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THE GENERAL IMPLICATIONS OF BIOTECHNOLOGY IN THE AGRICULTURAL INDUSTRY

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ABSTRACT

Reference is made to the newer technologies that are classed under the general heading of biotechnology and some of the objectives being pursued in the application of these technologies which have a relevance to agriculture are briefly outlined. Stress is laid on the need for further basic understanding of cellular and molecular biology before the full potential of biotechnology can be realised. In the final part of the paper the overall role that biotechnology may play in the agricultural industries of developed and developing countries is commented upon.

INTRODUCTION

Biotechnology, defined as the application of biological organisms systems or processes to manufacture and service industries (Anon, 1980) comprises a number of technologies based upon increasing understanding of biology at the cellular and molecular level. Some of the technologies are listed in Table 1; undoubtedly others will follow as knowledge advances.

TABLE 1

Some of the more recent techniques associated with biotechnology (Dunhill and Rudd, 1984)

Recombinant DNA manipulations
Monoclonal antibody preparation
Tissue culture
Protoplast fusion
Protein engineering
Immobilized enzyme and cell catalysis
Sensing with the aid of biological molecules

That the agricultural industry of the UK is important to the national economy is reflected in data relating to output for the year 1983, the last year for which complete statistics are currently available (HMSO, 1985). Total agricultural output of £11.19 billion comprised £2.98 billion as farm crops, £1.15 billion as horticultural crops, £4.02 billion as livestock and £3.05 billion as livestock products. In terms of gross domestic product, GDP, (gross output - gross input), the figure for 1983 is £5.27 billion or 2.0% of total UK GDP. During 1983 home produced food represented some 62.7% of all food consumed in the UK or 78.1% of all indigenous-type food consumed. If the agricultural and food processing industries are combined they contribute to just over 7.0% of UK GDP, some two-thirds of which relates to processes beyond the farm gate. In the EEC context and relating to the year 1979 (EEC, 1982) the agricultural and food processing industries represented some 7.0% of the EEC GDP output with each industry contributing approximately equal proportions. From the foregoing it is not surprising that the application of the newer biotechnologies to these industries is receiving a great deal of attention currently.

APPLICATIONS OF BIOTECHNOLOGY TO AGRICULTURE

Table 2 details some of the objectives currently being pursued in the application of biotechnology to agriculture. Up to the present it is true that successful commercial application has been limited - the development of certain vaccines and, also of novel protein sources for inclusion in human and farm livestock diets are examples. In certain cases the successful outcome will most likely have to await the development of additional powerful technologies based on further advances in fundamental knowledge of cellular and molecular biology. Brief mention will now be made concerning some of the projects listed in Table 2.

TABLE 2

Some applications of biotechnology to agriculture

Crops:	Improvement in	(i)	yields
		(ii)	ratio of primary to secondary products
		(iii)	nutritive value of products
		(iv)	physical properties - i.e. breadmaking
		(v)	resistance to insecticides and pesticides
		(vi)	capacity to resist stress
Livestock:	Improvement in	(vii)	disease control
		(viii)	efficiency of reproduction
		(ix)	yields of livestock products, i.e. meat, milk, wool, eggs
		(x)	composition of livestock products - i.e. leaner meat
		(xi)	feed value of low quality feeds - i.e. straw
	Introduction of	(xii)	novel protein sources
Man:	Introduction of	(xiii)	novel protein sources for man - i.e. meat analogues

BIOTECHNOLOGY AND CROPS

Plant breeding

It is widely accepted that one of the reasons for the markedly enhanced production of cereal grains during the last forty years has been the availability of greatly improved varieties of seed developed by the application of classical genetics and plant breeding. In England and Wales barley yields increased by 84% from 2.30 t/ha to 4.2 t/ha over the thirty year period from 1947 (Silvey, 1978). From a study of some 37 varieties of spring barleys grown in this country during the 100y period to 1980, Riggs et al (1981) concluded that the genetic contribution to annual grain yield increase over the period 1958-1980 was 0.84%. In the USA annual yields of maize have increased by 1.0% during the 50 year period from 1930 of which some 70% is attributable to genetic improvement, the remainder to improvements in non-feed factors such as fertiliser practise and pesticide usage (Frey, 1985). Similar results have been obtained from crops in different regions of the world, productivity increases ranging from 0.5 - 2.0% per annum.

