4.
Improving Crop and Animal Growth

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BOVINE SOMATOTROPIN

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ABSTRACT

The development of a new agricultural product based on recombinant DNA technology requires the coordination of multiple scientific, engineering, regulatory and legal efforts. Where competitive factors make rapid progress essential, extensive use is made of external as well as internal expertise. The development of bovine somatotropin illustrates the complexities and pitfalls inherent in such a project and the approaches Cyanamid has used to deal with them.

I am pleased to be with you today to talk about a global research and development project involving a product from the recombinant DNA technology that is presently involving five Cyanamid locations, seven universities in the U.S. and overseas, six contract research agreements with private companies and several consultants, largely from academic institutions.

In research, one can distinguish between discovery, which can be called the act of invention, and innovation or development which can be defined as the actions which lead to commercialization of the discovery. Cyanamid's agricultural, chemical, and medical research organizations are divided into discovery and development functions, and certain criteria must be met before a project moves from one phase to another. Since the basic scientific capability, particularly in molecular biology, was not present within Cyanamid at the time of this project's inception and since time was of the essence, the company availed itself of outside technology to complement internal resources. Thus, the initial inventions came from outside rather than from in-house discovery organizations. The purpose of this paper is to exemplify the act of innovation by describing the actions taking place in American Cyanamid Company that will lead to the commercialization of bovine somatotropin in dairy cows. Hopefully, this will suggest or stimulate academic institutions and upcoming research companies into thinking about new ways to move their discoveries into the marketplace.

First, a little history on bovine somatotropin. Its effects on body growth and increase in milk production have been known for 50 years. Increased milk production has been identified as the largest potential agricultural market from the new genetic engineering technology; global sales projections have been as high as one billion dollars and 100,000 kg of bovine somatotropin. Because of this, it has attracted at least six industrial and pharmaceutical companies who are working in this area. It also is recognized that it is a high risk project because the various governmental agencies have treated milk, if you will pardon the pun, as a "sacred cow", and because of the

excess milk production that exists in the United States and Europe. Production surpluses are not a unique situation in the development of new products to improve agriculture, but milk production has been influenced as much by politics as by economic conditions in the market. In the U.S., we have price supports, and in the EEC, quota systems. These potential problems have not deterred many companies because bovine somatotropin works every time in dairy cows; increases in milk production as high as 40% have been realized.

Before I get into the details of the project, I want to talk a little about the human safety aspects of bovine somatotropin, also known as bovine growth hormone, as this appears to be a major concern of many people on the continent. They are lumping all hormones into the same basket.

Bovine somatotropin, a peptide hormone, should not be equated with steroid hormones when considering the implications of the use of hormones in food-producing animals for the following reasons:

1. Steroid hormones, estrogens and androgens are active when given orally. Peptide hormones, such as somatotropin, are not active when given orally. Proteins are digested by endogenous proteases into small peptide fragments and to amino acids in the gastrointestinal tract.

2. Steroid hormones are active across a wide range of species. Somatotropins are largely species specific. Bovine somatotropin has been shown to have little or no activity in humans or other primates when administered parenterally.

3. Some steroid hormones have been shown to be carcinogenic when administered to rats and mice in chronic toxicology studies.

Of course, some of the steroids, such as estradiol, are natural hormones found in mammals, including humans. The increase in levels of these natural endogenous hormones in edible tissues of treated steers is miniscule compared with the human production of endogenous estrogens.

The somatotropin saga in American Cyamanid's Agricultural Division really began several years ago with the strategic decision to place major emphasis in the discovery area on Nutrition and Physiology with the objective of improving animal production efficiency and carcass quality and increasing milk production. In addition to the somatotropins, major efforts are underway with animal hormone-releasing peptides, partitioning agents that result in leaner meat, and antibiotics. Cyanamid's discovery people are working with compounds obtained from synthetic chemistry, soil antibiotic discovery programs, peptide synthesis and, most recently, recombinant DNA technology. With the advent of the latter technology, it became apparent that the somatotropins could be produced in large quantities and at low cost and would have a major impact on animal performance, a key strategic area for us. Until recently, the supply of the somatotropins has been limited, since these substances could only be ontained by extraction from

pituitary glands of sluaghtered animals and at a lower purity.

All of you, I'm sure, are familiar with the basic prinicples of protein production using recombinant DNA, in which a plasmid containing foreign DNA and a suitable expression system is introduced into a microorganism. The key components of the plasmid are shown in Figure 1. In Figure 2, a schematic representation of the processes involved leading to the biosynthesis of protein is illustrated.

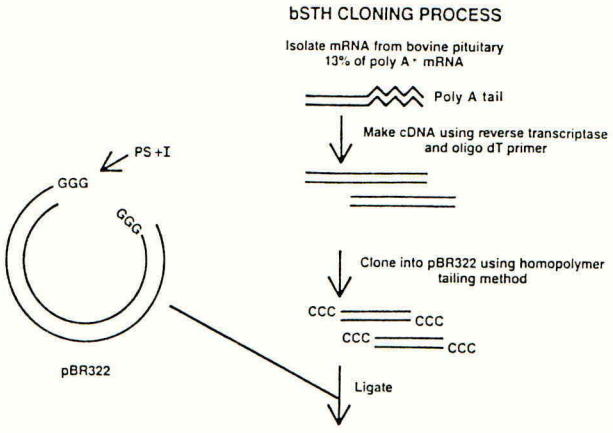

Fig. 1a

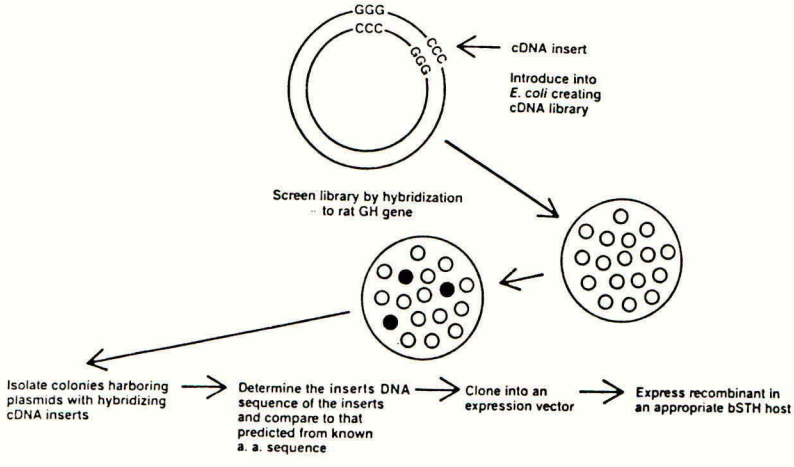

Fig. 1b

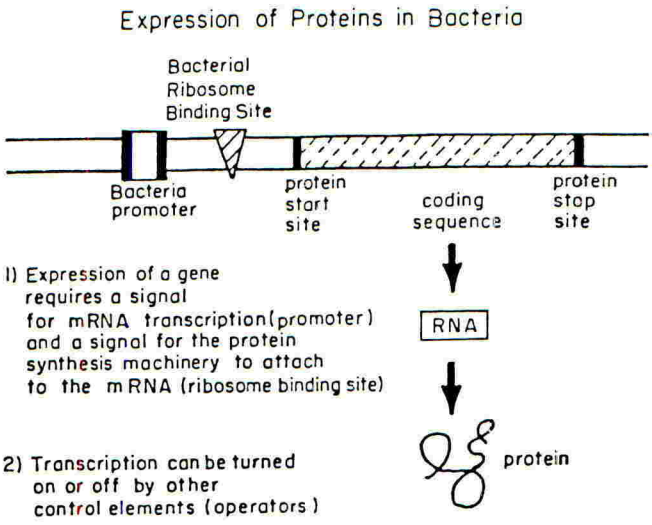

Fig. 2

It was apparent that we had a late start on this project, and because of the absence of an in-house molecular biology department, it was unlikely that we could carve out a major proprietary position without outside help. We, therefore, proceeded to use these outside sources of technology, and we found an ample supply of companies wanting to work with us. This approach works in many areas, as no matter how large the research budget is, industrial firms can't pursue all attractive projects.

After having had working relationships with two genetic engineering companies, a new relationship was established with a firm that, in addition to high producing strains, had an immediate supply of biosynthetic bovine somatotropin. The lack of product had prevented our initiating lactation, target animal safety, and sustained-release delivery studies. Thus, a contract was negotiated starting in September 1983 and signed in December 1983 with Biotechnology General, a biotechnology firm in Rehovot, Israel founded by staff members from the Weizmann Institute. With first-generation clones, a feasible laboratory isolation process, and the ability to supply somatotropin, they brought tremendous assets to the effort. We critiqued the technical and economical feasibility of the entire process, and evaluated potential patent problems. Data were also available that showed their recombinant bovine somatotropin had equivalent activity as measured by milk production to the natural product. It has turned out to be an excellent marriage between two companies - one very strong in molecular biology and protein chemistry and the other having a successful track record in developing agricultural products.

A comparison of the key factors impacting on the manufacture of human vs. animal products is shown in Figure 3. The human products are characterized by high value, low volume, and the delivery to the host patient of very small quantities. Production economics are generally not a controlling factor. Agricultural products are characterized by opposite attributes. Thus, the fermentation process must be run with Escherichia coli strains achieving high levels of expression and grown at high cell density to minimize the capital investment. The fermenter must achieve high oxygen transfer rates and high rates of heat transfer.

PRODUCTION FACTORS

SUBUNIT ANTIGEN, BLOOD FACTORS, INTERFERON		ANIMAL SOMATOTROPINS
LOW	PRODUCTION VOLUME	HIGH; 000'S OF KG
CAN BE HIGH	PRODUCTION COST	MUST BE LOW, $1 PER GM
LOW	NEW CAPITAL REQUIREMENTS	HIGH, UP TO M$100
MODERATE	GENE EXPRESSION	HIGH, 20%
COMPLEX; EXPENSIVE	GROWTH MEDIUM	INEXPENSIVE
BATCH, LARGE NUMBER OF STEPS TO OBTAIN HIGH PURITY	ISOLATION/PURIFICATION	CONTINUOUS, MINIMIZE CAPITAL INTENSIVE STEPS
SIMPLE	DELIVERY SYSTEM	COMPLEX

Fig. 3

Continuous fermentation improves productivity and reduces the peak oxygen transfer and heat transfer requirements. Expensive chemicals cannot be used for induction and cell kill. High throughput continuous centrifuges are required and expensive purification processes, such as gel and affinity chromatography, cannot be tolerated. Also, expensive enzymes and buffers must be eliminated, and high dilutions avoided. With these in mind, significant improvements have been made over the process disclosed in the Biotechnology General initial patent application. The major steps in the process are shown in Figure 4.

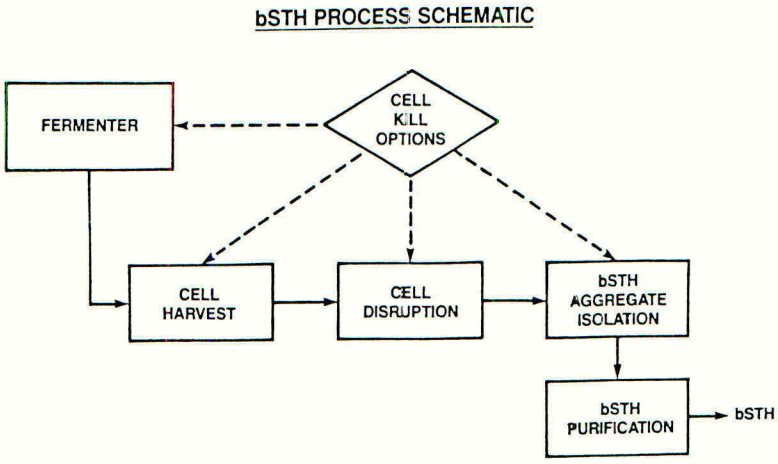

Fig. 4

As project manager, I have been required to coordinate the efforts at many locations and through several line organizations This can be the most difficult part of such a matrix management. Figure 5 indicates the major groups involved in this effort.

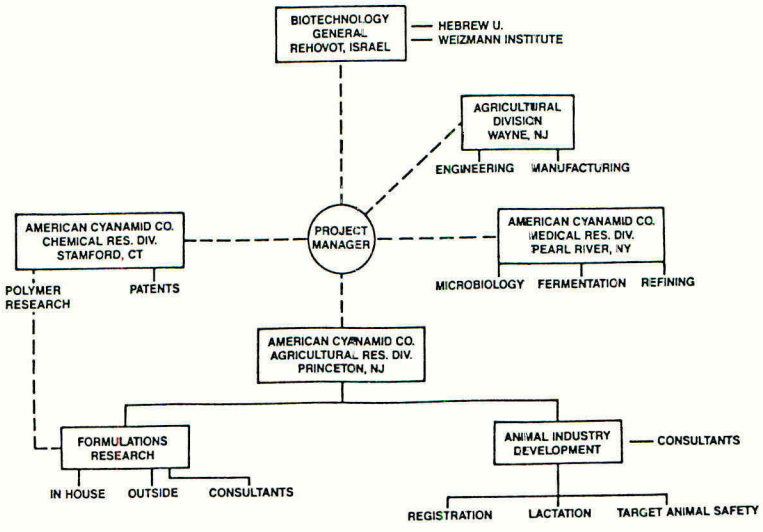

Fig. 5

Our bovine somatotropin research strategy is shown in Figure 6. During 1984, Biotechnology General continued to improve upon the expression plasmids and isolation-purification process, and supplied us with the required quantities of somatotropin from their pilot plant to conduct the trial work. At the same time, we were making major renovations at our fermentation and refining pilot plants in Pearl River, NY to satisfy the containment and process requirements. By the end of 1984, we were in a position to transfer the process technology from Biotechnology General to Cyanamid in a facility that was equipped with prototypes of commerical equipment, and in some units, to the size of equipment in a commercial plant.

