of the microorganisms and to rule out their metabolites or possible mutagenicity into toxigenic forms. Depending upon the nature of the raw materials, the media and the conditions of processing, analyses for the possible presence of impurities or contaminants such as solvent residues, heavy metals, fluoride, etc., should precede any toxicological feeding studies.

2.5.1.6 Multilevel feeding of test protein

When graded levels of the test protein are fed as supplements to a natural-type diet, it is not possible to maintain isonitrogenicity. From the toxicological standpoint, however, it is preferable to insure a sufficient level of complete protein in the basal diet rather than to adjust for differences in nitrogen content. This complicating factor can be avoided by using a semisynthetic type of diet in which casein constitutes the single basal protein, provided it is present in all diets at nutritionally-adequate levels.

2.5.1.7 Comparability of test and basal diets

To the extent that the test protein source contributes high levels of lipid, ash or indigestible cellulosic material, adjustments may be required to balance out these factors in the test and control diets.

2.5.1.8 Highest feasible feeding level of test protein

In contrast to safety evaluation studies of food additives generally, it is not feasible to include large multiples of levels of proteinaceous foods in experimental diets employed in toxicological evaluations. Nevertheless, as high a level as possible should be included, keeping in mind the fact that excessive amounts of even high-quality proteins, such as casein, may depress growth and food efficiency.

2.5.2 Testing protocols. The need for and experimental design of a safety evaluation study in animals depend upon the novelty of the protein as a food for man, in respect to both its source and method of production. The following discussion assumes that the test material is sufficiently different from conventional forms of dietary protein to require rather thorough toxicological appraisal before conducting feeding studies on human subjects.

Furthermore, it is assumed that any nutritional evaluations which may have been conducted suggest that the test material is sufficiently safe to warrant more extended studies.

The protocol for a safety evaluation program requires decisions with respect to:

2.5.2.1 Choice of animal species

Rats are by far the best single species. Mice are also used but less is known of their nutritional requirements and their size precludes obtaining sufficient blood or urine for examination. Among the nonrodent species, beagle dogs, rhesus monkeys and miniature pigs have been used for short-term but not for chronic (life-cycle) studies.

2.5.2.2 Composition of basal diet

As a basal diet for short-term, e.g. 3-month tests, either a synthetic-type (casein-starch) or a commercial natural-type ration is suitable. For long-term, e.g. 2-year, studies the latter is preferred. In either case the basal ration must satisfy all nutritional requirements for the species in question.

2.5.2.3 Dietary level of test protein

The test protein should be fed at two or three graded levels, the highest being determined by its nutritional adequacy as a sole source; for example, 50 per cent of the total protein of a test diet, provided it does not lower the overall biological value. If the test protein has been shown to be nutritionally complete, it may be fed as a replacement for casein in a synthetic-type diet, at least in a short-term toxicity test.

2.5.2.4 Equilibration of test and control diets

If the test protein product has a high ash content, e.g. certain fish protein concentrates, or is otherwise unbalanced with respect to one specific nutrient(s), it may be necessary to equalize the level of this component in the test and control diets to facilitate proper interpretation of the responses.

2.5.2.5 Age, weight, sex and method of assigning animals to groups

Rodents are usually started on tests at or shortly after weaning and are assigned to groups of equal size, balanced with respect to litter distribution, sex and average weight. For short-term tests, groups consist of 10 to 15 animals of each sex, but for long-term tests at least twice this number is recommended. In the case of the larger mammals, the groups should include 3 and 6 of each sex for short- and long-term studies, respectively.

The use of neonatal animals as toxicological test subjects has been suggested for the evaluation of protein which might be included in the diets of infants. It has been demonstrated that newborn animals have not the capability of the adult for enzyme induction and hence lack the ability to metabolize or detoxify foreign substances.

2.5.2.6 Individual or group housing

Animals should be housed individually during the initial period of rapid growth so as to permit reliable measurement of food consumption. Caging in pairs or larger groups may be resorted to at later stages. Metabolism cages should be used during periods when urine or feces are collected.

2.5.2.7 Maintenance and sanitary controls

The animal quarters should be maintained in a sanitary condition and controlled with respect to temperature and humidity. The environment should be kept insect-free without the use of pesticidal aerosols. Cages should be washed preferably at weekly intervals.

2.5.2.8 Nature and frequency of observations

The gross inspections, clinical laboratory tests and pathological observations, and their frequency in both short- and long-term toxicological studies, are illustrated in the following publication.

