EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

REPORT OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON THE USE IN ANIMAL FEED OF PROTEIN-RICH BIOMASS DERIVED LARGELY FROM CELLS OF METHANOTROPHIC BACTERIA GROWN USING NATURAL GAS AS A CARBON SOURCE

(adopted on 22 October 1999)

1. TERMS OF REFERENCE

The Scientific Committee for Animal Nutrition (SCAN) and the Scientific Committee for Food (SCF) are requested to give their opinion on the following questions:

(1) Does the protein-rich product of fermentation from natural gas obtained by the culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans* and *Bacillus firmus*, the living cells of which have been killed, have a nutritional value for piglets, pigs for fattening and chickens for fattening because it provides nitrogen or protein?

(2) Does the use in these animal species of the protein-rich product defined above pose any risks for humans (consumer or user) or to animal health?

2. BACKGROUND

The use in animal nutrition of protein-rich products derived largely from bacteria of the *Methylococcaeae* has been examined on two previous occasions jointly by SCAN and the SCF. The first opinion, published in September 1985, considered the use of a product "Pruteen" produced by growth of a *Methylophilus* sp. in methanol. This was followed in 1993 by a request for registration of a second protein-rich product "BioProtein" which consisted of the heat-killed cells of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans* and two species of *Bacillus* (*B. brevis and B. firmus*) grown using natural gas as carbon source. SCAN and SCF considered this in 1994 and, after supplementary data requested by the two committees had been received, an opinion was expressed on 28th April 1995. BioProtein, in modified form, is also the subject of the current opinion.

In the opinion published in 1995, SCAN and the SCF concluded that BioProtein had an acceptable, but not exceptional, value as a protein source in animal nutrition. The product was considered to carry no microbiological risks or any appreciable risk to livestock, provided that the maximum level of incorporation in rations judged prudent by the Committees was not exceeded. No risk to individuals consuming the products of animals fed the protein source was identified and there were no adverse effects on the organoleptic quality of animal products. It was recognised that, in common with most other protein products, there was a risk to the health of workers

of sensitisation by inhalation and the possibility of respiratory allergic reactions in susceptible individuals. In the view of the committee, this risk could be minimised if normal precautions were taken when handling the product.

However, because a growth depression was noted in some target species fed high concentrations of BioProtein and because of an absence of data on the metabolism of cyclopropanoics and other non-identified fatty acids present in the product and possibly present in the animal product lipids, the Committees judged that it would be prudent to limit the conditions of usage as follows:

– Growing pigs from 25 kg up to a 100 kg live weight.

– The quantity of inclusion in complete feedingstuffs not to exceed:

- 8% for piglets starting at 25 kg
- 8% for veal calves starting at 80 kg
- 19% for salmon fish in fresh water
- 33% for salmon fish in seawater

It was also recommended that the amount of protein provided by the product should be expressed as percentage of the total protein content of the compound feedingstuffs and declared on the label or packaging.

The present opinion considers a supplementary Dossier on BioProtein produced in support of an extension of approval in the annex of Directive 82/471/EEC to include chickens for fattening and pigs from piglets to slaughter weight. Notification is also included of small changes in the chemical composition of the final product thought to result from an increase in steady-state growth rate during fermentation and the exclusion of *Bacillus brevis* from the inoculum. Primary consideration is given to the new data provided and reference is made to data previously considered only when this has a bearing on the present extension of use.

3. OPINION OF SCAN

3.1. Changes to the method of production

A typical analysis of the natural gas used for the production of BioProtein shows that methane provides 91% by volume, ethane 5%, the remainder being hydrocarbons of higher molecular weight. Methanotrophic bacteria such as *Methylococcus capsulatus*, oxidise methane for energy and biomass production with carbon dioxide as end product. Ethane and the higher hydrocarbons are partially oxidised to the corresponding alcohol, aldehyde and acid, but are not utilised by methanotrophs for growth. Metabolic intermediates from the higher hydrocarbons and products of cell lysis are inhibitory and have to be removed from the fermentation. This is achieved by inclusion of the heterotrophic bacterium *Alcaligenes acidivorans*. Under steady state conditions, the fermentation consists of approximately 88% methanotrophic and 12% heterotrophic bacteria.