Conventional plant breeding suffers from a number of drawbacks (Shaw, 1984) - particularly in its reliance on the natural process of fertilization to introduce modifications to the genetic make up of a plant and the

restriction of the gene pool to the range of plants which are sexually compatible. The application of genetic engineering technology will circumvent such restrictions and allow plant breeders access to a much more diverse range of genes. Both Shaw (1984) and Frey (1985) have emphasised that the new technologies will only add a new dimension to plant breeding not replace it and, that currently, successful application of the new technologies is being hampered by the lack of a sound understanding of the genetic processes concerned with crop productivity. Frey (1985) considers that genetic improvement in the major agricultural crops up to the end of the present century will be mostly the result of conventional plant breeding practise, the major contributions of biotechnology to crop improvement occuring in the 21st century.

Most of the genetic improvements in grain crop yields obtained during the last 4-5 decades have been associated with increases in harvest ratio defined as the ratio of primary (i.e. grain) yield to total crop yield (grain plus straw). For example in the studies on spring barleys by Riggs et al (1981) the harvest ratio for the spring barley Chevalier, introduced before 1900 was 0.322; that in the variety Proctor introduced in 1953 was 0.415 while for the variety Triumph, introduced in 1980 the ratio is 0.495. There is evidence that for some crops, grown under carefully controlled conditions, record yields, close to calculated potential yields have been obtained (Blaxter, 1982). The establishment of a plant breeding industry based on a deep understanding of cellular and molecular biology offers the possibility to overcome such limits to production. For example the pathway of photosynthesis in temperate plants involves fixation of carbon dioxide under the action of the enzyme ribulose-1, 5-biphosphate carboxylase. It has been suggested (see Shaw, 1984) that small alterations in the characteristics of the enzyme via changes in the coding sequence of the appropriate gene may result in enhanced efficiency. Another possibility could be the replacement of the three carbon pathway of fixation characteristic of temperate region plants by the four-carbon pathway of carbon dioxide fixation to phosphoenol pyruvate which exists in certain tropical fodder plants.

Nitrogen-fixation

Another, potentially very significant approach is that of introducing symbiotic, nitrogen-fixation into non leguminous plants. There is much speculation whether such plants would be less productive than their nitrogen-dependant relative, due to the high-energy requirement for nitrogen-fixation, (Shaw, 1984); this may not prove to be such a very significant factor in food production in the Third World countries where the lack of dependance on fertilizer nitrogen could be very important. In addition their use in developed countries might reduce the problems associated with nitrate pollution.

Increased disease resistance

Additional improvements are likely to be associated with increased, and longer-lasting resistance to crop pests in plants and also increased resistance by plants to cost-effective herbicides and pesticides. The technique of in vitro tissue culture and selection has already allowed Molecular Genetics, Inc. to develop maize plants that are resistant to a family of herbicides which act by inhibiting an enzyme in the pathway of synthesis of the three branched-chain amino acids. Tissue culture selection of material in which the enzyme's specific binding properties are modified have rendered the herbicides non-toxic to the plants (Frey, 1985).

Improved quality of plant product

Another important area in which plant biotechnology is likely to have a considerable impact on the agricultural industry is in the development of plant varieties whose seed proteins have been modified to the needs of man. One such example is the improvement in breadmaking quality of high-yielding, home grown wheats which is largely, though not entirely dependant upon the characteristics of the gluten proteins present in the seed endosperm (Miflin et al, 1983). Marais Huntsman is an example of such a wheat variety that is used for animal feed; if used for breadmaking it produces loaves of low volume and irregular crumb structure (Ellis, 1984).

A second such objective is to improve the nutritive value of the cereal proteins for man - and also pigs and poultry, by alteration in overall amino acid composition of the proteins present; cereal proteins are limiting in lysine (Shewry et al, 1984) and to a lesser extent threonine. It is often overlooked that cereal proteins provide the major dietary source of protein for man.