TABLE I.

TYPICAL CRITERIA USED IN TOXICOLOGICAL EVALUATIONS

Observations	Frequency	
	Short-term	Long-term
Physical appearance	daily	daily
Behavior	daily	daily
Body weight	weekly	weekly
Food consumption	weekly	weekly
Hematology		
Hemoglobin	0, 4, 8, 12 weeks	1, 3, 6, 9, 12, 18, 24 months
Hematocrit		
Leukocytes, total and differential		
Platelets		
Reticulocytes		
Blood chemistry		
Glucose	as above	as above
Urea nitrogen		
Protein, total		
Albumin/globulin ratio		
Triglycerides		
Cholesterol		
SGOT, SGPT		
Alkaline phosphatase		
Uric acid		
Allantoin		
Urine		
Volume, pH, sp. gr.	as above	as above
Glucose		
Protein		
Ketone bodies		
Bile		
Occult blood		
Sediment		
Autopsy (dead or sacrificed animals)		
Gross pathological examination	terminal	
Organ weights		
Liver, kidney, heart, brain, spleen, gonads, pituitary, adrenals, thyroid		
Histopathology		
20+ organs and tissues		
Electron microscopy of liver and kidneys		

5.2.9 Functional or metabolic studies

special cases, tests for hepatic, gastro-testinal or renal function, metabolic alance studies, or neurological or behavioral ests may be suggested. Periodic ophthal-ological examinations should also be conducted.

ingle cell proteins are known to contain a igh level of nucleoprotein, in some cases s much as 12 to 15 per cent. The effect of igh levels of intake on uricogenesis in man therefore must be considered. Since the available evidence indicates that all animal species except man and the anthropoid apes metabolize purines past the uric acid stage to allantoin, this phenomenon can best be studied only in man (Dalmatian dogs excrete uric acid not because of the lack of the uricolytic enzyme but because of a low renal th[illegible]hold). Allantoin levels in blood and urine should be included in toxicological studies of single cell protein.

2.5.2.10 Reproduction and lactation: multigeneration studies

The degree of novelty of a potentially important food item, both as to source and method of production, should determine the need for reproduction and lactation studies in animals. While they may not be indicated in the case of a fish or cereal protein concentrate, such tests should be included in a protocol for safety evaluation of a single cell protein. Though questions have been raised concerning the application of rat reproduction data [illegible] the human species, this problem is not peculiar to reproduction phenomena. Chronic toxicological assessments may be designed to include multigeneration studies according to Figure I.

The horizontal lines represent the generations of rats through their successive matings and the dotted vertical lines indicate litters; termination of a litter or a generation is shown by the letter T and the figures indicate the number of weeks elapsed at each stage, beginning with the first mating in F_0.

The observations recorded in reproduction studies include the proportion of successful matings, the number and weight of young born and their ability to survive and grow during the normal lactation period. Certain of these parameters are expressed in terms of indexes for fertility (the proportion of matings resulting in pregnancies), gestation (the proportion of pregnancies resulting in live litters), viability (the proportion of pups born which are alive at 4 days of age) and lactation (the proportion of pups alive at 4 days which survive to weaning) (27).

2.5.2.11 Duration of test period

Short-term toxicological tests generally run from 3 to 6 months; long-term tests from 1 to 2 years. If potential carcinogenicity is suspected, studies should be designed to include larger than usual groups of rats or mice and the test periods should run at least 2 or 1.5 years, respectively.