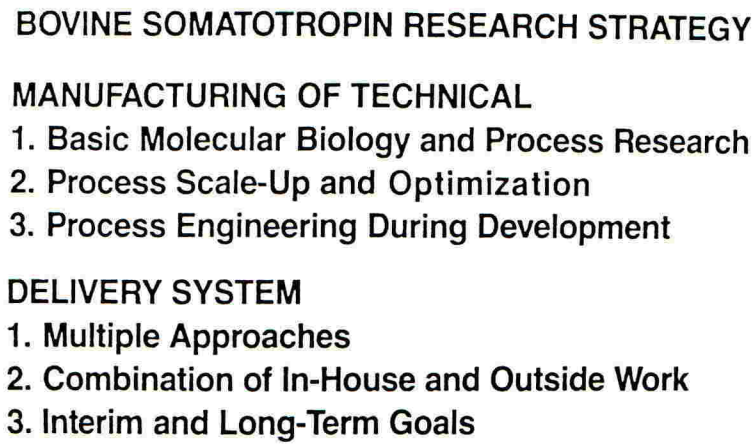

Fig. 6a

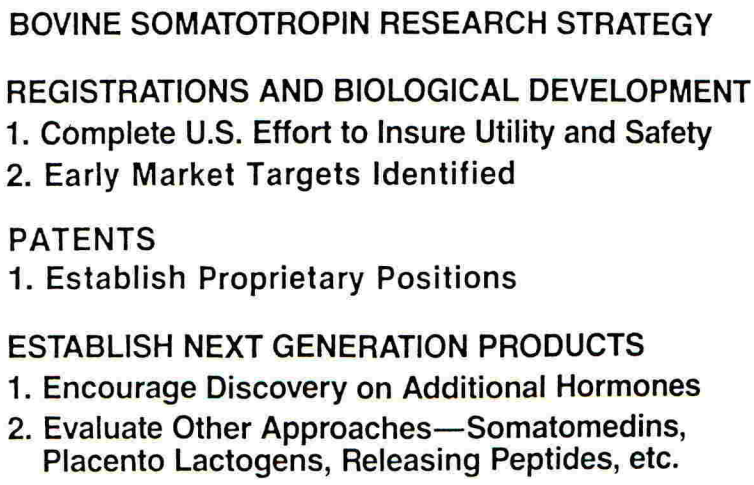

Fig. 6b

Biotechnology General has used the staff at the Weizmann Institute and Hebrew University as consultants and "hands on" work in genetic engineering and protein chemistry. At Pearl River, we have several consultants working on protein assays, protein purification and fermentation. In addition, we are working with numerous equipment suppliers as the scale of the commercial plant for bovine somatotropin will be the largest plant ever constructed for a product from recombinant DNA technology.

The Agricultural Division's Engineering Department was requested to develop material balance and equipment flow sheets based on pilot plant experience and estimate the capital investment of a world scale plant. With the information generated, we identified the capital intensive areas of the process and in one case, a piece of equipment that was not technically feasible on the scale of operation projected. Priorities in the pilot plant program were set to achieve our objective of being the minimum cost producer. The pilot plant in Pearl River continues to operate, producing product for the field trials and delivery system requirements and obtaining information on improving yields, scale-up and equipment reliability.

The bovine somatotropin delivery system could very well determine which company will have the dominant position in the market. Since a daily injectable is workable but not an ideal system for dairy farmers, a sustained-release system is a critical requirement. Our objective is to design a parenteral formulation capable of releasing bovine somatotropin to dairy cattle for up to 30 days. The task for such a development is formidable because of the criteria shown in Figure 7.

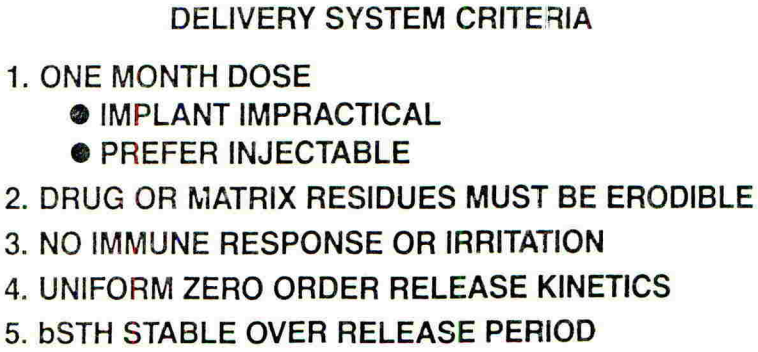

Fig. 7

We have taken a multi-prong approach because of the difficulty of the task and the projected utilization of somatotropins in other animal species. In addition to a group dedicated to this effort using several approaches at Princeton, Cyanamid's Chemical Group Laboratory in Stamford, CT and the Davis & Geck Laboratory in Danbury, CT are involved in the project. Also, we have contracted for research on delivery systems with six outside companies. Feasibility studies on other approaches have been done, and outside consultants used. Initial screening is done in hypox rats, secondary screening in sheep by monitoring blood serum levels, and the proof of the pudding is performance in the dairy cow. Patent applications have been filed on several of our systems.

We have obtained an INAD from the FDA and an Animal Test Certificate from the British regulatory agency, which means the milk produced during our lactation studies can be sold and used in human consumption. Lactation and target animal testing is being done at the Agricultural Center in Princeton, NJ, four full-scale lactation trials are underway at universities in the United States, two studies are in progress in Great Britain, and one in Canada. Critical items related to the lactation and safety studies are determining the long-term effects of administering bSTH to dairy cows. These items are illustrated in Figure 8. It is known that high-producing cows experience some of these problems. and it is expected at high increases in milk production from the use of bovine somatotropin, some of these responses will be observed. We feel good about the results obtained to date, and many more trials are scheduled to start this fall.

BIOLOGICAL DEVELOPMENT

MILK PRODUCTION

- Determine optimum bSTH daily dose; critical to design of delivery system.
- Determine nutritional requirements for increased milk production and returning the dairy cow to the correct energy balance at the end of the lactation period.
- Is burnout a problem, particularly at high doses? Number of lactation periods a cow will produce milk effectively.
- Quality of Milk
- Determine optimum cost-effective period during the lactation period for administering bSTH.
- Health problems, possible toxic effects.
- Immune response to bSTH.

Fig. 8a

BIOLOGICAL DEVELOPMENT

REPRODUCTION

- Breeding.
- Carrying calf to terminal pregnancy.
- Rebreeding.

Fig. 8b

The patent area for this new biotechnology is a potential minefield, and the number of patent applications appears to be rising exponentially. Patent filings made as long as ten years ago are still under examination.

As may be seen in Figure 9, many patents have been issued and applications filed on gene-expression systems, and attempts have been made to obtain dominating patents through broad claims. In large, the European and U.S. Patent Offices have not allowed broad claims and, thus, there appear to be a variety of efficient expression systems for a particular protein. The exceptions to this narrow view in claim breadth are very early applications, such as the Cohen-Boyer patents.

PATENTS

- GENE EXPRESSION SYSTEMS
  - c DNA
  - CONSTRUCTION PLASMIDS
  - EXPRESSION PLASMIDS
- COMPOSITION OF MATTER
- USE
- PROCESS
- DELIVERY SYSTEMS

Fig. 9

Composition-of-matter patents have been filed for numerous proteins in order to obtain a dominating position, but these nave been difficult to obtain. The patent offices appear to have taken the position that unless the recombinant protein has some unexpected advantages over the natural protein, claims will not be allowed. There have been a few exceptions to this, such as recombinantly-derived proteins difficult to isolate, characterize and study in the natural form.

Very few use patents have been filed and issued. A composition-of-matter patent is preferred and usually more enforceable, particularly since many of the uses of such proteins have been known for years for the equivalent natural products.

Process patents are generally of less value unless they can be policed through some unique property of the ísolated product. Also, alternative routes are usually available.

Sustained-release delivery systems will be major factor in who dominates the market, and a strong patent position will be critical in maintaining a competitive advantage.

As project manager, my major contributions have been to set priorities; insure that nothing drops in a crack; obtain tne physical, financial and personnel resources needed to carry out the project and, also, to convince our top management we have a winner. In addition to frequent informal meetings and visits with the people involved, a project meeting is held every other week in Princeton with representatives from the key areas to bring everyone up to date, resolve problems by appropriate actions, and set priorities. In addition, we plan meetings in Rehovot, Israel every 2-3 months and their key scientists visit us in between these visits.

We have now reached the stage that there is close involvement with Cyanamid marketing and manufacturing people on a global basis, inside and outside engineering organizations and, of course, we are heavily involved with the Patent Law Department and outside counsel since we live in a litigious society.

In summary, as shown in Figure 4, a broad range of institutions, consultants, and academics are playing a critical role in our project. We believe that this demonstrates both the complexities of innovation and the variety of talents and skills that can make a contribution in the process of innovation. Hopefully, this will suggest some new approaches to commercialization of biotechnology. For Cyanamid, it has also been an excellent training ground for the development of products from this new technology.

FERMENTATION PRODUCTS AS ANIMAL FEED ADDITIVES

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INTRODUCTION

Fermentation products have long been recognised as invaluable aids to the livestock producers of the world. As therapeutic antibiotics, such as penicillins and tetracyclines, they have been successfully used for individual animal treatment via injection, or for multiple animal treatments via drinking water or feed systems. These products were initially developed for animals as an extension to their use in human medicine. Over the past twenty years however, fermentation products have been introduced specifically for animals as prophylactic and most importantly as growth promoting agents. These products promote the efficient production of meat under intensive conditions. In this strict economic sector the fermentation derived products have to be considerably more effective than the older alternative synthetic products, as they are in general considerably more expensive to produce. Growth promoting and prophylactic agents require routine daily dosage to produce the effects; growth promoters are fed for life, prophylactic agents are given for between three days and life. The route via feed is the most practicable to provide this constant mass medication.

The U.K. broiler industry provides a good example of the reasons for feed medication. Over 400 million birds are grown each year, and most on farms having 100,000 or more birds. The feed route gives a dose related to the size of the bird, with no extra work for the farmer. Any other route would be more complex and costly.

It is possible for medicinal agents to be added via drinking water systems, but this is not a convenient route except for short term medication of therapeutic or prophylactic medicines. All other methods of administration, however sophisticated in terms of multiple dosing, are on a single animal basis.

Most growth promoters and prophylactics (the anticoccidials) are sold as free sale feed additives (FSFA). The E.E.C. regulatory system for FSFA dictates the products and levels to be used in feed for each species and controls the dilution steps in feed. The product distribution pathways follow accordingly. Therapeutic agents are controlled by National regulatory bodies and the veterinary surgeon plays a control role in their use. An E.E.C. veterinary directive is in existence and in future will impact on the national registrations and control of medicinal feed additives.

FEED MEDICATION TYPES

Animal feed medication can be separated into the above three types and, although these appear to be distinctly different, there is appreciable overlap.

Veterinary therapeutics: these are high potency antibiotics which are used in a manner similar to human pharmaceuticals.

Prophylactics: these vary from high potency wide spectrum to relatively weak narrow spectrum antibiotics, they are given not only to prevent the development of diseases and growth of parasites, but also to avoid detrimental effects on animal growth. In the major group, the anticoccidial agents, the antimicrobial effect is incidental to the antiparasitic effect, but can provide some growth promotional effect.

Growth promoters: the antimicrobial effects of these compounds alter the composition of the flora and fauna of the gut and hence promote better feed utilisation. In some cases this means faster growth but the main benefit is feed saving. These two parameters are normally measured by the rate of weight gain and feed conversion ratio or F.C.R. All of the current FSFA products are not used in human medicine and most have narrow ranges of activity.

The bulk of feed additives are used as growth promoters or prophylactic agents with over a thousand tonnes sold per annum in the U.K. alone. In terms of sales, the fermentation products outweigh synthetic chemicals by a ratio of over ten to one.

The following list gives some of the large number of additives in the marketplace, covering the varying disease challenges, housing and feeding conditions - especially for growth promotion in pigs.

Typical Feed Additives and Their Uses

Active Ingredient	Species	Use
Apramycin*	Pigs,	Antibacterial, vs. enteritis
Avoparcin	Pigs, Broilers, Cattle	Growth Promotion
Bambermycin	Poultry, Pigs, Cattle	Growth Promotion
Chlortetracycline*	Calves, Pigs, Poultry	Antibacterial, wide spectrum
Levamisole	Cattle, Sheep	Anthelmintic
Lincomycin*	Pigs	Antibacterial, vs. dysentery
Olaquindox	Pigs	Growth Promotion
Monensin	Broilers	Anticoccidial
	Cattle	Growth Promotion
Narasin	Broilers	Anticoccidial
Salinomycin	Broilers	Anticoccidial
Sulphamethazine*	Cattle, Sheep, Pigs,	Antibacterial, vs. atropic rhinitis
Tylosin	Pigs	Growth Promotion
*	Pigs	Antibacterial, prevention and control of dysentery
Virginiamycin	Pigs, Broilers, Turkeys	Growth Promotion
Zinc Bacitracin	All	Growth Promotion

* Items which require veterinary prescription

ANIMAL FEED TYPES

There are three broad types of feedstuff manufactured in the industry; mineral/vitamin supplements, protein concentrates and finished feeds; however, there is considerable variation within each type, especially in concentrates for cattle.

The mineral/vitamin supplement contains most of the essential minerals and vitamins. It is an integral part of the other two types of feed, and constitutes a 0.2 to 3 percent of the final ration. Medicinal additives especially growth promoters and prophylactics tend to be added at this stage.

The protein concentrate is a concentrated high protein ration which would form twenty to forty percent of the complete feedingstuff.

Finished feed is a complete ration which can be fed throughout life. It contains all the essential minerals, vitamins, proteins and other nutrients. A typical composition is given by the following example of a pig starter feed:

Ingredient	Percentage
Barley meal	13
Soya meal	16
Wheat meal	31
Wheatfeed	18
Maize meal	11
Fishmeal	3
Meat and Bone Meal	3
Fat	2
Limestone	1
Molasses	0.5
Mineral Vitamin Supplement	1.5
Declared analysis	
Oil	4.8
Protein	18.5
Fibre	4.6
Metabolisable Energy	11.6 MJ/kg

Finished feeds and concentrates are often pelleted. This prevents ingredient segregation problems and gives a higher density diet. Considerable feed wastage by some animals is also avoided. Virtually all feeds for chickens are pelleted, ensuring that their strict nutrient requirements are met. About fifty percent of pig rations are pelleted with about ten percent for cattle. The pelleting process involves treatment of the feed with steam and then the resulting hot wet mash is forced through a circular die by one or more rollers. The pellets are then cooled/dried in a counter current of air.

Medicinal additives can be added to all the types of feed and they will undergo the various mixing, transportation, and other processes to which the feed is subjected.

The table below gives approximate percentages of each type of feed which are purchased by meat producers in the U.K.; these contain the medicinal additives(s). The concentrates and supplements are incorporated into a complete ration. For pigs and some cattle this involves feed mixing on farm, but top dressing and free access are also used for cattle.

% Types of Feed Produced by Compounders for Farmers

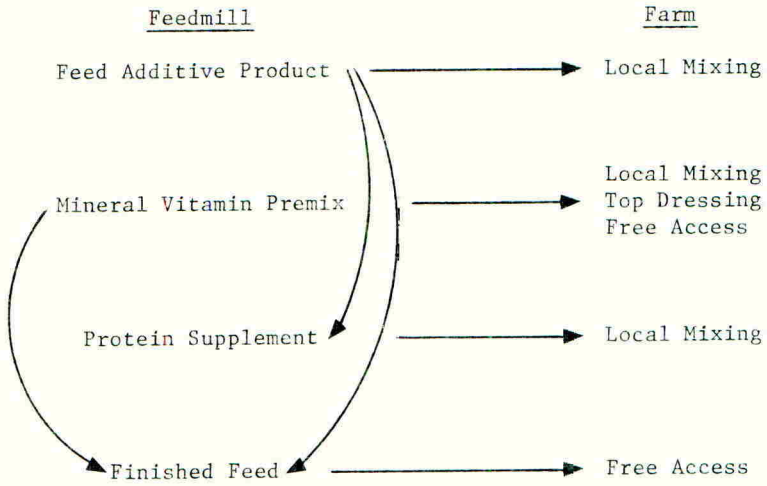
	Chickens	Pigs	Cattle
Finished Feed	100	50	20
Concentrate	-	30	30
Mineral/Vitamin Supplement	-	30	40
Blocks or Liquid Feeds	-	-	10

The differences not only reflect the differing intensities of production between these animals, but also the strict adherence to their nutri tional requirements. The balance of the feed for those animals fed concen trates/supplement etc. will be grass, silage or cheaper, locally available cereals/by-products.