Improved resistance of plants to stress

The potential for improved crop plant productivity arising from the presence of mycorrhizal fungi is being investigated. Such fungi benefit their symbiotic host plants by facilitating water and nutrient uptake, particularly of phosphorous and some of the trace elements, and increased resistance to stress. The subject with reference to biotechnology and in particular genetic engineering has been recently reviewed by Hirsch (1984).

BIOTECHNOLOGY AND THE LIVESTOCK INDUSTRY

Disease control

Vaccines for the prevention of colibacillosis scours in piglets and calves, developed by application of recombinant - DNA technology are already on the market (Storm, 1984) while vaccines for use against foot and mouth disease are in the process of development (Delente, 1984). Currently work is in progress on the production of a novel 'live' vaccine against infectious bronchitis virus in poultry again based upon the newer technologies (Brown and Boursnell, 1984).

Harnessing the production of microbial metabolites in the control of livestock disease is already playing an important role in the maintenance of livestock health; examples are the use of monensin isolated from Streptomyces cinnamonensis as a coccidiostat for poultry and the avermectins, isolated from Streptomyces avermitilis as very effective anthelminties.

Reproduction

Improvements in efficiency of reproduction in farm livestock involving techniques such as super ovulation, embryo transfer and cloning are already having an impact in the livestock breeding industry.

Livestock production

One of the best known examples relating to biotechnology and livestock production relates to the use of recombinant DNA derived bovine growth hormone (somatotrophin) to increase milk production from the dairy cow. Bauman and colleagues (see Bauman and McCutcheon, 1985), in experiments with lactating cows yielding in excess of 9000l/lactation observed that daily injections of the manufactured growth hormone extending over a 188 day period increased milk yield over that of controls not receiving the hormone

by 23-41% depending upon the level of the hormone administered. There were no significant changes in milk composition and no adverse effects noted arising from the daily hormone administration. The extent to which recombinant DNA derived bovine growth hormone becomes a part of accepted dairy cow management is, as yet, a matter of conjecture. Currently considerable research effort is being expended in the development of a satisfactory and publically acceptable method of administering the hormone without recourse to daily injections.

Daily growth hormone administration to young stock has been shown to increase daily liveweight gain and feed conversion in cattle, sheep and pigs (for references see Armstrong, 1985); with pigs and most probably with ruminants, the increased daily gain is associated with higher content of protein at the expense of fat.

A second technique for enhancing live weight gain in farm livestock is that of autoimmunisation against the hormone somatostatin (somatotrophin release inhibiting factor). In experiments with lambs so immunised, the treatment induced a considerable increase in growth rate resulting in a 20% reduction in rearing time to slaughter, a 27% improvement in feed utilization and no detrimental effects on body composition (Spencer, 1985).

In the far longer term, means of growth stimulation through modification of the mammalian genome, as demonstrated in mice by Palmiter et al (1982) has great implications for world livestock production. The same is true for wool growth in sheep and studies are well advanced in the initial and essential task of characterizing the individual genes of the wool keratin gene complex (Ward et al, 1982).

Recombinant DNA technology and rumen bacteria

The rumen microbial population plays a major role in the digestion of feeds by cattle and sheep. Considerable interest is currently being shown in the potential of the more recent techniques for modifying the rumen microbial ecosystem cosystem with the objective of increasing efficiency of production of the host animal (see Table 3).

TABLE 3

Some potential objectives in attempting to modify components of the rumen ecosystem (Armstrong & Gilbert, 1985)

Enhance cellulolytic activity
Introduce the capability to cleave ligno-hemicellulose complexes
Reduce methane production
Decrease proteolylic and/or deaminase activities
Increase biuretase activity
Increase microbial production of specific amino acids
Introduce the capability for N-fixation

Armstrong and Gilbert (1985) have stressed the technical difficulties to be overcome in attaining any of these objectives and also that, even should appropriate modifications to specific bacteria be achieved it is uncertain whether the modified organism would compete successfully with the unmodified organism for survival in the complex rumen microbial ecosystem.

Improvement in nutritive value of low quality feeds

The introduction of enzymes capable of degrading or at least modifying the ligno-hemicellulose complexes of the mature plant cell wall into individual species of microorganisms already possessing strong cellulolytic properties may provide a means of improving the digestibility and hence nutritive value of the very considerable amounts of straw associated with cereal grain production. This objective is one of a number that may result from basic studies in progress on the problem of lignin biodegradation (Wallace et al, 1983).