In the original formulation, two species of *Bacillus* (*B. firmus* and *B. brevis*) were included as heterotrophs able to complement the activity of *Alcaligenes acidivorans* and to occupy any nutritional niche which would favour growth of other contaminating spore-forming bacteria. Strains of *B. brevis* are known to produce the peptide antibiotics gramicidin and tyrocidine. Although the strain originally used in the production of BioProtein apparently did not produce detectable concentrations of these antibiotics, routine testing for their presence would have been needed as part of quality control. In addition, recently developed mammalian cell cytotoxicity assays suggest that some strains of *B. brevis* produce enterotoxins damaging to human health (Beattie and Williams, 1999). This possibility was recognised in the previous SCAN opinion and led to a request for an oral test for toxin production. Exclusion of *B. brevis* from the inoculum was found to make no apparent difference to the stability of the fermentation or the composition of the final product. Consequently, this strain has been omitted from the product in its present formulation. The second *Bacillus* strain, *B. firmus,* which is not considered a potential pathogen, has been retained and contributes approximately 1% by weight to the final product. Heat-killed cells of *B. firmus* have been reported to be immunomodulatory in the pig and to stimulate anti-infectious immunity (Trebichavsky *et al.*, 1997).

3.2. Chemical composition

The gross composition of the present formulation is little changed from the earlier product. The mean crude protein value (N x 6.25) is 70.6% with a range of 69.3-71.7% although this overestimates the true protein content because of the presence of nucleic acids. Mean values for the other major constituents are fat 9.8% (9.2–10.6%), ash 7.1% (6.9- 7.3%) and N-free extractive 11.8% (9.6-13.6%). The major difference between old and new formulations is the nucleic acid content and, consequently, the phosphorus content. The present product has a mean nucleic acid concentration of 10.2% (9.1-11.4%), which is substantially greater than that found in the product previously considered by SCAN and the SCF (approximately 7% of dry matter). This change is said to have resulted from a doubling of the steady-state growth rate (0.05 h^{-1} to 0.1 h^{-1}). It is a common observation that, at slow growth rates, a change in growth rate produces a shift in the RNA: DNA ratio (Nomura *et al.,* 1984). Virtually all of the observed increase in nucleic acid concentration was accounted for by an increase in RNA (from an average 4.8% to 7.9%), with the concentration of DNA staying essentially constant.

Any differences in fatty acid composition produced by the changes to the manufacturing process were minimal and, consequently, introduced no new issues relating to product safety. The presence of C15:0, C17:0 and cyclopropane fatty acids was considered by the previous SCAN/SCF who concluded that no accumulation of these fatty acids occurred in body fat of target species and that, in salmon, there was no evidence of interference with the synthesis of the nutritionally important n-3 fatty acids. The latter observation is of significance since the capacity of cyclopropanoics in which C9 or C10 is included in the ring to inhibit desaturase activity in mammals is well recognised (Fogerty *et al.*, 1972; Raju and Reiser, 1973). Membrane-

associated triterpenoid lipids (hopanoids) are reported to occur in some bacteria (Kannenberg and Poralla, 1999) including *M. capsulatus* (Tippelt *et al*., 1998). The full function of these hopanoids, their metabolic fate when ingested and any possible affects on the organoleptic qualities of animal products are unknown at this time.

The product description sets the upper limits for iron and copper of 310mg and 110mg kg^{-1} dry product respectively, although the observed mean values are somewhat lower. Both metals are essential to the functioning of a number of important metalloproteins and hence the growth of *M. capsulatus*. Methane monooxygenase (EC 1.14.13.25), the enzyme responsible for the initial hydroxylation of methane, occurs in two forms in *M. capsulatus*. The particulate, membrane-bound form is Cu (I) dependent with 12-15 copper ions per protein sub-unit (Nguyen *et al*., 1998) while the soluble form contains only iron (Fe (II)/Fe (III)) (Davydov *et al*., 1999). The fact that these two metals are integral to the product and cannot be readily reduced in concentration has given rise to some small concern. In particular, the feeding of BioProtein to lambs and sheep is recognised as inadvisable because of the copper content.