INCORPORATION OF FEED ADDITIVES

The table below gives a breakdown of the chain of mixing steps which could be carried out involving feed additives.

Feed Medication Routes



The therapeutic drugs tend to be mixed directly into final feed, either on farm or at the feedmill. These drugs can only be used on prescription by a veterinary surgeon. The prophylactic and growth promoting agents tend to be mixed into the mineral vitamin supplements, which are then mixed into protein concentrates or finished feeds; normally at feedmills, but sometimes on farm. These feed compounding steps can be carried out on different sites by different manufacturers.

DEVELOPMENT OF A NEW ACTIVE INGREDIENT

The identification and development of a new medicinal feed additive is similar to that of a new pharmaceutical drug. One major difference is that it is possible to test the drug in the target species at an earlier stage, and follow on to limited efficacy studies in that species, prior to obtaining an Animal Test Certificate. A second major difference is that more extensive toxicology studies are involved to ensure safety to the target species, humans and to the environment.

There are again three main types of fermentation product developed for medication of animals via feeds, these are:

a) Purified fermentation products: normally crystalline materials recovered by solvent extraction procedures, e.g. lincomycin.

b) Chemically modified fermentation products: purified antibiotics, which have had a side chain cleaved off and then replaced with another, thus giving modified or improved efficacy, e.g. procaine penicillin.

c) Dried broths: these contain essentially all the solids remaining from fermentation, e.g. monensin. The bulk of the water is removed by:

   i) Filtration/centrifugation - this also removes aqueous soluble materials.

   ii) Total evaporation - using azeotropic, reduced pressure or spray drying technique.

The fermentation stages for these products are very similar. Pure cultured organisms are grown under sterile conditions in large stirred tanks (these can have capacities of 20,000 gallons or more) containing various nutrients in an aerated aqueous medium. Strict controls on parameters such as air-supply, temperature and pH are made to ensure maximum rate of growth. When optimum growth has been achieved the fermentation process is terminated.

The recovery processes for the purified and all the chemically modified products tend to be product specific and relatively expensive. These are the high potency antibiotics which are used for therapy.

The recovery processes for the dried broths have common features in that the initial fermentation broth is normally a weak slurry, containing live organisms, residual ingredients and by-products from fermentation. Residual lipids create particular problems relating to flowability. Free-flow throughout the drying process from slurry to dry powder is maintained by the addition of manufacturing aids, at what can be high levels. These also promote more rapid and efficient removal of water. The particular aid or aids are chosen to suit the form of raw material to be produced, but could be of vegetable origin, such as maize or soya by-products, or mineral, such as silicates or limestone. The dried broth type products fall into the growth promotion/prophylaxis areas and form the major group of feed additives. These products tend to be lower potency antibiotics with a narrower range of antimicrobial activity.

The raw material should be produced in a form:
(a) suitable for mixing into the various types of feedstuff,
(b) stable in all these feed types,
(c) as safe as possible for personnel involved at all stages from manufacture to end use. There is a trend towards granulation of medicinal active ingredients and products to improve their safety by reducing the level of dust and thus reducing the potential for dermal, ocular and inhalation toxicity. Safety of other species is also increased by reduced potential for carryover. Granulation can also improve stability and flowability; however, if the particle size is too large, toxicity or efficacy problems may ensue due to the increased probability of having high and low concentrations within the final ration. This effect will vary between species where cattle may eat twenty kg of feed per day and young chicks only ten grammes.

After the discovery, testing and pilot raw material production, the early development of a new medicinal feed additive involves the confirmation that the form of the raw material is satisfactory. This is done mainly by stability testing. The active ingredient within the raw material itself, and in the presence of certain feed components must show good stability under normal and some accelerated conditions. It must then show acceptable flowability and potential mixing characteristics.

MEDICINAL PREMIX PRODUCT DEVELOPMENT

Medicinal feed additives are normally marketed as premixes. These are simply a mixture of active ingredients and suitable edible inerts of cereal, vegetable or even mineral origin. (There are however examples of totally granular premixes, which are in effect standardised granulated raw materials). The following are typical examples of these edible inerts or premix diluents (sometimes known as carriers):

Limestone	Soya meal
Maize meal	Soya bean mill run
Maize gluten meal	Wheat middlings

These premix diluents vary widely in physical nature, nutritive value and cost. Not all diluents, however, are suitable for all products, or are universally acceptable to Regulatory Authorities. The following list gives the most important parameters used in assessing a new premix diluent, before any testing is undertaken.

1. Must be acceptable to Regulatory Authorities
2. Cost - must be reasonably low
3. Particle size - must be within acceptable range
4. Long term availability/quality - suitable quality material in adequate quantities must be consistently available.

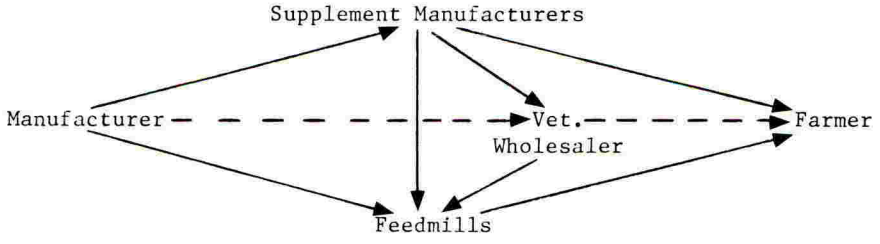
The premix development process initially involves the manufacture of small scale trial mixes for evaluation of appearance, dusting potential, flowability and segregation properties. The desired premix concentration varies widely between products and end uses e.g. virginiamycin 50% (chickens, pigs etc.) to halofuginone 0.6% (chickens), with alternative product concentrations often being available. Large customers tend to prefer higher concentration premixes to reduce their storage volume with lower concentration premixes being available to local or "on farm" mixers to improve product dispersability into feed . Lower concentration premixes also tend to be supplied when the final use level is low e.g. halofuginone premix (0.6%) provides a first step down to the recommended final feed level of 3 ppm.

Larger scale mixing and stability studies on the premix itself and on mixtures with mineral vitamin supplements, concentrates and feeds then follow. These studies provide confirmation that the medicated premix will be stable in its final pack for its declared shelf life, that the product will give homogenous mixtures at all stages of the mixing cycle and that unacceptable losses of active ingredient do not occur during feed pelleting or during the storage of intermediate or complete feeds.

DISTRIBUTION OF MEDICATED FEED ADDITIVES

The following table illustrates the routes of distribution of free sale feed additives from manufacturer to farmer.

Distribution of Medicated Feed Additives



The feed additive manufacturer normally sells to major feed supplement manufacturers. These manufacturers then pass on either the premixes, or supplements containing these premixes to their own feed manufacturing plant, smaller feed compounders, large farmers or veterinary wholesalers.

The medicated premix can be incorporated into mineral/vitamin supplements or protein concentrates at different sites and by different companies, prior to incorporation into finished feeds.

Veterinary prescription items follow similar pathways except that the major proportion of the premixes are sold direct to veterinary wholesalers and on to veterinary surgeons. These premixes are normally added direct to the finished feed on receipt of the prescription, without going via the intermediate supplement steps.

FUTURE DEVELOPMENTS

The medication of animals with fermentation products via feeds will continue to provide benefits in animal husbandry for some time ahead. In the existing range of products there will be an increased trend towards granulated products to reduce carryover of active ingredients into other rations within feedmills and also to reduce toxic hazards associated with dust. New fermentation and other products for existing and new uses will continue to be introduced despite increased regulatory and consumer pressure group demands, and development costs. These new products will show new/broader/increased efficacy leading to increased cost benefits for the farmer.

The introduction of specific growth hormones and similar products, beginning in the mid 1990's, is likely to give benefits in addition to those gained by current growth promoters and is thus unlikely to affect their sales; however, other developments of products such as monoclonal antibodies are likely to reduce prophylactic and therapeutic uses in the longer term.

MOLECULAR GENETICS OF SYMBIOTIC NITROGEN FIXATION

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The ability to fix atmospheric nitrogen by biological means is restricted to relatively few bacterial species. Many of these bacteria fix nitrogen, not alone, but in association with higher plants; in such a symbiotic arrangement the host obtains a supply of ammonia and the bacterium occupies a niche in which it receives the energy supply required to drive the energy-expensive reduction of ammonia. These symbiotic relationships include those between blue-green algae and the water-fern Azolla, those between the Actinomycete Frankia and various trees such as Alder and those between Rhizobium and legumes.

In molecular terms, very little is known of the means by which the prokaryote specifically recognizes and invades its respective host-plant. However, there has been a growing interest in approaching this problem by identifying and analysing the relevant symbiotic genes and their gene products. Because of the agricultural importance of the Rhizobium-legume interaction, the most detailed studies have been conducted on this system. Because bacteria are simpler than plants our knowledge of the symbiotic genes of Rhizobium is better than that of the plant genes. There have been several recent advances in the analysis of the Rhizobium genes that are involved in nodulation, host-range specificity and nitrogen fixation. By dissecting the structure, organization and regulation of these genes, a better understanding of their functions may be forthcoming.

In the fast-growing Rhizobium species, which nodulate temperate legumes such as clover, alfafa, peas and field beans, the genes for nodulation and nitrogen fixation are clustered on large symbiotic plasmids. One such plasmid, pRL1JI, was isolated from a strain of R. leguminosarum (which nodulates peas) and in this case the genes that determined the ability to nodulate peas were located between two groups of genes required for nitrogen fixation. The study of the nitrogen-fixation (nif) genes in Rhizobium has been greatly facilitated by the fact that at least some of them are very similar to the corresponding nif genes in the enteric free-living nitrogen-fixing bacterium Klebsiella pneumoniae.

In Klebsiella, 17 nif genes have been identified which are variously involved in nif gene regulation, the synthesis of the iron-molybdenum cofactor (FeMoCo) on which N_2 is reduced, electron transport and the structural nitrogenase enzyme proteins. Using DNA sequence comparisons, Rhizobium species have been shown to contain the nifHD&K genes (nitrogenase proteins) nifA (regulation) and nifB (synthesis of FeMoCo). Further studies may well demonstrate similarities between other Rhizobium and Klebsiella nif genes.

Concerning the genes (called nod genes) for nodulation and host-range specificity, an important observation is that, despite the morphological complexity of the infection process, only a few genes on the symbiotic plasmid are required for nodulation. In the R. leguminosarum symbiotic plasmid pRL1JI, less than 10kb appears to be required for nodulation and for the

determination of host-range specificity for peas. This region has been analysed by DNA sequencing (and thus identifying genes as long open reading frames), isolation and characterization of mutants and by studying the regulation of the nod genes.

AZOSPIRILLUM : A POTENTIAL BIOFERTILISER FOR GRASSES

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ABSTRACT

Nitrogen-fixing spirillum-like bacteria isolated from the root zone of many plants, in particular from grasses, have been classified in a new genus Azospirillum. Genetic analysis of nitrogen fixation was essentially initiated in A. brasilense Sp7, where some genetic tools and mutants are available. The nitrogenase structural genes (nifHDK) were cloned using homology to Klebsiella pneumoniae probes. Several mutants impaired in the regulation of nitrogen fixation were isolated and the role of the glutamine synthetase in the regulation of nif expression will be discussed. Very little is known on the molecular biology of the association of Azospirillum with plants. No differentiated structure is formed during the association, and host-bacterium specificity is difficult to establish. Effect of Azospirillum inoculant resulted in enhancement of lateral root proliferation that was attributed to phytohormone production. It is not clearly established as yet, whether the effect on crop productivity is due to nitrogen fixation or to growth hormone production.

INTRODUCTION

Azospirillum has attracted considerable attention as a possible source of biological fertilizer. The potential agronomic importance of the bacterium was raised when Döbereiner and Day (1976) reported its association with the roots of grasses from various geographical origins. The bacteria, first described in 1922 by Beijerinck and rediscovered in 1963 by Becking, were called under the name of Spirillum lipoferum. Taxonomic studies led to the creation of new genus: Azospirillum. This genus, defined by Tarrand et al. in 1978, comprises two species: A. brasilense and A. lipoferum. A third species A. amazonense was recently discovered (Magalhaes et al. 1984).

PHYSIOLOGICAL PROPERTIES

Physiological properties of Azospirillum spp. were recently reviewed (Okon 1985). The bacteria are gram-negative aerobes, curved rod shaped, with a polar flagellum and containing globules of poly-betahydroxybutyrate. They have a DNA base composition of 69-71 moles per cent G+C. The bacteria can grow on organic acids such as malate. Strains of A. lipoferum can utilize a large number of carbohydrates including glucose, which is not used by A. brasilense. A. amazonense can utilize saccharose. Studies on hydrogen metabolism in Azospirillum revealed the existence of a H_2-uptake hydrogenase activity. Autotrophy and methylotrophy were also demonstrated. Azospirillum spp participate in all steps of the nitrogen cycle except nitrification. In particular most strains are denitrifiers. Ability to fix nitrogen in pure culture was established by the ^{15}N isotopic method. Nitrogen fixation occurs only under microaerobic conditions.

OCCURENCE AND ROLE OF PLASMIDS

Plasmids of various molecular weight have been discovered in many diazotrophs e.g.: Azotobacter, Anabaena, Frankia, Rhizobium (reviewed by Elmerich, 1984). In most cases, phenotypes associated with plasmids are not known. In rhizobia, functions related to symbiotic nitrogen fixation were shown to be plasmid borne (see Johnston, this volume). All Azospirillum strains, examined so far, contained at least one plasmid (Franche & Elmerich 1981, Plazinsky et al. 1983). The two taxonomic groups, A. brasilense and A. lipoferum, cannot be differentiated on the basis of their plasmid content, and no phenotypic property was demonstrated as plasmid-borne. Plasmid location for nif genes was not observed in Azospirillum (Elmerich 1983, Plazinski et al. 1983). However, it is tempting to speculate that functions related to bacteria-plant associations might be present on Azospirillum plasmids.