Novel food (protein) sources

The introduction during recent years of a number of protein sources originating from the growth of microorganisms has considerable significance for the feed industry. The most well known is 'Pruteen', developed by ICI and based on the microorganism Methylophilus methylotrophus grown on methanol as a substrate (Rodgers, 1984). Efficiency of growth of the microorganism has been improved by the use of recombinant DNA technology to introduce a more efficient pathway for the incorporation of ammonia, used as the nitrogen source for microbial protein synthesis. The product is becoming increasingly used by the feed industry as a source of protein in diets for broilers, piglets, pre-ruminant calves and fish.

In recent times ICI have linked up with RHM to commercially develop a protein rich, mycelial food produced by the continuous fermentation of a selected strain of Fusarium graminearum growing on a glucose substrate derived from wheat, maize or any other high starch crop. The product is capable of being processed into a meat analogue and is currently being test marketed as a major component of cold slicing meats, pies etc.

BIOTECHNOLOGY AND THE AGRICULTURAL INDUSTRY

It is clear from the foregoing that the potential contribution of the present technologies and those yet to be developed to world food production is very great. With reference to the intensive agricultural industries of developed countries such as the USA and those of most of the EEC countries there are already surpluses of most food products that can be considered indigenous to their climates and the question often posed is why add to the surpluses? There are at least two factors that will encourage the application of new developments.

The first is that their application should further increase the efficiency of food production using the term in the economic sense to mean providing food at the lowest possible cost. The data shown in Table 4 indicate that during the past four decades, agricultural developments have steadily reduced the cost of basic food commodities. In these developed countries the application of biotechnology should have as its primary goal no change in output but rather the same output but at lower cost.

TABLE 4
Average prices standardized to 1980 money values
paid to UK farmers for certain products (Jollans, 1985)

Commodity	1940	1960	1980
Wheat £/tonne	178.71	135.81	99.30
Barley £/tonne	284.31	141.24	92.84
Potatoes £/tonne	78.10	65.96	51.20
Milk p/litre	23.32	16.72	12.64

A second factor relates to the likely impact that further knowledge concerning the relation between nutrition and human health may have on future food habits. Some of the changes in UK food habits proposed in the NACNE (1983) and COMA (1984) reports, particularly those relating to a reduction in total fat intake and the proporation of saturated fatty acids can well be assisted by application of biotechnology to, for example, the production of meat. An additional impetus will derive from the capability of improving the nutritive value of cereal and legume proteins.

With reference to the agricultures of the Third World countries, the ever-increasing rise in world population cannot be ignored. Recent estimates of a world population growth rate of 2.0%/annum implies a 50% increase in population by the end of the century (Blaxter, 1982). Biotechnology must have a very significant role to play in rendering more productive the agricultural industries of Third World countries.

There is a further aspect upon which comment is justified. Taking the UK agricultural industry as an example of an intensively managed one it has been shown (White, 1978) that, to the farm gate, the industry utilises some 4% of the country's total fossil fuel consumption, this value reaching 16% if the additional components of food processing and packageing (7%) and food storage and distribution (approximately 5%) are also taken into account.

As Blaxter (1982) has pointed out if one considers the whole food producing chain together then for every joule of food energy actually consumed by the population some 10 joules of fossil energy is used; this last mentioned is a finite resource based upon the harvest of past solar radiation. The very considerable advances being made through the application of biotechnology in the production of alternative fuels, such as ethyl alchohol from biomass, the last mentioned being defined as plant organic matter derived from photosynthesis implies that the agricultural industry could become an 'energy' producer contributing to the partial replacement of fossil fuel reserves.

Finally it is certain that the agricultural industries in many parts of the world will increasingly make a major contribution to the production of crops destined for, and in certain instances specifically designed for a 'vegetable plant' based chemical industry.