3.3. Nutritional value and studies with target species

The nutritional characteristics of the earlier BioProtein product were described in the previous SCAN/SCF report and compared to other protein sources commonly used in animal feed. Although the true digestibility of protein determined in the rat was lower (78.5%) than other protein sources, BioProtein was considered to have a high biological value and a well-balanced amino acid profile. Any concerns introduced by the low availability of cystine were considered to be offset by the higher content of digestible methionine. The composition of the present formulation does not differ greatly from that considered previously and these conclusions are considered equally applicable to the new product.

3.3.1. Pigs of all ages

The previous SCAN/SCF report reviewed data on protein digestibility in pigs (72%), a net energy determination (8.96 MJ kg^{-1}), and the results of a 28 day feeding trial with weaned piglets (initially of 28 days of age) when 4, 8 or 12% of the product was included in the diet. Inclusion of the product to a maximum of 8% of diet, as a replacement for other sources of protein, did not produce any adverse effects on performance or the health of the piglets. Feed conversion was less efficient when BioProtein was included at 12% of diet.

With respect to the extension of use sought for the product, new data has been provided by the Company from two production experiments. The first made with growing-finishing pigs and the second with weaning piglets completes the experimental coverage of the period from weaning to slaughter. The two studies made with grower-finisher pigs each involved 48 animals divided into six experimental groups taken from about 25kg to slaughter at approximately 100kg liveweight. In the first study, animals were restriction fed a barley-soybean diet providing 15% or 17% crude protein, or

the same diets with BioProtein replacing half of the soybean lysine or all of the soybean meal. In the second experiment, the basal diet was based on barley, tapioca meal and soybean providing 15% crude protein with test diets in which BioProtein again replaced half of the lysine from soybean or all of the soybean meal. In this study half of the animals were restriction fed whilst the remainder had free access to feed.

In the first experiment, animals fed BioProtein as a total replacement for soybean had a significantly ($P<0.05$) reduced daily weight gain compared to control animals over the first eight weeks. This difference was less evident from eight weeks onwards but the overall result was that the experimental animals took on average an additional seven days to reach slaughter weight. This difference was not seen in the second experiment in which there were no significant differences between control and experimental groups. No adverse effect on the health of animals was seen in either experiment and there was no effect of treatment on liver weight.

Substitution of 4, 8 or 12% BioProtein for the protein sources (soybean meal, fish meal, meat and bone meal) of a conventional weaning diet resulted in a significantly ($P<0.05$) higher feed intake by piglets fed at the higher levels of substitution compared to controls. However, although piglets fed diets containing 8 and 12% BioProtein had a numerically higher average daily weight gain this did not reach significance and overall there appeared a small negative effect on the efficiency of feed conversion.

Data on the ileal and whole tract digestibility of nitrogen and individual amino acids in 35kg pigs is presented in the Dossier prepared in support of the extension of use. However, it is evident from the published version of this data (Skrede *et al*., 1998) in which mention is made of *Bacillus brevis*, that this work was done with the older formulation of BioProtein and has already been reviewed in the 1995 opinion.

3.3.2. Chickens for fattening

Data from three types of studies are presented in support of the use of BioProtein in diets of chickens for fattening – production trials, a feed preference study and data on nitrogen and amino acid digestibility. The latter formed part of the work reported by Skrede *et al*. (1998) and was based on the older formulation.

A preliminary feeding study was made with 120 chicks assigned to each of six treatment groups. These were fed a cereal-soybean basal diet or test diets in which BioProtein at 2, 4, 6, 8, or 10% of diet progressively substituted for the soybean. Small adjustments were also made to the content of wheat and rendered fat to provide the same proximate composition and metabolisable energy content. Inclusion of 6-10% BioProtein significantly ($P<0.05$) reduced weight at 21 days and 8-10% inclusion at 35 days. Feed to gain ration was significantly improved by the presence of BioProtein at concentrations >2% which implies some reduction to intake although intake data was not provided. Similar responses were seen in a larger scale trial. This involved 1024 day-old chicks divided between four high protein diets (~23% crude protein) formulated to meet the recommendations for commercial starter

feed, and four low protein diets (~21% crude protein) corresponding to a grower/fattening feed. In both diet groups, soybean in the control diet was replaced by 3, 6 or 9% BioProtein. The presence of 6-9% BioProtein in the high protein diet and 3-9% in the low protein diet significantly ($P<0.01$) depressed weight gained after 14 days. Older birds, however, evidently better tolerated BioProtein. After five weeks there was no difference in the weight of birds fed 6% BioProtein compared to control birds, although 9% inclusion still resulted in significantly ($P<0.001$) lower liveweights. Feed intake was also lower in the presence of BioProtein but this depression only reached significance ($P<0.01$) with 9% BioProtein. Little effect on feed conversion efficacy was seen, except in the presence of 9% BioProtein when there was a significant ($P<0.01$) improvement.