GENETICS OF NITROGEN FIXATION

Most of what is known on genetics and regulation of nitrogen fixation was initially established in Klebsiella pneumoniae oxytoca M5a1. In this bacterium, a cluster of 17 nif genes, localized on the chromosome and organized in 7 or 8 transcription units has been identified (see figure 1) (reviewed by: Dixon 1984, Elmerich 1984). The structural genes for the nitrogenase nifHDK, are highly conserved as shown by DNA-DNA hybridization (Ruvkun & Ausubel 1980). Genes homologous to nifHDK were cloned from several diazotrophs including several rhizobia (Johnston, this volume).

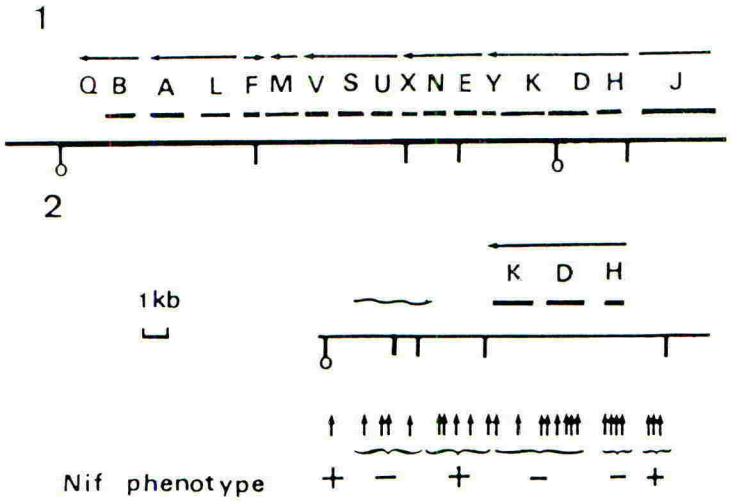

Fig. 1. Physical map and nif gene organization of K. pneumoniae (1) and A. brasilense Sp7 (2). Restriction sites : EcoRI; HindIII, horizontal arrows : transcription units, boxes : relative size of the genes products when known, vertical arrows : location of the Tn5 insertions which were isolated in the Sp7 genome; the corresponding Nif phenotype is mentioned below, waved line : newly identified nif loci.

Using the nifHDK cluster of K. pneumoniae as a probe, homology was detected with several strains of Azospirillum (Quiviger et al. 1982). With A. brasilense Sp7, the probe hybridized with a single 6.7 kb EcoRI fragment. Recently, a 5.8 kb fragment adjacent to the nifHDH cluster was cloned. Isolation of Nif$^-$ mutants was performed by Tn5 site-directed mutagenesis, by using the procedure of Simon et al. (1983). A set of Tn5 insertions were isolated in Escherichia coli in plasmid pSUP202, containing the cloned fragments carrying the nifHDK cluster and the adjacent region of strain Sp7. The localization of the Tn5 insertions obtained is shown in Figure 1. Plasmid pSUP202 cannot replicate outside enteric bacteria. Thus, Tn5 insertions were recombined in the Azospirillum genome and the resulting Nif phenotype was determined (see figure 1). As expected, insertions in nifH, nifD, and nifK led to a Nif$^-$ phenotype. Insertions in nifH were polar on nifD and nifK, as determined by genetic complementation and by gene-product analysis. Thus the nifHDK genes are transcribed as a single operon in Azospirillum. Some of the insertions in the 5.8 kb fragment led also to a Nif$^-$ phenotype suggesting, as in K. pneumoniae, the presence of nif genes in the region adjacent to nifHDK.

REGULATION OF NITROGEN FIXATION

In E. coli, Salmonella and Klebsiella, the structural gene for glutamine synthetase, glnA, belongs to a complex regulon (Merrick 1983, Dixon 1984). This regulon contains the ntrBC genes, the products of which are responsible for the transcriptional activation of a number of operons involved in nitrogen assimilation. Regulation of nitrogen fixation in K. pneumoniae, involves two mechanisms (Merrick 1983, Dixon 1984) : i) a nif specific regulation through the products of nifL and nifA, acting respectively as repressor and activator of the other nif operons ; ii) a non-nif specific regulation through the products of ntrBC genes. Some of the glutamine auxotrophs of K. pneumoniae have a Nif$^-$ phenotype, as a consequence of a mutation in the ntrBC genes or as a result of a polar effect of glnA mutation on ntrBC expression.

Azospirillum mutants impaired both in glutamine synthetase activity and nitrogen fixation were isolated (Gauthier & Elmerich 1977). In particular strain 7029 of Sp7 has a Gln$^-$ Nif$^-$ phenotype, and thus resembles to glnA or ntrBC mutants of K. pneumoniae. More recently, Pedrosa and Yates (1984) isolated Nif$^-$ mutants of Sp7 whose nitrogen fixation was restored by plasmid pGE10 which contained the glnAntrBC regulon of K.pneumoniae. The authors proposed that Azospirillum contains genes with functions analogous to K. pneumoniae ntrC gene.

In order to clone the glnA gene of strain Sp7, a library of total DNA of A. brasilense Sp7 was constructed in the broad host range vector pVK100 (Knauf & Nester 1983). A plasmid, designated pAB44, that restored by complementation a Gln$^-$ Nif$^-$ phenotype to the Gln$^-$ Nif$^-$ mutant 7029, was isolated. The glnA gene was localized on subclones of pAB44 by Tn5 insertion and DNA hybridization with a K. pneumoniae glnA specific probe. No expression of pAB44 was found in E. coli or K. pneumoniae Gln$^-$ mutants. However, plasmid mutants which restored glutamine independant growth were obtained in E. coli strain ET8051, that carries a glnAntrBC deletion. It is thus tempting to speculate that plasmid pAB44 carries the equivalent of the ntrBC genes. However, pAB44 did not complement the mutant strains isolated by Pedrosa and Yates. This raises the question of the

localisation of the ntrBC genes in Azospirillum. Further experiments are in progress to answer this question.

ASSOCIATION WITH PLANTS

Process of the association.

Azospirillum spp. were isolated from the rhizosphere of a large number of monocotyledon and of some dicotyledon plants. (see reviews by: Döbereiner & De-Polli 1980, Van Berkum & Bohlool 1980, Elmerich 1984). In most cases, the bacteria were isolated after surface sterilization of the roots. No differentiated structures were formed, but pictures of root hair deformation were reported (Patriquin et al. 1983). It appeared that Azospirillum could invade the cortical and vascular tissues of the host (Döbereiner & Day 1976, Patriquin & Döbereiner 1978). Studies on adsorption of A. brasilense strains to roots of Pennisetum americanum (pearl millet) revealed a preferential colonization by this species as compared to other genus (Umali-Garcia et al. 1980). Bacteria were located mostly in the mucigel. In addition, protease-sensitive nondialyzable-substances, which might be considered as "lectin-like" compounds, were isolated from the root exudates (Umali-Garcia et al. 1980). Azospirillum produced enzymes with pectinolytic activity, which might favor invasion of the root tissues (Umali-Garcia et al. 1980, Tien et al. 1981). After Azospirillum inoculation on plant roots a large enhancement of the number of lateral roots and of root hairs was observed, (Tien et al. 1979, Umali-Garcia et al. 1980, Kapulnik et al. 1981a). This proliferation was concomittant to an increase in mineral uptake (Lin et al. 1983), and was attributed to phytohormone production rather than to nitrogen fixation (Tien et al. 1979, Lin et al., Okon 1985). At the moment no real specificity of association between the bacteria and the plant has been demonstrated but there are indications that some specificity could exist. Baldani and Döbereiner (1980) showed that most of the strains isolated from maize were A. lipoferum and that most of the strains isolated from wheat or rice were A. brasilense Nir$^-$, suggesting a difference of specificity between the two species towards C_4 and C_3 plants (Döbereiner & De Polli 1980). Further experiments are required, in particular with bacterial mutants, to determine the basis of the recognition process.

Efficiency of the association.

Effect of Azospirillum inoculant on plant productivity gave variable results. Increase in dry matter and total nitrogen content was observed in a few cases, e.g. for pearl millet (Smith et al. 1976); maize (Nur et al. 1980, Rennie 1980); setaria (Nur et al. 1980); sorghum and wheat (Kapulnik et al 19881b). In other cases, non-significant or negative effects were noted (Smith et al. 1976, reviewed by Boddey & Döbereiner 1982). The ^{15}N isotope dilution method has been applied to estimate the amount of plant nitrogen derived from nitrogen fixation (% Ndfa), in a few cases (Rennie & Rennie 1983). For maize, inoculated with Azospirillum, a value of 12 % was reported (Rennie 1980). For some wheat genotypes the values determined ranged between 15 and 30 % (Rennie et al 1983). Thus selection of plant genotypes for increased nitrogen fixation should open fruitful perspectives.

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5.
Microbial Products in Crop and Animal Production

Chairman:

Dr J. R. CORBETT
FBC Ltd, Saffron Walden

POSSIBILITIES FOR USING GENETIC MANIPULATION TO PRODUCE NEW BIOACTIVE MOLECULES FROM STREPTOMYCETES

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Notwithstanding the great importance of filamentous fungi as producers of the classical penicillins and cephalosporins, members of the genus Streptomyces (and some other related genera of the Actinomycetales) continue to yield the vast majority of new bioactive molecules for use, not only in chemotherapy but, increasingly, also as agents with other agriculturally relevant properties. The avermectins (from S. avermitilis) represent a striking recent example of an important new class of compounds with novel applications as antihelminthic agents; bialaphos, a metabolite of S. hygroscopicus with herbicidal properties, may soon pioneer another new class of applications. Undoubtedly, we have not yet seen the full capacity of these filamentous sporulating bacteria to synthesise complex and useful organic molecules. Nevertheless, the isolation and screening of natural isolates as the sole means of discovering chemical novelty must inevitably be subject, eventually, to a law of diminishing returns. How can genetic manipulation help to widen the search for new bioactive molecules?

Ever since Behrens et al. (1948) showed that the type of penicillin produced by Penicillium chrysogenum could be altered by the feeding of unnatural side-chain precursors to the fermentation ("directed biosynthesis"), various other approaches have been taken to induce microorganisms to produce novel antibiotic compounds. These include "mutasynthesis" (reviewed by Daum & Lemke, 1979), in which an unnatural precursor is fed to an idiotroph genetically blocked in antibiotic biosynthesis; and "hybrid biosynthesis" (Omura et al., 1980), in which a wild-type strain is fed with such a precursor in the presence of an enzyme inhibitor (such as cerulenin for polyketide antibiotics) which phenotypically blocks antibiotic biosynthesis in the recipient strain, thus favouring incorporation of the fed compound.

A different approach is to produce, by recombination between two pre-existing strains, a novel culture capable of synthesizing a "hybrid" antibiotic without precursor feeding. Although it might in principle be simplest to generate such a recombinant by natural mating (which is normally a plasmid-mediated conjugation in Streptomyces: Hopwood et al., 1984) or by protoplast fusion (Hopwood et al., 1977), in practice recombination in inter-specific matings or protoplast fusions is found to be infrequent and unpredictable (in contrast to the situation with intra-strain fusions in which recombination is extremely frequent). Probably the main reason is the lack of close DNA base-sequence homology between species, so that recombination is very inefficient (but there may be other contributory factors such as DNA restriction or mutually lethal effects of the antibiotics produced by the two parents). Nevertheless, there are already a few reports of the production of new antibiotics by recombinants generated by matings (Schlegel & Fleck, 1980; Mazierès et al., 1981) or fusions (Hotta et al., 1985). A much more widely applicable route to the generation of recombinants between species (and even very distantly related organisms) uses recombinant DNA techniques, capitalising on the excellent possibilities for gene cloning on plasmid and phage vectors now available in Streptomyces (Hopwood et al., 1985b). These

allow the isolation of genes, including groups of linked genes for whole antibiotic biosynthetic pathways (Malpartida and Hopwood, 1984; Chater and Bruton, 1985), by direct shotgun cloning into Streptomyces hosts, as well as the comparatively easy transfer of the cloned genes into other antibiotic-producers on broad host-range plasmids or phages. The first examples of "hybrid" antibiotics to be produced by this approach (Hopwood et al., 1985a) involved the transfer of the whole set, or parts of the set, of genes for the biosynthesis of actinorhodin, cloned in S. coelicolor A3(2), into the producers of medermycin (Streptomyces sp. AM7161) or granaticin (S. violaceoruber Tü22) on SCP2*-based vectors to generate mederrhodin A and dihydrogranatirhodin. In one case the new antibiotic differed from that produced by the recipient strain by addition of a side group, while in the other the new compound was a hybrid molecule in respect of the stereochemistry of the chromophore.

Recombinant DNA techniques have many advantages over mating or protoplast fusion in the search for genuinely "hybrid" antibiotics whose structure results from the co-operation of biosynthetic enzymes coded by genes from two parents. Not least is the possibility of determining unambiguously the genotype of the recombinant strain and thus the contributions of donor and recipient to the structure of the novel metabolite, leading to useful predictions for further work. Even more important is the wide scope of the "crosses" that can be performed. Genes from other prokaryotes appear to be expressed from their own transcription and translation signals in Streptomyces (Bibb & Cohen, 1982). It is therefore very unlikely that significant barriers to expression will be encountered in gene transfers between various actinomycetes. The use of expression vectors which put the introduced DNA under the transcriptional or translational control of vector signals, and which are now being developed for Streptomyces, will soon allow the effective use in Streptomyces of genes also from lower eukaryotes (most of which are unlikely to contain introns: otherwise cDNA clones could be substituted for genomic clones). Thus the possibilities for the recombination of genes for antibiotic biosynthesis will become enormous.

In the published examples of novel antibiotics produced by mating or protoplast fusion, evidence that the new compounds were in fact "hybrid" compounds in the sense used above is slight. On the contrary, the most likely explanation (and this must certainly account for the production of novel rifamycins by the mating of closely related derivatives of the same strain of Nocardia mediterranei: Schupp et al., 1981) is an alteration of the regulatory controls present in one member of the partnership by the introduction into it of genetic material from the other. In this way, a compound not normally produced, but within the genetic capability of one strain to produce, is revealed. This explanation implies that actinomycetes have a considerable unexpressed capacity for antibiotic production. A striking illustration of the expression of a "silent" gene recently resulted from the transfer of DNA from a producer of actinomycin, S. antibioticus, into a non-producer, S. lividans. Either of two unrelated genomic fragments from S. antibioticus caused the expression of a normally unexpressed gene for phenoxazinone synthase in S. lividans (Jones & Hopwood, 1984).

The extent of the potential for revealing novel biosynthetic capabilities by activating "silent" genes through gene cloning remains to be seen. However, it may be significant that streptomycetes have some 2-3 times the amount of unique DNA sequences possessed by Escherichia coli or Bacillus subtilis (Antonov et al., 1978). Nor are the relevant genes necessarily totally "silent" under all conditions in the natural strain - otherwise it

would be hard to rationalise their continued presence generation after generation. It is more likely that they are expressed, but only at a low level and at a specific "window" in the life cycle, when their products may sometimes play a role as hormone-like signals, with effects on the morphogenesis of the colony. Thus these genes are normally under a very stringent regulation which makes their products inaccessible to the usual screening regimes unless regulation is perturbed by the introduction of foreign genetic material.