2.5.2.12 Teratogenic or mutagenic studies

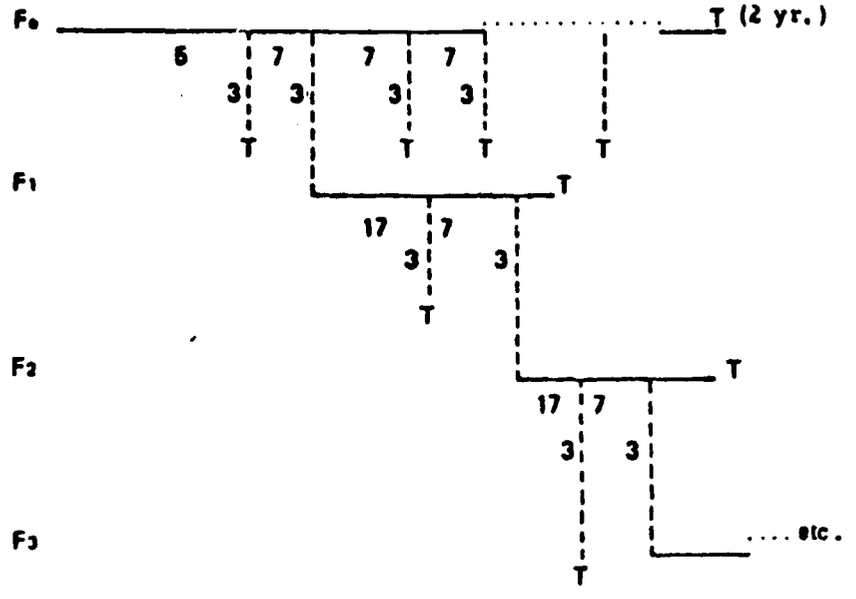
Recent emphasis on teratogenic and mutagenic investigations is in part, at least, a response to public and political pressure. However, without belittling the significance of genetic aberrations as an aspect of toxicity, further research is needed to establish the validity of present procedures for teratologic and mutagenic tests for assessing safety under use conditions in man. Current methods are being applied largely for the purpose of accumulating data to establish their relevance to human safety evaluation.

If the source or nature of the protein product is so unusual as to demand more extensive study, or if abnormal responses are seen in reproduction studies (2.5.2.10), the possibility of teratogenic or mutagenic effects may be considered.

Teratologic studies are conducted in rats and

FIGURE 1.

CHRONOLOGICAL SCHEME OF REPRODUCTION AND LACTATION



rabbits with particular attention to the time and duration of test dosage before or during gestation. Litters are delivered by cesarean section and responses are observed in terms of implantation sites, resorption, survival of the pups and examination of the soft tissues and of skeletal structure after clarification and staining.

Cytologic tests in tissue culture for evidence of chromosomal aberrations in animals have recently been emphasized for the investigation of potentially mutagenic drugs and pesticides. However, the significance of such tests as applied to the intact mammalian species has yet to be established. So far as is known, no proteins included in the human diet have been investigated for possible mutagenicity.

2.5.2.13 Statistical analyses and interpretation of findings

In the interpretation of the responses to toxicological tests, the statistical significance of differences in responses between test and control groups plays an important role. Hence the size of experimental groups as well as the quantitative rating of both objective and subjective observations are particularly relevant. However, whatever statistical probability is adopted as the basis for defining significance, the chance that a single group may deviate from the norm without actually indicating a biological aberration cannot be ignored. Judgement founded on experience of the investigator and past performance of the particular strain and colony of animals must be given due weight. Interpretation of experimental findings should take into account the quantitative relationship of the test vs. use conditions of the product under investigation, interspecies variations, the limited number and variety of observations incorporated into the safety evaluation program and the relative size of the test and human populations.

For a discussion of the basic procedures involved in safety evaluation of food components, reference is made to the reviews of the United States Food and Drug Administration (28), the

Food Protection Committee of the National Academy of Sciences/National Research Council (29) and the reports of the Joint Expert Committee on Food Additives of FAO/WHO.

Reports of investigations submitted for review and evaluation by the PAG must include full details and data for control as well as test groups and appropriate statistical analysis of the findings. Brief descriptions of the observations and conclusions will not be acceptable.

3. Conclusions

The objective of the preclinical study is achieved when it has been determined that the levels and conditions of feeding of the novel dietary component are sufficiently s[illegible] to warrant a cautious program of study in human subjects.

4. References

1. Carpenter, K.J. 1960. Estimation of available lysine in animal-protein foods. Biochem. J. 77:604-610.

2. Boyne, A.W., K.J. Carpenter and A.A. Woodham. 1961. Assessment of laboratory procedures suggested as indicators of protein quality in feeds. J. Sci. Food Agr. 12:832-848.

3. Carpenter, K.J., and B.E. March. 1961. The availability of lysine in peanut biscuits u[illegible] in the treatment of kwashiorkor. Brit. J. Nutr. 15:403-409.

4. DeGroot, A.P., and P. Slump. 1969. Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr. 98:45-56.

5. Sheffner, G.A., G.A. Eckfeldt and H. Spector. 1956. The pepsin-digest-residue (P.D.R.) amino acid index of net protein utilization. J. Nutr. 60:105-120.