The palatability of feed containing BioProtein was further assessed in a preference test. Day-old chicks (180) were offered in one pen a choice between a low protein diet or a test diet in which 6% BioProtein substituted for part of the soybean component. Birds in a second pen were given the same low protein diet containing 3 or 9% BioProtein. No diet preferences were observed over a 24-day period.

The apparent digestibility of individual amino acids was measured in four adult birds fed a diet containing BioProtein as the sole source of protein. Amongst the essential amino acids, arginine showed a significantly ($P<0.05$) higher mean digestibility (86.2%) than the total amino acid nitrogen (80.5%). Significantly lower digestibility values were observed for phenylalanine (73.8%), threonine (75.8%), tryptophan (75.4%) and a number of non-essential amino acids.

3.4. Product safety – cyclopropane fatty acids

The fatty acids of lipid tissue samples taken at slaughter weight of chickens fed diets containing 0 - 9% BioProtein and pigs fed diets containing 0 - 10.7% BioProtein were analysed for cyclopropane fatty acids. Both species evidently metabolised cyc17:0 fatty acids since neither species accumulated cyclopropanoics in storage fat. Pigs appeared more effective than chickens as concentrations detected in body fat were at the limits of detection and were independent of intake. Concentrations of cyclopropane fatty acids in the depot fat of chickens fed BioProtein were greater than in birds fed diets free from the product and, although low, related directly to intake.

3.5. Product quality

A sensory analysis of meat from chickens fed diets containing BioProtein found significantly ($P< 0.05$) less intensity of odour and a less rancid flavour compared to meat from chickens fed the control diet without BioProtein. No other product attributes differed significantly. Carcasses of pigs slaughtered at approximately 100kg were evaluated one day after chilling. No significant differences in dressing percentage, meat area in cutlets or percentage lean meat was seen in animals fed diets containing BioProtein when compared to control animals. However, a significant ($P<0.05$) increase in backfat thickness and improvement in fat firmness and colour was found in animals whose diet contained BioProtein.

3.6. Other aspects of consumer and environmental safety

Microbiological and environmental safety, the results of feeding studies in laboratory animals and the results of various *in vitro* toxicity tests were assessed by SCAN and the SCF and their conclusions published in 1995. Based on the information available at that time, it was concluded that the product posed no appreciable risks for livestock, individuals consuming the products of animals fed BioProtein, those handling BioProtein, or to the wider environment. No additional information from the company, from scientific literature published subsequently, or from elsewhere would suggest a need to revise or modify these conclusions.

3.7. Conclusions and recommendations

3.7.1. Product composition and safety

SCAN is of the opinion that:

omitting the strain of *B. brevis* from the fermentation is a sensible precaution and one that has no adverse implication for any previous assessment of product safety.

the changes to the composition of the product do not introduce any previously unrecognised risks and do not warrant any revision of the previous assessment of product safety. The proposed change to the Declaration of Contents to replace "not for ewe milk replacers" with "not to be fed to sheep or lambs" is considered an improvement and an added safeguard.

the slightly higher concentration of iron in the new product poses no danger to veal calves

3.7.2. Extension to conditions of use

The product was well tolerated and utilised by pigs and poultry and no significant problems of palatability or effects on the health of target species were detected. However, it is evident that at the higher inclusion levels tested, BioProtein can result in a depression of growth and a reduction in feed conversion efficiency. SCAN therefore recommends that the inclusion of BioProtein in the total feed should not exceed:

– 8% for piglets and fattening pigs to slaughter weight

– 6% for chickens for fattening.

SCAN recognises that at, or close to, these maximum inclusion levels small negative effects on intake, growth or feed conversion efficiency may occur with some diets and feeding systems. The maximum inclusions levels are intended to provide the flexibility to define an optimum concentration, whilst ensuring that should this optimum be exceeded any unforeseen reduction in performance is small and not detrimental to the health and wellbeing of the target animal.

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