Genetic manipulation as an approach to the discovery of new bioactive compounds is still in its infancy. Undoubtedly it will lead to the discovery of significant numbers of novel compounds in the near future as its various possibilities are explored. It will be interesting to see what proportion of these new molecules are useful, and whether this proportion differs from that amongst natural compounds discovered by traditional methods.

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CHEMISTRY AND BIOLOGICAL ACTIVITIES OF AVERMECTIN DERIVATIVES

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ABSTRACT

The avermectins constitute a group of eight closely related macrocyclic lactones with potent antiparasitic activities. Their discovery, isolation and structure determination are reviewed. Hydrogenation of the 22,23-double bond led to ivermectin, used as an endo- and ectoparasiticide for animal health. Certain aspects of the structure-activity relationship of chemically modified avermectin derivatives are discussed. Avermectin B_1 is in development as an agricultural acaricide and insecticide. Chemical modifications of the structure resulted in a substantial increase of insecticidal potency against certain lepidoptera. The role of the avermectins in the GABA-modulated opening of chloride channels of the nervous system is reviewed.

DISCOVERY, ISOLATION AND STRUCTURE DETERMINATION

The contributions of microbiology to the practice of medicine through the discovery and development of a number of potent antibacterial antibiotics is widely recognized. A major effort concerned with the discovery of new structures with antibacterial activities and the chemical modification of existing natural products is still ongoing. Although these antibiotics have found widespread use as growth promotants in the animal health area, only more recently were microbial products produced primarily for animal growth or agricultural purposes, for instance monensin as a coccidiostat for poultry and a growth permittant for ruminant animals.

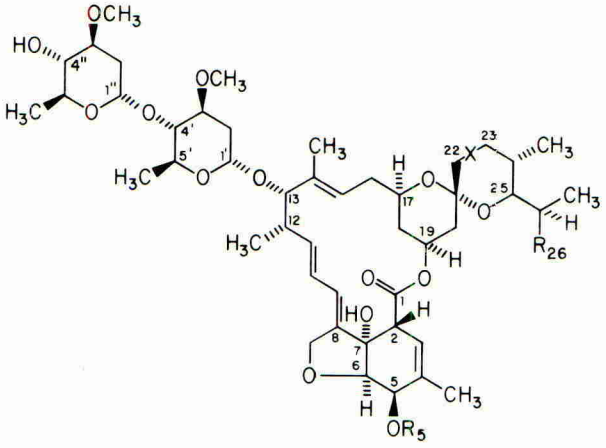
For some time, in vitro antibacterial assays were available to serve as efficient test systems for the detection of microbial metabolites with these activities in large-scale screens. In vitro assays for the detection of fermentation products with anthelmintic activities, however, have shown little success due to the large number of toxic compounds or the high dilution of the fermentation broths. Several in vivo anthelmintic assays were available and were used successfully in the discovery of synthetic anthelmintic agents but could not be readily adapted to the screening of fermentation broths. A special assay using mice infected with the nematode Nematospiroides dubius and fed with a mixture of lab chow with the crude fermentation products was then developed for the screen at Merck.

Within a relatively short time, a culture obtained through a collaborative effort from the collection of the Kitasato Institute showed a positive result. There were no parasite eggs in the feces of the mice and no worms could be found after autopsy. This culture with the Kitasato number OS-3153 was given the product number C-076 and the Merck culture collection number MA-4680 (NRRL 8165) and was eventually named Streptomyces avermitilis. After the activity of the culture was confirmed at 50 ml of broth per 25 g of feed, a titration down to one-eighth of the original concentration showed full activities on each level and suggested a very high potency for the new metabolite. It was

later estimated based on HPLC analyses that the original fermentation batches contained at least 9 µg/ml of the active principles. This yield was quickly raised to 120 µg/ml by optimizing the culture medium. Then a high producing strain (MA 4848) was obtained by u.v. irradiation, which produced 500 µg/ml of total avermectin compounds (Burg et al. 1979; Campbell et al. 1984). Since then, over 40,000 actinomycete cultures were tested in the same assay without yielding one capable of producing avermectins (Campbell et al. 1983).

Extensive use of modern chromatography methods and materials resulted in the isolation of a mixture of four closely related homologous pairs of active substances, which were named A_1 (5%), A_2 (16%), B_1 (20%) and B_2 (15%). The crude fermentation extract could be split by partition on a Sephadex LH-20 column into a fraction A (23% A_1 and 64% A_2) and fraction B (57% B_1 and 43% B_2). Two additional partition chromatographies with slightly changed solvents resolved fraction A into components A_1 and A_2 and fraction B into B_1 and B_2. The important B_1 component could also be obtained by direct crystallization (Miller et al. 1979).

Avermectin Structures



components A : $R_5 = CH_3$ components 1 : X = -CH=CH-

components B : $R_5 = H$ components 2 : X = $-CH_2\overset{OH}{\overset{|}{C}}H-$

components a : $R_{26} = C_2H_5$
components b : $R_{26} = CH_3$

Avermectin B_1 (Abamectin): $R_5 = H$
$R_{26} = C_2H_5$ and CH_3
X = —CH=CH—

22,23—Dihydroavermectin B_1 (Ivermectin): $R_5 = H$
$R_{26} = C_2H_5$ and CH_3
$X = —CH_2—CH_2—$

The structure determination of the avermectins proceeded rapidly by relying mainly on the powerful physical methods, in particular, high resolution mass spectrometry and ^{13}C nuclear magnetic resonance spectroscopy. The mass spectra showed immediately that the four components A_1 through B_2 isolated so far were not completely pure but that each of them contained up to 20% of a homolog missing one CH_2 group, bringing the number of avermectin components to eight. The partial structures deduced by these two methods pointed to a relationship with the previously described milbemycins (Mishima et al. 1975; Takiguchi et al. 1980) if allowances were made for characteristic changes at carbon atoms 13, 22, 23 and 25 of the sixteen-membered macrocyclic lactone skeleton. The avermectins have a common disaccharide substituent at C-13, either a double bond at C-22,23 or an axial hydroxy group at C-23, and sec-butyl or isopropyl groups at C-25 for the components "a" and "b" of the homologous series. The only remaining variable substituents are the C-5 methoxy or hydroxy group for A and B components. High resolution (300 mHz) ^1H-nmr spectra and certain chemical reactions served to refine the structures. These were confirmed by X-ray crystallography, which in combination with the known absolute configuration of the levorotatory L-oleandrose obtained from the avermectins served to fix the absolute stereochemistry of the avermectin structures (Albers-Schonberg et al. 1981; Springer et al. 1981).

BIOSYNTHESIS

The 29 carbon atoms numbered 1 through 24 and the methyl or methylene groups 4a, 8a, 12a, 14a and 24a are derived from seven acetate and five propionate units. Seven of the nine oxygen atoms of the A_2 or B_2 components, with oxygens at C-6 and C-25 the exceptions, are also contributed by these acids. This shows that the C-22,23 double bond of the "1" components originates by dehydration of the C-23 alcohol, and that the avermectins are not derived from milbemycins by hydroxylation of the C-13 position. Carbon atom 25 and the attached sec-butyl group of the "a" components or isopropyl group of the "b" components derive directly from 2-methylbutyrate or isobutyrate respectively, which originate from L-isoleucine and L-valine precursors (Cane et al. 1983; Chen et al. 1983). The avermectin A series of compounds are obtained from the B series by transfer of the methyl of S-adenosyl methionine by an enzyme avermectin B O-methyl transferase (Schulman et al. 1985).

BIOLOGICAL ACTIVITIES

A battery of in vivo anthelmintic assays maintained by parasitologists at Merck made use of the purified natural avermectin derivatives and discovered activities at the very low levels of 0.05 to 0.1 mg/kg against a wide spectrum of economically important nematodes, but not cestodes or trematodes (Egerton et al. 1979). A laboratory animal test also demonstrated very interesting insecticidal properties against an ectoparasitic insect species upon systemic application to the host (Ostlind et al. 1979). Avermectin B_1 as the most interesting component was further tested and found effective upon oral administration in sheep and oral and parenteral application in cattle against all important gastrointestinal parasites at doses from 0.1 to 0.025 mg/kg.

Comparisons of the anthelmintic spectrum of B_1 and B_2 by oral and parenteral routes revealed some subtle differences of activities (Egerton et al. 1978). Avermectin B_1 was more effective than B_2 on oral treatment of sheep and cattle for adult Haemonchus contortus and H. placei while B_2 was more

effective than B_1 for Cooperia oncophora on parenteral injection in cattle. In particular, efficacy against Cooperia species decreased markedly upon parenteral treatment with B_1. A close look at the structures of avermectin B_1 and B_2 shows that the change from a C-22,23-double bond to a saturated carbon-carbon bond with a C-23 hydroxy substituent has a marked effect on the spatial arrangement of the spiroketal structure part. It became, therefore, very interesting to modify these structural features, especially converting the two trigonal carbon atoms 22 and 23 of B_1 to tetrahedral carbons without the 23-hydroxy group of B_2.

STABILITY AND CHEMICAL REACTIVITY

Before we could begin with chemical modifications, we had to learn something about solubilities, stabilities and chromatographic behavior of the avermectins. As shown already by our isolation chemists, thin layer and column chromatograhies on silica gel can be applied to the avermectins without any decomposition to reveal often remarkable powers of separation, and reverse phase hplc can separate the homologous mixtures of the C-25 sec-butyl and isopropyl avermectins. The avermectins dissolve in most organic solvents but have an extremely low water solubility of about 0.006 to 0.008 ppm. For the identification and characterization of synthetically modified avermectin derivatives, the fragmentation patterns of the mass spectra and high field ^1H nmr spectroscopy were extensively used.

The complex structures of the avermectins possess a number of features which command caution in the use of chemical reagents. The cyclohexenediol appears labile to dehydration and subsequent aromatization, a reaction which was developed into a sensitive fluorescent assay (Tolan et al. 1980). Certain basic reaction conditions lead to epimerization of the C-2 substituent and migration of the 3,4-double bond into conjugation with the lactone carbonyl, to aromatization and eventual total destruction (Pivnichny et al. 1983). Acidic conditions lead to the loss of the acid-sensitive 2-deoxy sugars, yielding monosaccharides and aglycones in good yield (Mrozik et al. 1982). Mild oxidation reagents like MnO_2 convert the allylic 5-hydroxy group to a ketone. This ketone can be reduced stereospecifically back to the natural 5-β-alcohol with $NaBH_4$ and, using $NaBT_4$ as reducing agent, this two-step reaction gives us radiolabeled avermectins B_1 (Chabala et al. 1981). The carbon 8a as part of an allylic ether system is subject to radical autoxidation, and a 8a-hydroperoxide is formed under certain conditions which are not fully understood. This hydroperoxide presumably is an intermediate to the equilibrium mixture consisting of the 8a-hydroxy analog and, after opening of the hemiacetal, the aldehyde which was also observed as a soil metabolite (Bull et al. 1984). The 8,9,10,11-diene is the chromophore, which allows simple detection on tlc plates or hplc via its u.v. absorption but also makes the avermectins subject to photoreactions. Irradiation in solution in a quartz flask causes rapid cis-trans isomerization of the 8,9 and to a lesser degree, the 10,11-double bond. Apparently the sixteen-membered lactone ring is flexible enough to allow such a drastic change in the conformation. Extended irradiation leads to complete destruction of the avermectin molecule. Although this chemical sensitivity must result in reduced persistence as a pesticide, it has the advantage that the environment does not become contaminated with long-lasting, toxic residues.

IVERMECTIN FOR ANIMAL HEALTH APPLICATIONS

As stated above, we were interested in the selective reduction of the C-22 double bond in the presence of four additional double bonds. Closer inspection of the structure reveals that the 22-double bond is the only disubstituted cis double bond in the molecule, a condition for which catalytic hydrogenation conditions using Wilkinson's homogenous rhodium catalyst was known to be highly specific. Indeed an excellent yield of the 22,23-dihydroavermectin B_1 was obtained with only about 4% of a 3,4,22,23-tetrahydro over-reduction by-product. This compound proved to be best suited as a systemic endo- and ectoparasiticide for animal health and was widely tested under its code number MK-933 or generic name ivermectin (Chabala et al. 1980; Egerton et al. 1980). In 1984 sales exceeded $100 million for the parenteral formulation IVOMEC for cattle (Vagelos 1985). It is fully effective against essentially all of the gastrointestinal nematode species considered pathogenic or economically important to cattle at a dose of 0.2 mg/kg or less but is not active against tapeworms or flukes. The most exciting properties, however, are its insecticidal and acaricidal activities upon systemic application. At the anthelmintically effective single subcutaneous dose of 0.2 mg/kg, ivermectin is highly effective in cattle against mange mites, many important tick species, suckling lice, and parasitic larvae (grubs) and dung-dwelling larvae of flies. Formulations of ivermectin for sheep, horses, swine and dogs are currently being evaluated. A summary of parasite genera susceptible at least at one point in their life cycle to ivermectin is shown in tabular form in Table 1 (Campbell and Benz 1984).

STRUCTURE-ACTIVITY RELATIONSHIPS

We were interested to see if the biological activities rested in a distinct part of the large molecule or if the whole structure was required. Reactions mentioned so far, such as oxidation of the C-5-hydroxy group, methylation of the C-5-hydroxy group (avermectin "A" series), epimerization at C-2, double bond shift from 3,4 to 2,3, hydrogenation of the 3,4-double bond, or aromatization all lead to less potent, often practically inactive, derivatives. Careful acetylation of avermectin B_1 gave a mixture which was separated into 4",- 5-mono- and 4",5-diacetates. Like methylation of the C-5-hydroxy group, acetylation reduces activity 4-8 times, while it is apparent that acetylation as well as other substituents at the C-4" position can be tolerated with neither any loss nor any increase in anthelmintic activities (Mrozik et al. 1982). We found in the bulky tert-butyldimethylsilyl group a way to specifically protect the C-5-hydroxy group, introducing the desired substituent at C-4"-OH and then removing the protecting group by mild hydrolysis, as shown in the example of the 4"-O-phosphate ester of avermectin, which in the form of its monosodium salt affords a highly active, water-soluble derivative with certain advantages for parenteral formulations. Careful reaction of unprotected B_1 with the bis(2,2,2-trichloroethyl)-phosphorochloridate gave the 5-O-monosubstituted derivative, and subsequent zinc reduction liberated the free phosphate ester of the C-5-hydroxy group. The latter has surprisingly very low in vivo activity, since we expected that ubiquitous phosphatases would remove the phosphate groups readily. An in vitro experiment carried out with a commercially available alkaline phosphatase showed, however, that of the two phosphate esters, only the C-4"-phosphate group was hydrolyzed rapidly (Pivnichny 1984). We believe, however, that in general it is not necessary to remove acyl or alkyl substituents from the C-4"-hydroxy group in order to have activity.