6. Sheffner, G.A. 1967. In vitro protein evaluation. In Newer methods of nutritional biochemistry (A.A. Albanese, ed.), Vol. III, Academic Press, New York, N.Y., U.S.A.

7. Mauron, J. 1970. Nutritional evaluation of proteins by enzymatic methods. In Evaluation of novel protein products (A.E. Bender et al., eds.). Proceedings of a symposium held in Stockholm, Sweden, 1968. Pergamon Press, New York, N.Y., U.S.A.

8. Mitchell, H.H., and R.J. Block. 1946. Some relationships between amino acid contents of protein and their nutritive value for the rat. J. Biol. Chem. 163:599-620.

9. Block, R.J., and H.H. Mitchell. 1946. Correlation of the amino acid composition of proteins with their nutritive value. Nutr. Abstr. Rev. 16:249-278.

10. Oser, B.L. 1951. Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietet. Assoc. 27:396-402.

11. Oser, B.L. 1959. An integrated essential amino acid index for predicting the biological value of proteins. In Newer methods of nutritional biochemistry (A.A. Albanese, ed.), Vol. I. Academic Press, New York, N.Y., U.S.A.

12. NAS/NRC. 1963. Evaluation of protein quality. Publication 1100. National Academy of Sciences/National Research Council, Washington, D.C., U.S.A.

13. FAO/WHO. 1973. Energy and protein requirements. Report of a Joint Expert Committee. FAO Nutrition Meetings Report Series No. 52; WHO Technical Report Series No. 522. Food and Agriculture Organization, Rome, Italy, and World Health Organization, Geneva, Switzerland.

14. Campbell, J.A. 1961. Methodology of

protein evaluation. PAG Document R. 10/ Add. 37. Protein Advisory Group of the United Nations System, United Nations, N.Y. 10017, U.S.A.

15. AOAC. 1965. Official methods of analysis. Tenth ed. Association of Official Agricultural Chemists, Washington, D.C., U.S.A.

16. Bliss, C.I. 1952. The statistics of bioassay. In Vitamin methods, Vol. II. Academic Press, New York, N.Y., U.S.A.

17. Hegsted, D.M., and Y. Chang. 1965. Protein utilization in growing rats. I. Relative growth index as a bioassay procedure. J. Nutr. 85:159-168.

18. Thomas, K. 1909. Biological value of nitrogenous substances in different foods. Arch. Anat. Physiol., pp. 219-302.

19. Allison, J.B., J.A. Anderson and R.D. Seeley. 1946. Determination of the nitrogen balance index in normal and hypoproteinemic dogs. Ann. N.Y. Acad. Sci. 47:245-271.

20. Allison, J.B. 1959. The efficiency of utilization of dietary proteins. In Protein and amino acid nutrition (A.A. Albanese, ed.). Academic Press, New York, N.Y., U.S.A.

21. Mitchell, H.H. 1924. A method of determining the biological value of protein. J. Biol. Chem. 58:873-903.

22. Mitchell, H.H., T.S. Hamilton, J.R. Beadles and F. Simpson. 1945. The importance of commercial processing for the protein value of food products. I. Soybean, coconut, and sunflower seed. J. Nutr. 29:13-25.

23. Njaa, L.R. 1963. A study on the Mitchell method for determination of the biological value of protein. John Griegs Boktrykkeri, Bergen, Norway.

24. Irwin, M.I., and D.M. Hegsted. 1971. A conspectus of research on amino acid requirements of man. J. Nutr. 101:539-566.

25. Oser, B.L. 1970. The safety evaluation of new sources of protein for man. In Evaluation of novel protein products (A.E. Bender, et al., eds.). Pergamon Press, New York, N.Y., U.S.A.

26. NAS/NRC. 1966. Toxicants occurring naturally in foods. Publication 1354. National Academy of Sciences/National Research Council, Washington, D.C., U.S.A.

27. Oser, B.L., and M. Oser. 1956. Nutrition studies on rats on diets containing high levels of partial ester emulsifiers. II. Reproduction and lactation. J. Nutr. 60:489-505.

28. United States Food and Drug Administration. 1959. Appraisal of the safety of chemicals in food, drugs and cosmetics. Assoc. Food Drug Officials United States, Austin, Texas.

29. NAS/NRC. 1970. Evaluating the safety of food chemicals. National Academy of Sciences/National Research Council, Washington, D.C., U.S.A.