TABLE 1

Systematic listing of genera of parasites (nematodes and arthropods) known to be susceptible, in at least some developmental stage, to the action of ivermectin

Class: NEMATODA

Order: Rhabditida
- Superfamily Trichostrongyloidea: Cooperia, Haemonchus, Hyostrongylus, Nematodirus, Nematospiroides, Ostertagia, Trichostrongylus.
- Superfamily Strongyloidea: Ancylostoma, Bunostomum, Chabertia, Cyathostomum, Cylicodontophorus, Cylicocyclus, Cylicostephanus, Gaigeria, Cyalocephalus, Oesophagodontus, Oesophagostomum, Poteriostomum, Stephanurus, Strongylus, Triodontophorus, Uncinaria.
- Superfamily Metastrongyloidea: Dictyocaulus, Metastrongylus.
- Superfamily Rhabditoridea: Strongyloides.

Order: Ascaridida
- Superfamily Ascaridoidea: Ascaridia, Ascaris, Heterakis, Parascaris, Toxascaris, Toxocara.
- Superfamily Oxyuroidea: Aspiculuris, Oxyuris, Syphacia.

Order: Spirurida
- Superfamily Spiruroidea: Draschia, Habronema.
- Superfamily Filaroidea: Brugia, Dipetalonema, Dirofilaria, Litomosoides, Onchocerca, Parafilaria, Setaria.

Order: Enoplida
- Superfamily Trichuroidea: Capillaria, Trichinella, Trichuris.

Class: INSECTA

Order: Diptera
- Suborder Cyclorrapha: Chrysomyia, Cuterebra, Dermatobia, Gastrophilus, Hypoderma, Lucilia, Oestrus, Glossina.

Order: Phthiraptera
- Suborder Anoplura: Haematopinus, Linognathus, Solenoptes.
- Suborder Mallophaga: Bovicola (Damalinia).

Order: Siphonaptera
- Family Pulicidae: Xenopsylla.

Class: ARACHNIDA

Order: Acarina
- Suborder Mesostigmata: Ornithonyssus.
- Suborder Trombidiformes: Psorergates.
- Suborder Sarcoptiformes: Chorioptes, Otodectes, Psoroptes, Sarcoptes.
- Suborder Ixodoidea: Amblyomma, Boophilus, Dermacentor, Haemaphysalis, Hyalomma, Ornithodorus, Rhipicephalus.

From W. C. Campbell and G. W. Benz, 1984. Ivermectin, A Review of Efficacy and Safety, J. Vet. Pharmacol. Therap. **7**, 1-16.

Since the major structural differences between avermectins and milbemycins are confined to the C-13-substituent, we converted ivermectin and other avermectins step by step into 13-deoxyavermectin aglycones, which are closely related to the milbemycins. Mild acid hydrolysis gave the monosaccharides and more forcing conditions gave the aglycones (Mrozik et al. 1982). Then the selective deoxygenation of the aglycones at C-13 should provide the desired derivatives. It was again possible to protect the reactive C-5-hydroxyl group in the presence of the apparently less reactive C-13-hydroxy group. Although the allylic C-13-hydroxy group did not yield stable sulfonate esters needed as leaving groups, reaction conditions were found to give the desired 13-β-chloro derivatives without isolation of the intermediate o-nitrobenzenesulfonate. Reductive dehalogenation with tri-n-butyltin hydride in the presence of a radical initiator and removal of the protecting group yielded the 13-deoxyaglycones. In most biological assays, especially in vivo systems, loss of one sugar causes loss of potency; loss of both sugars generally causes a substantial reduction of activity. Removal of the 13-hydroxy group from the aglycones, however, often restores significant anthelmintic activities. The 13-hydroxy group is apparently detrimental to the biological activity (Table 2).

AGRICULTURAL APPLICATIONS

Avermectin B_1 (abamectin) was selected for development as an agricultural pesticide since it is the most potent acaricide and insecticide of the naturally-occurring avermectins. It is highly toxic to the two-spotted spider mite at .02 to .03 ppm and to other mites including the citrus rust mite, citrus red mite and the strawberry mite at 0.02 to 0.24 ppm in laboratory studies (Putter et al. 1981). In a bait formulation, it is active at 125 mg/ha against the imported red fire ant (Lofgren and Williams 1981). It also has insecticidal activity against a number of pests of economic importance with somewhat reduced potency against the Southern armyworm and the corn earworm (Dybas and Green 1984). Certain derivatives, however, notably the monosaccharide and the 13-deoxy aglycone of 22,23-dihydroavermectin B_1 (ivermectin), exhibit improved activity against the Southern armyworm in a laboratory feeding assay. Further modifications at the disaccharide part of avermectin B_1 were carried out in order to explore this lead. Noting that many antibacterial 14- or 16-membered macrolides contain an amino substituent, we chose to introduce an amino group at the C-4" position in order to explore its effect on the activities and spectrum, expecting differences due to absorption, penetration, distribution and binding of a basic avermectin derivative. We were very gratified to see a more than one-hundred-fold increase in the activity against the Southern armyworm for one of these derivatives—the 4"-epi-amino-4"-deoxyavermectin-B_1. In a number of small-scale field tests, this derivative showed excellent control of armyworm species on celery, corn, alfalfa and chrysanthumum and of corn earworm on soybeans at 0.02 lbs/acre.

MODE OF ACTION

Strong evidence has been accumulated suggesting that avermectins and presumably milbemycins exert their antiparasitic effects by stimulating chloride ion conductance of axons mediated by the putative inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Wang and Pong 1981). Since GABA is a neurotransmitter in the peripheral nervous system of a large number of nematodes, acarids and insects but is mainly concentrated in the central nervous system of vertebrates, this mode of action can be responsible for the selective toxicity

TABLE 2

Biological Activities of Avermectin Derivatives

Avermectins	In vivo anthelmintic assay ED_{90} (mg/kg))[a]	Two-spotted spider mite assay EC_{90} (ppm)[b]	Southern armyworm assay EC_{90} (ppm)[c]
B_1 (abamectin)	0.03	0.03	8.0
B_1-monosaccharide	0.1	< 0.01	8.0
13-deoxy-B_1-aglycone	0.06	< 0.005	-
22,23-dihydro-B_1 (ivermectin)	0.03	0.05	8.0
22,23-dihydro-B_1 monosacchardie	0.1	0.1	0.5
22,23-dihydro-B_1 aglycone	0.2	> 0.1	-
13-deoxy-22,23-dihydro-B_1-aglycone	0.06	0.05	0.5
4"-amino-4"-deoxy-B_1	HA[d]	0.25	0.1
4"-epiamino-4"-deoxy-B_1	HA[d]	0.25	0.02
4"-epiacetylamino-4"-deoxy-B_1	HA[d]	0.25	0.5
4"-epiamino-4"-deoxy-22,23-dihydro-B_1	HA[d]	1.25	0.5
4'-epiamino-4'-deoxy-22,23-dihydro-B_1 monosaccharide	A[d]	> 0.05	0.1
4'-epiamino4'-deoxy-B_1-monosaccharide	A[d]	> 0.25	> 0.5

a) Trichostrongylus colubriformis in gerbils (Ostlind and Cifelli 1981). b) Tetranychus urticae (Putter et al 1981). c) Spodoptera eridania (Putter et al. 1981). d) HA = highly active and A = active in a related in vivo anthelmintic assay.

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toward parasites, provided that the avermectins do not penetrate the blood brain barrier readily. Adult Ascaris become immobilized within a few minutes upon injection of 1.5 µg of avermectin B_1 retaining normal muscular rigidity capable of a reversible muscular tetanus upon injection of 50 µg of acetylcholine (Kass et al. 1980). By use of a carefully prepared Ascaris section containing one dorsal excitatory motor neuron with its muscle connected through one commisure to the ventral inhibitory neuron it was shown that avermectin B_1

blocks the signal transmission from the interneuron to the excitatory motor neuron but has little effect on neuromuscular transmission of excitatory motorneurons alone. This action can be reversed by picrotoxin, which is known to close the chloride channels and reverse the actions of GABA (Kass et al. 1984). A high affinity binding site for [^{3}H] avermectin B_1 which is associated with the GABA-benzodiazepin receptor of the chloride ion channel ionophore has been demonstrated in rat brain membranes. Avermectin B_1 stimulates the high affinity GABA binding and the binding of diazepam and related structures to this protein (Pong and Wang 1982; Drexler and Sieghart 1984). The stimulation of [^{3}H] flunitrazepam binding and the displacement of [^{3}H] avermectin B_1 from rat brain sites correlates to a great extent with the anthelmintic potency of a number of avermectin derivatives.

The GABAergic mode of action of the avermectins constitutes a previously unknown mechanism for insecticidal and anthelmintic action, perhaps with the exception of the weakly anthelmintic piperazine and therefore no cross resistance to existing pesticides can be expected. The avermectins are also reported to bind to glycine receptors (Graham et al 1982) and interfere in the reproduction of several insect species, i.e., the red imported fire ant (Glancey et al. 1982). These actions are not readily understood so far and further research on the effects of avermectins is ongoing (Olsen and Snowman 1985; Wright et al. 1984).

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MICROBIAL INSECTICIDES

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Abstract

Biological control of pests has a long and somewhat varied history. The use of microbes as pesticides, which is more recent, has had some notable successes. Examples are given of bacteria, fungi and viruses which have been commercialized, frequently after skillful production and formulation efforts. In the longer term it is likely that genetically engineered microorganisms will be highly specific and highly effective at pest control; there are however, many issues which will receive thorough public discussion before this happens. In the shorter term, the biotechnological revolution will be applied to the more cost-efficient production of naturally occuring microbes and to obtaining a better understanding of their mode of action.

Introduction

Microbial control of insects is not new. Neither is the concept of biological control of pests, weeds and diseases. Nevertheless, revolutionary techniques in biotechnology offer tantilizing new prospects for the future. Before discussing the future however, a brief review of the past will help to put the possibilities into context.

Biological control has a long history, reaching into the undated past when the Chinese used Pharoah's ants to control pests of stored grain. Others date the start of biological control to the domestication of the cat.

The introduction of an exotic species to control a pest, the classical form of biological control, is claimed to date from 1762 when the Indian Mynah was brought to Mauritius to control the Red Locust. Subsequent attempts at introduction occasionally had unwanted results; the introduction of cats to control rats on Ascension Island in 1815 resulted in the extermination of virtually all the sea birds on the island.

Other attempts were made nevertheless and the successful control of cottony cushion scale by Australian ladybirds in California in 1888 encouraged practitioners to continue to develop biological pest control by the introduction of exotic enemies.

"Introductions" are not theoretically limited to insects and vertebrate predators; the use of the myxomatosis virus to control rabbits in Australia suggests that microbes may be able to play similar roles. However, introductions have two fundamental limitations:

i. they have an extra dimension of unpredictability because an organism, hitherto unknown, is being brought to a new area which it might find fertile in unanticipated ways and;

ii. there is no clear way to make a profit from one-of introductions.

Research and development therefore remains wholly in the public sector.

There are two other main types of biological control, inundation and augmentation, both of which may be practiced with microbes or other organisms. They are essentially self-descriptive, inundation involving the use of very large numbers of organisms over a short time to suppress or destroy a population, augmentation being the supplementing of an existing natural population which has the power to control a pest or disease.

Both of these methods are employed in the control of insects by microbes. Many of the microbes used to suppress insect populations for the duration of crop would devastate the insects anyway, unfortunately a little later than is desirable for crop protection. The numbers of propagules are therefore augmented at an early stage in the season and suppression of the pests begins and climaxes earlier, resulting in protected crop. To the present time most microbial insect control has aimed to bring forward the time when the pathogen controls the population rather than to introduce a new factor into the pest control system.

Bacteria, fungi and viruses are the microorganisms most commonly researched as potential microbial pesticides.

BACTERIA

Over 90 species of bacteria which infect insects have been described; at present there are commercial products based on at least two although others have been produced experimentally. Bacillus thuringiensis (Bt) species are used for the control of caterpillar pests, mosquitoes and blackflies, Bacillus popilliae for the control of Japanese beetles. Bacillus sphaericus is considered to be a highly promising pathogen for mosquito control but is not yet in commercial production.

Bt was discovered in silkworms in Japan in the early part of the twentieth century. It first became a commercial product in France in 1938 and has been on sale ever since. The bacterium produces a proteinaceous crystal which becomes insecticidal after digestion in the alkaline environment found in the mid-guts of susceptible species. The crystal varies amongst the many serotypes and isolates of Bt producing a varied spectrum of activity against different insects. The two commercial strains are 'Kurstaki', used against caterpillars, and 'israelensis' (H-14), effective against mosquitoes and blackfly.

In addition, some strains of Bt produce a soluble toxin called beta-exotoxin which is highly toxic to house-flies and does not need to be ingested. Unfortunately, the beta-exotoxin has slight mammalian toxicity and, apart from Finland, no products containing the beta-exotoxin are marketed in the west.

Production of Bacillus thuringiensis

Bt is produced in liquid fermentations after which it is concentrated and formulated into a variety of liquid and powder forms to be used.

Although the fermentation of Bt presents few conceptual problems, its cost will determine the competitiveness of the company making it. Costs can be held down by the use of inexpensive raw materials, by faster fermentations, and by simplification of the concentration steps used after the fermentation. Our experience has been that costs have been reduced by at least 30% over the last two years and innovative process steps presently at the large scale proving stage should reduce them by a further 30-50%.

Formulation is crucial to the commercial success of a product. At Microbial Resources, Bt H-14 is made into five separate products:

i. a wettable powder for formulation at the point of use,
ii. a flowable liquid for general use,
iii. an emulsion for rapid dispersion over large areas of water,
iv. a sand-granule for aerial application through foliage,
v. an ultra-low volume liquid for efficient applications by aircraft when there is no canopy.

In addition to making a product easy to use, formulation of microbial products is critically necessary for their stability. With no additives Bt H-14 will lose half its activity in 0.05 months at 42 C. Initially it was shown that this could be increased 10 fold; now, formulated H-14 will last 300 times longer at 42 C than unformulated.

The development of low cost production methods and successful formulation of products such as microbial insecticides represents the commercial cutting edge of biotechnology.

The Effect of Biotechnology

Substantial contributions can also come from fundamental biotechnological research:

i. Increases in potency by modification of the plasmid complement of the bacterium, which controls the synthesis of the protein both reduce the cost of production and provide the starting place for new, more easily used formulations.

ii. Changes in the spectrum of activity of the toxic protein by genetic engineering can open wholly new markets.

iii. Production of asporogenic strains may reduce production costs by avoiding the 'waste' of metabolic energy on spore production and may make the product more acceptable in certain countries.

Microbial Resources conducts its own programme of research into more cost-effective products based on Bt and other bacteria.

FUNGI

Over 400 species of fungi are known to attack insects and mites. Fungi usually infect their hosts by direct invasion and are therefore able, unlike most bacteria and viruses, to attack pests without first being ingested. In addition, by their ability to sporulate on the dead bodies of their hosts, fungi often control not just the insect they land on, but the entire host population for some extended period. The timely application of sufficient fungal propagules at an early stage can control pests to sub-economic levels for the duration of the crop. However, to be effective, fungi have fairly stringent requirements for humidity and temperature. If the humidity falls, or the temperature varies too far from the fungus' optimum, control of pests may be sluggish or non-existent and the desired epidemic (termed 'epizootic') may fail to develop.

Nevertheless, fungi have great promise as economical pest control agents. Metarhizium anisopliae is the best known of all entomopathogenic fungi and was the first fungus to be produced on a large scale. It infects a wide range of species and has been successfully commercialized by a number of small companies in Brazil for the control of spittle-bug in pasture and in sugar cane; it has been combined effectively with a virus to obtain control of the rhinocerous beetle on palms in the Pacific.

Another extensively researched fungus is Beaveria bassiana; a product has been produced in the USSR to control Colorado Potato Beetle. Experiments in the United States have shown good control of this beetle to be possible. Verticillium lecanii has been produced commercially in the U.K. for several years. Strains of this fungus can control aphids and whitefly, sometimes for months at a time. Hirsutella thompsonii was produced briefly in the United States by Abbott Laboratories for the control of mites in citrus; environmental conditions did not favor the growth of the fungus and it has not yet found a place in citrus pest control programmes. Nomuraea rileyi was investigated for many years by researchers in the U.S. primarily in collaboration with Abbott. This fungus can be very effective against caterpillars particularly in soybeans.

In addition to these, fungi from the Entomophthorales show good potential. Research at Rothamsted over many years has shown one particular strain to be effective against aphids in outdoor crops. These fungi are particularly difficult to grow outside of their inside host; recent work however has shown that it is possible to produce infective propagules in vitro so a product based on this fungus may not now be far off.

Fungi, like bacteria are produced by fermentation. However, there is often a preference for growth on some sort of solid medium rather than in submerged culture. This is because the spores produced by fungi in solid culture are usually much more durable than those from a liquid. Unfortunately, solid culture is slower and more expensive than liquid culture so a compromise must be found. This compromise can be eased by skillful formulation which can extend the half-life of a fragile spore produced in liquid from 2 weeks at 4 C to 11 months.

Although there is extensive knowledge and expertise in the genetics of fungi important to existing industries like pharmaceuticals and brewing, relatively little is known about why one strain of an entomopathogen generates a successful epizootic while another, equally pathogenic strain, does not. We believe that the future development of successful fungal products for insect control rests as much with the geneticists, biochemists, physiologists and ecologists as it does with the fermentation and formulation specialists and we pursue our own programme for this purpose.

VIRUSES

The potential of viruses as pesticides will be reviewed in the next talk by Dr. Entwistle. Therefore this talk will be confined to our own experience with a viral product and my own view of the future.

Dr. Entwistle's laboratory, the NERC Institute of Virology in Oxford, spent a number of years characterizing the action of Neodiprion sertifer Nuclear Polyhedrosis Virus; the U.S. Forest Service did similar work and also extensive toxicological studies of the virus, studies probably not strictly justified by its commercial potential. Our own contribution was to provide a formulation for the commercial product based on our previous experience and to assist in obtaining registration for the product in the U.K. and the U.S.A., again based on previous experience.

The product, named VIROX after the telex number of the Institute where it was researched, has been very successful and we are pleased to be associated with it.

There are many more viruses that are known to be active against insects than can be produced and commercialized as cost-effective pest control agents. One of the key reasons for the lack of cost-effectiveness is that viruses must usually be produced in vivo, i.e. in the host. The biotechnical revolution would be making a major contribution to insect control if a way could be found to reduce viral production costs sufficiently to allow the use of larger, more effective quantities.

In addition, now that detection methods have become as sensitive and effective as they have, should the spectrum of viruses considered as potential microbial pesticides be extended from the groups focussed on until now. Toxicological studies that would have been impossible several years ago because they involved attempts to recover the virus from animal tissues using a bioassay as a detection method, now should be relatively simple using DNA and other probes.

Additional references on this subject may be found in:

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THE POTENTIAL OF VIRUSES AS PESTICIDES

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ABSTRACT

Viruses are strong regulators of insect populations and have often been identified as key determinants of the periodicity of upsurges of pests of such relatively undisturbed habitats as forests and pastures. Habitat disruption by normal agricultural practices generally diminishes the epizootic capacity of insect viruses but nevertheless economically telling epizootics do occur as for instance in Trichoplusia ni on cabbages and Phthorimaea operculella on potatoes.

The natural epizootic status of a virus is, however, far from being a good indicator of its potential as a pest control agent. For instance the granulosis virus of Carpocapsa pomonella, Codling moth, is never epizootic and has seldom been recognised in the 'wild' but when sprayed on populations of neonate larvae it can provide very good control.

Viruses from eleven separate families occur in insects. Nine of these families include viruses with vertebrate hosts and it is unlikely that their insect-pathogenic members will be considered for use as pesticides until their possible impact on vertebrates has been better explored. The remaining two families are the Nudaurelia β viruses and the Baculoviridae of which the latter appear to be the most frequent and to have the better characteristics for employment as pesticides.

Baculoviruses (BVs) are particularly frequent in Lepidoptera (440) and Hymenoptera Symphyta - sawflies (25) - and less so in other insect orders. The virus particle is rod-shaped (250 μm long by 45 μm diameter) and the genome consists of circular double-stranded supercoiled DNA. There are three subgroups, A, the nuclear polyhedrosis viruses (NPV) in which groups of virus particles are occluded in proteinaceous polyhedral inclusion bodies (PIBs); B, the granulosis viruses (GV) where the ovoid inclusion bodies each contain only one virion and C, the Oryctes types where no inclusion body is formed. Inclusion bodies are virus coded. The principal susceptible life stage is the larva which is infected following ingestion of virus. Under the alkaline conditions in the gut inclusion body protein disintegrates and releases virions to infect midgut secretory cells and other tissues. Inclusion body production is commonly $>1 \times 10^9$ (NPV) and $>1 \times 10^{11}$ (GV) per larva whilst LD_{50}s for neonate larvae can be <10. However, decreasing susceptibility accompanies larval growth. Inclusion body BVs have considerable environmental stability unlike viruses in subgroup C where infection depends on close contact between individual insects. BVs do not affect beneficial insects and tend to have narrow host ranges (with some exceptions) and so make good discriminative pesticides.

Methods of use range from classical biological control to spraying which is the most effective and by which research during the past 30 years has shown it is possible to control over 40 species of Lepidoptera and diprionid sawflies.

Some very broad generalisations can be made about dosages. For instance the numbers of NPV PIBs currently required per hectare per year are, cotton -

1.5×10^{13}; other field crops (cabbages, clover, maize, etc.) - 1.0×10^{13}; broadleaved trees - 5×10^{12}; coniferous trees - 4.5×10^{11} (Lepidoptera) and 5×10^{10} (sawflies). The ultimate validity of these averages depends on future developments in formulation and application which in relation to both viruses and other insect pathogens are severely underdeveloped technologies.

Prime requirements are to develop a range of formulations compatible with various application techniques and to incorporate sufficient amounts of substances providing production against ultra violet (uv) radiation in droplets from 25-250 µm diameter and this with volumes from 8.2×10^{3} - 8.2×10^{6} μm^{-3}, a range of x1000. DNA is readily damaged by solar uv B wavelengths (290-315 nm) and though BVs stand high in the hierarchy of resistance of insect pathogens to uv nevertheless pure deposits on leaves can lose most of their activity in a day of direct exposure to sunlight. In reality the complex architecture of plants provides some escape which can be extended by incorporation of uv absorbent (e.g. carbon, some benzyl ring compounds etc.) and reflectant (e.g. titanium dioxide, naphthatriozole stilbene derivatives, etc.) substances into formulations. Among crop plants the chemical nature of the surface of cotton seems uniquely injurious to BVs and presents special formulation problems.

BVs are particulate entities not amenable to the considerable dilutions at which many soluble chemical insecticides can be used so that for even the most infective-viruses this imposes constraints on spray volume and droplet size. Efficient spray application depends on even distribution of inclusion bodies between droplets, which can only be achieved by producing droplet spectra of narrow size range (controlled droplet application - cda) and ensuring that the inclusion bodies do not aggregate. Spray techniques should aim to achieve optimal coverage of neonate larval feeding sites. Greater attention to these questions is likely to provide progress in the BV control of 'difficult' insects as is currently being demonstrated by the Institute of Virology's (IOV) work on Panolis flammea, Pine beauty moth, where the amount of plant surface tissue, and hence of PIBs, ingested is little and the site of larvae feeding is spatially restricted, both considerations theoretically dictating the need for a very high PIB dose but which studies on coverage and formulation are showing it is possible to circumvent.

A major ultimate economic consideration in the use of BVs is the cost of their production for which two approaches are available. It is possible to culture viruses in vivo in many Lepidoptera which can be fed on semi-synthetic diets under controlled and sterile conditions. Such a methodology can be industrialised as has been done for the NPV of Heliothis spp., cotton bollworm, and is being done for C.pomonella GV. The high original cost of production in vivo can be reduced. For instance, largely due to diet and labour refinements the production costs of Lymantria dispar, Gypsy moth, NPV were reduced between 1975-81 from 10 to 0.4 cents per 1×10^{9} PIBs. In principle, however, industry favours the growth of BVs in vitro in insect cells in large scale fermenter cultures. This may be the real future of BV production but some problems need first to be solved. At present multiply enveloped NPVs (in which several nucleocapsids share a common virus membrane) grow best in cell culture but few singly enveloped ones and GVs can be so cultured. In addition insect cells tend to be damaged by the forces required to support them in suspension in large volume cultures. Media development is required, especially to find a replacement for expensive foetal bovine serum. Despite these problems a recent evaluation viewed the possibility of achieving economic in vitro production very favourably.

The wider use of viruses for insect pest control will depend on development of several areas. Problems of formulation, application and production have already been mentioned. Virus control of Coleoptera and Diptera, for instance, will mainly be achieved by development of members of families other than the Baculoviridae and a prime requisite here will be demonstration of safety for man and other non-target organisms. It has been shown that modification of the NPV genome is possible. Incorporation of a gene coding for production of an interferon into the NPV of Autographa californica (AcNPV) with heavy production of interferon in insect cell culture has been achieved. The same might usefully be done with other genes, e.g. that coding for production of the delta endotoxin which is the main active component of the insect pathogenic Bacillus thuringiensis, to achieve more rapid death of insects than results from NPVs per se. Other possibilities of 'improving' the BV genome for pesticidal purposes are under investigation in laboratories around the world and there will also no doubt be further work on the use of the now well understood AcNPV as an expression vector.

Commercialisation of BVs is growing and, for instance, the NPV of Neodiprion sertifer, Pine sawfly, came to the UK market, as a result of IOV's developmental work, in 1984 since when it has been sprayed on >6000 has. The limited market outlet imposed by the narrow host range of insect viruses and the current unavailability of in vitro methods of production, however, appears to restrict the interest of major agro-chemical companies and to indicate that for the next few years development and production will principally reside with public research organisations and smaller, biotechnology companies.

VACCINES AND THEIR APPLICATIONS TO THE AGRICULTURAL INDUSTRY

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1. Introduction

To discuss the application of "biotechnology" to vaccines and hence to the agricultural industry it is necessary, in the first instance, to understand clearly the nature of the activities which are construed as biotechnology. Secondly, the relation of such biotechnological activity to the design and implementation of new vaccines will be described. As vaccines play a role in the enabling of the intensification of livestock farming, as well as in lowering the overall disease load to which the nation is exposed, an overview of these vaccine controllable disease is required. Finally, the present and future situations will be reviewed in a way which combines the three main aspects of this talk, viz; Biotechnology, vaccines and the Agricultural Industry.

2. Biotechnology

There are many definitions of biotechnology[1] and yet there is more confusion as to what it is and what is involved in the practice of that discipline. The reason for this is that biotechnology, as an activity, is practiced in University and Polytechnic laboratories, government and private research institutes and large and small industries, who are each involved to some extent in research, development, testing, evaluation and production exercises. Clearly what goes on in industry (or a University joint owned Company) is better defined as PROCESS BIOTECHNOLOGY. This is simply described as a discipline which enables its exponents to convert raw materials to final products when either the raw material and/or a stage in the production process involves biological entities (Fig. 1). Biotechnology in the broad, however, involves the cloners, the hybridisers and the molecular and cell biologists. This also is a discipline in its own right. It involves the conjoint interaction of two identifiable sub-components; bioScience and bioTechnology. Such an interaction is SYNERGISTIC as each activity feeds, enables and promotes the other's activity. The manner of this interaction can be seen from a perusal of Fig. 2. It is therefore difficult to understand those who would have Science done in Universities, Research and Development (presumably that's Technology) done in Research Institutes, and Industry would be left with the job of Production, Marketing and Profit-Making. The real and, the author holds, desirable situation is that each of the facilities which affect work in the area of biotechnology should be allowed to take their work through to the production stage. Were a differentiation (or, at least,

a conceptual polarisation) to be adduced to this situation, then the universities and polytechnics would be free to "work in any subject area" while the research institutes would have their areas delineated either by the availability and requirements of private funders or by direction resulting from a definition of national needs. Industry would "do its own thing" while at the same time joining with the universities/polytechnics and research institutes at the stage when products are seen and markets are in the process of development. In this way new enterprises would be started and the successful ones either bought up by the major companies, and if strong enough allowed to develop to become major industries themselves.

2.1 Biotechnology in the development of Vaccines

In Tables I and II the basic laboratory tools of the biotechnology trade are depicted. Many of them have been developed during the last 10 years. The pace of development has not decreased. Machines which cost many tens of thousands of pounds five years ago are now available for less than ten thousand pounds and whereas the best sequencing and expression vectors were once acquired with difficulty via personal contact, many such vectors or vehicules are now available from the regular suppliers of laboratory biochemicals.

On the Industrial front, developments in capability have kept pace (though lagging behind) work in the laboratories. Whereas traditional, up to the mid 1970's, scale-up methods have relied on multiple process systems, wherein the scale-up of the system involved an increse in the number of units of production of the same size as the laboratory equipment, modern systems, based on unit systems concepts increase the size of the apparatus. This leads to greater production efficiencies through a) decreased labour requirements, b) overall increase in reliability, reproducibility and contamination-free processes, and c) the use of opportunities for monitoring and hence controlling the environment of the production vessel. The involvement of computers and direct digital control (DDC) systems in this area not only increase the range of monitoring and control options available, it also does it at reduced cost, increased flexibility and improved reliability over equivalent analogue or analogue-digital systems (Table III). Further discussion of the applications of the various fermenter technologies may be found in a number of recent reviews (2). Such techniques are presently in use for the production of the newer vaccines (see Section 4 below).

2.1.1 Routes to a defined polypeptide vaccine

There is little doubt that an ideal vaccine is one in which each of the constituent parts is defined exactly in terms of molecular composition and in quantity. Interactions between components germane to the performance of the vaccine should also be known. While the major portion of contemporary vaccines are whole organisms or derivatives therefrom, the ability to produce a molecularly defined material did not until recently exist. However, were it possible to produce a prophylactic material (made by chemisynthesis), (unlike prophylactically administered antibiotics), which works by harnessing the animal's (or human's) immune system to develop an effective and

long-lasting protection against infection, then the route to a tightly defined vaccine would be open. Recent procedures schematically defined in Table IV have resulted in potential successes in two or perhaps three human vaccines (Neisseria gonorrhoea, malaria and possibly hepatitis B), and are under intensive investigation for the new veterinary vaccine for foot-and-mouth disease.

3. Vaccines and Infectious Diseases

Disease does not respect the boundaries set up by nation states. Therefore it is well to bear in mind the international disease scene when considering any particular disease at a defined location (Table V). Furthermore, the methods used for disease control and elimination have been most effective in either eradicating or controlling a variety of clearly delineable diseases from the food animal communities of the UK (Table VI). However, the era of approaching a single disease with a single vaccine within a comprehensive control strategy of movement limitations, slaughter or stamping out systems along with diagnostic and monitoring, veterinary and investigatory infrastructure is inappropriate. For today we are presented with complex disease syndromes which stem from multiple infections. So tomorrow's vaccines will not only be more sophisticated from a molecular standpoint - they will also be complex mixtures of immunogens derived from an organismal range spanning the phyla between helminths to viruses. A list of important disease syndromes is presented in Table VII. Breakdowns of the organisms involved in the syndromes of Table VII are presented in Table VIII, IX and X. It is clear from Table XI that the need to do the utmost to control disease is paramount as the intensity of the farming units increases. Under such circumstances a non-debilitating illness which spread through a herd, flock or battery would have a serious economic impact even were it to decrease feed conversion efficiency by as little as 2 - 5%. On this score there is clear evidence that about $30 million spent in the 10 year long research and development of a vaccine to protect chickens against Marek's disease yielded an annual dividend of $170 million in improved yield, after a significant use of the vaccine had been implemented (3).

Clearly the UK is continually under threat from diseases which have been eradicated locally and even nationally but which yet prevail in neighbouring countries, particularly those of the nearest neighbour across the Channel. Amongst such diseases are those highlighted in Table XII. Of these diseases African Swine Fever presents the greatest threat for, as yet, it has not been possible to develop a reliably effective vaccine. Whether any of the methods referred to in Section 4 below will be developed to a useful level remains to be seen.

4. Biotechnology makes Vaccines for Agriculture

Present routes to effective and putative vaccines have been diagrammed in Figure 3. With regard to the diseases of Table XII, there is an attenuated live virus vaccine to protect pigs against Aujesky's disease which is less than totally effective; it is used in The Netherlands. A number of vaccines are available for protecting animals against infection with the Rabies virus yet the main problem is in the elimination of the virus from the wild (feral) animal population.

Experiments using freshly severed chicken heads injected with live, attenuated, Rabies virus and left for feral foxes in Switzerland has created an immunised fox population. However, there is a danger of reversion to virulence on passage of the live virus in the fox population so that such an approach may not be optimal. Foot-and-mouth disease vaccines are held to be capable of further development (Table XIII), so much so that much work has been expended on making a genetically engineered or chemisynthetic peptide vaccine. However, such vaccines, at the time of writing, are still some way from being as effective as vaccines as the current killed virus vaccines.

5. Conclusions

Many of the latest techniques will be needed to make the vaccines which will be effective in reducing the economic impact of the disease syndromes. Molecular engineering is the approach most likely to yield vaccines against the rapidly changeable spots of the enteric bacteria while new technology will be used to exploit animal cells on the large scale to make the Herpes, vaccinia and picornaviruses based vaccines involved in economically important diseases of food animals.

Ironically, once a disease has been eliminated worldwide, or even locally, pressure is generated to cease vaccination routines. This enables the development of a disease free designation for a country or an area which enhances the possibilities for livestock and bloodstock trade. It also enables the pressure of importers of meat from areas where disease and/or vaccines prevail to be resisted. The conundrum created is that the better a vaccine is, the more likely it is to lead to disease elimination and with it is lost a profitable activity of a manufacturer. Evidently there is a need for government to recognise its responsibility to Society and its industry by creating the enabling conditions for the production of the optimised vaccines needed for the eradication of as many diseases as possible while enabling vaccine manufacturers to get on with the job of helping to eradicate "the next disease in line". That vaccines operate at the complex interface of three activities which are in themselves not simple (Figure IV) means that our thinking has to incorporate many diverse elements in the course of enabling new societies to enjoy the benefits of a more healthy way of life.

References

(1) O.E.C.D. Report "Biotechnology" 1982 p.21.

(2) Chapters 7 - 13 inclusive of "Animal Cell Biotechnology", Vol. I, Eds. R.E. Spier and J.B. Griffiths, Academic Press, 1985.

(3) In "Animal Disease Control, R.P. Hanson and M.G. Hanson, Iowa State University Press, 1982 p. 170.

TABLE I

ANALYTICAL TOOLS

(Laboratory)

SEPARATION TECHNIQUES
- Electrophoresis
- Chromatography
- Centrifugation
- Membrane

DETECTION/AMPLIFICATION
- Radio-labels
- Enzymes
- Blotting methods

COMPUTATIONAL ADVANCES
- Sequencing
- Comparisons
- Molecular graphics

SEQUENCE ANALYSERS
- Amino Acid

ENZYMES
- Restriction Endonucleases
- Proteases

MONOCLONAL ANTIBODIES

TABLE II

SYNTHETIC TOOLS

(Laboratory)

POLYPEPTIDE SYNTHESISERS

POLYNUCLEOTIDE SYNTHESISERS

GENE CONSTRUCTION METHODS

GENE AMPLIFICATION METHODS

SINGLE BASE CHANGE METHODS

TABLE III

SYNTHETIC TOOLS

(Manufacturing)

FERMENTER DEVELOPMENTS
- Air Lift
- Surface Adherent
  - Packed Beds
  - Microcarriers
- Trapped Systems
  - Gels
  - Containers
    - Micro
    - Macro
- Computers in
  - Monitoring
  - Control

TABLE IV

STEPS TO THE IDENTIFICATION OF AN IMMUNOGEN

- ISOLATE PUTATIVE IMMUNOGENIC PROTEIN FROM INFECTIOUS AGENT
- DETERMINE A NUMBER (>7^{O}F) CONSECUTIVE AMINO ACIDS
- SYNTHESISE A DNA POLYNUCLEOTIDE TO THE CODE OF THE SEQUENCED AA's
- USE SYNTHETIC DNA TO ISOLATE THE GENE FOR PROTEIN
- CLONE WHOLE GENE DNA TO ISOLATE GENE FOR PROTEIN
- SEQUENCE WHOLE GENE
- TRANSLATE DNA SEQUENCE TO AA SEQUENCE
- EXAMINE AA SEQUENCE FOR
  - HYDROPHILIC AREAS
  - AREAS OF VARIABILITY
  - BETA-TURNS
  - PROTEASE SENSITIVITIES
- CHEMISYNTHESISE PUTATIVE IMMUNOGEN
- EXAMINE REACTIVITY OF PUTATIVE IMMUNOGENS WITH MONOCLONAL ANTIBODIES WHICH NEUTRALISE INFECTIOUS DISEASE CAUSING AGENT
- EXAMINE EFFECTS OF SO SCREENED POLYPEPTIDES IN VIVO

TABLE V

VACCINES AND INFECTIOUS DISEASE

- WORLDWIDE VIEW
- DEVELOPED COUNTRIES
- UNITED KINGDOM SITUATION
  - TRADITIONAL
    - Single Disease
  - MODERN
    - Single disease and
    - Disease Syndromes

TABLE VI

INFECTIOUS DISEASES UNDER CONTROL

(United Kingdom)

- RINDERPEST	)	Diseases thought
- FOOT-AND-MOUTH	)	absent for the
- AUJESZKY'S	)	last two years
- SWINE VESICULAR	)	(1983 - 1985)
- RABIES	)	

- BRUCELLA	)	
- NEWCASTLE	)	
- BOVINE TB	)	Endemic diseases
	)	more or less

TABLE VII

DISEASE SYNDROMES

(BOVINES)

- RESPIRATORY DISEASE
- MASTITIS (UDDER INFECTIONS)
- ENTERIC DISEASE

TABLE VIII

SOME COMPONENTS OF THE RESPIRATORY DISEASE SYNDROME OF BOVINES

- MYCOPLASMA
- RESPIRATORY SYNCYTIAL VIRUS
- PASTEURELLA HAEMOLYTICA
- DICTYOCAULUS VIVIPARUS
- MUCOSAL DISEASE-BOVINE VIRAL DIARRHOEA
- INFECTIOUS BOVINE RHINOTRACHEITIS
- PARAINFLUENZA 3
- HAEMOPHILUS SOMNUS

TABLE IX

SOME COMPONENTS OF THE MASTITIS DISEASE SYNDROME OF BOVINES

- STREPTOCOCCUS AGALACTICAE
- STREPTOCOCCUS AUREUS
- STREPTOCOCCUS UBERIS
- ESCHERICHIA COLI
- BACILLUS CEREUS
- PSEUDOMONAS SPP.
- CLOSTRIDIUM PERFRINGENS
- CAMPYLOBACTER JEJUNI
- CORYNEBACTERIUM PYOGENES
- CORYNEBACTERIUM ULCERANS
- HERPES MAMILLITIS

TABLE X

SOME COMPONENTS OF THE ENTERIC DISEASE SYNDROM OF BOVINES

- ROTAVIRUS
- CORONAVIRUS
- ASTRAVIRUS
- MUCOSAL DISEASE-BOVINE VIRAL DIARRHOEA VIRUS
- SALMONELLA SPP.
- ESCHERICHAI COLI
- CAMPYLOBACTER JEJUNI
- CLOSTRIDIUM PERFINGENS
- CRYPTOSPORIDIUM
- COCCIDIA

TABLE XI

INTENSIFICATION OF LIVESTOCK UNITS

ANIMAL	SIZE OF UNIT	NUMBER OF UNITS (1974)	% INCREASE OVER 1972
CATTLE	500+	1097	+83
SHEEP	2000+	1476	+18
PIGS	5000+	56	+166
POULTRY (CHICKENS)	50000-500000 (1982)		

TABLE XIII

FOOT-AND-MOUTH DISEASE

VACCINE CAN BE IMPROVED

- INACTIVATION
- COLD CHAIN
- STORAGE
- LOWER ANTIGEN COST
  - WIDER USAGE
  - MORE ANTIGEN/DOSE
    - GREATER EFFICACY
      - LONGEVITY OF PROTECTION
      - SPEED OF PROTECTION
    - BETTER CROSS PROTECTION

TABLE XII

DISEASES WHICH POSE A THREAT TO THE UNITED KINGDOM

- AFRICAN SWINE FEVER
- FOOT-AND-MOUTH
- SWINE VESICULAR
- RABIES
- AUJESZKY's

FIG 1

PROCESS BIOTECHNOLOGY DEFINED

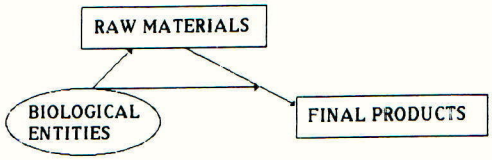


FIG 2

BIOTECHNOLOGY

A synergisitic interaction between bio-Science and bio-Technology

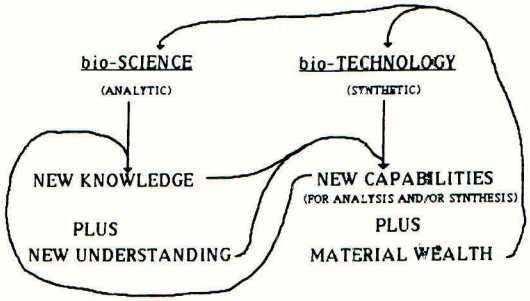
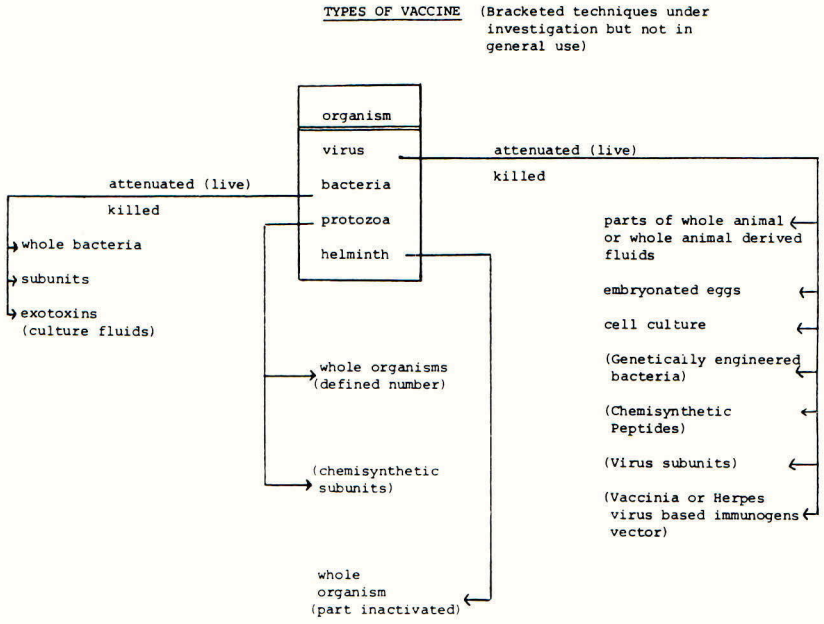
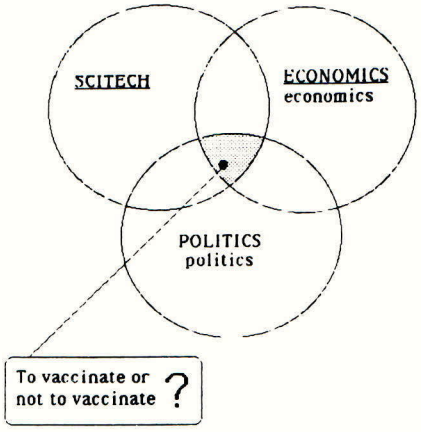


FIG 3



(Note: Often a vaccine is made by a number of different methods concurrently: a "horses for courses" situation.)

FIG 4



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BCPC Monograph No. 34 ISBN 0 901436 96 8

Edited by Peter D. Day

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