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A EUROPEAN VIEW OF BIOTECHNOLOGY IN AGRICULTURE

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(Opinions expressed here are those of the author alone; in no circumstances should they be taken as an authoritative statement of the views of the Commission)

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ABSTRACT

Europe's Common Agricultural Policy has encouraged agricultural biotechnology to such an extent that large surpluses of many foods have emerged. Thus land must now be progressively withdrawn from food production and used in some other way. Obvious opportunities exist to provide inputs to various industries (chemicals, pharmaceuticals, forest products ...) and since industry also provides inputs to agriculture interrelationships between the two will intensify. Advances in biotechnology will lead to the design, growth and processing of crops under unified control, irrespective of end use (food, feed, chemicals ...), and to optimisation of the overall system. Similar changes are taking place in the US and the Far East so Europe must compete, or risk having productive land fall idle. The Commission's Biotechnology Action Programme (1985-1989) should improve the context for such competition through European programmes of research and training and by coordination of policies, especially on "feedstock" prices, regulations and patents.

1. THE EMERGENCE OF PERSISTENT AGRICULTURAL SURPLUSES

From its inception the twin aims of Europe's Common Agricultural Policy (CAP) have been to improve security of food supply and to provide a satisfactory income for those involved in farming.

This policy has been astonishingly successful and Europe is participating strongly in the continuing shift, that is clearly discernible in the organised world, to higher productivity in all branches of agriculture. This shift is the result of applying a range of biotechnologies to the problems of plant and animal production. In achieving it the use of nitrogen fertiliser, prepared by the chemical industry, to permit enhanced photosynthetic carbohydrate, natural oil and protein synthesis in crops has been every bit as important, and is just as surely biotechnology, as the use of improved plant and animal varieties and chemical pest control.

As a result, higher productivity food surpluses are emerging in many places, particularly in Western Europe and North America with their nearly static populations. In a recent study carried out for the Commission Rexen and Munck (1984) predict an annual EEC cereals surplus of 58 million tonnes by the year 2000 on present trends and even more than this if quotas are enforced on animal production and improvements are made through new developments in genetics and plant husbandry.

Already the United States is leaving crop land idle and reducing prices to relieve its grain and oilseed surpluses.

The shift to higher productivity is not confined to the developed countries. Thus, according to the World Food Institute of Iowa State University, China's grain production has increased by 50% to 340 million tonnes in the past decade (Anon 1, 1984) and limited its import requirement while "The Economist" reported recently (Anon 2, 1985) that India is exporting wheat because no more storage space is available for its accumulated stocks, which could reach 30 million tonnes during 1985.

If these better organised developing countries no longer offer an expanding market for primary agricultural produce it still could be argued that surplus food will be needed as aid to combat famine in less well organised or naturally disadvantaged parts of the world. Indeed the Commission does already help in this way and will certainly continue to do so in the future. Such aid, however, will always be a short-term palliative. Commission policy is to couple it with longer term help to famine-affected countries designed to shift them towards self-sufficiency in food production as the only satisfactory long-term solution to famine. Broadly this policy is working well. The average annual supply of cereal grain per world inhabitant increased by 20% to 360 kg between 1960 and 1980 (Rexen and Munck, 1984).

2. SURPLUS LAND IN EUROPE: THE NEED TO FIND NEW USES FOR LAND

Thus Europe must now begin to take substantial areas of land out of food production. The rate at which this must be done can be reduced by import substitution particularly in seed oils (75% imported), oilcake (90% imported) and tropical produce, but it cannot be achieved faster than the necessarily slow development of new crop varieties, ways of cultivation and processing methods.

The emergence of surplus land in Europe presents an unprecedented challenge and opportunity for new economic developments. These can include tourism and recreation but there is scope especially for the development of manufacturing industry based on agriculture in its broadest sense. Europe, with its first-rate skills in the biological sciences and its powerful industry (food, chemical, pharmaceutical, forestry ...) has an outstanding opportunity to make use of an emerging domestic asset.

3. AGRICULTURE AND INDUSTRY TODAY

Industry already makes some of its products from agricultural raw materials, though during this century, and especially between 1945 and 1973 the tendency has been to use fossil hydrocarbon-based synthetics to replace natural products in fields such as dyestuffs, plastics, textiles, industrial solvents, specialty chemicals and pharmaceuticals. Throughout this period we have often learnt our chemistry from the living world and applied it in the factory. Thus today's highly profitable and effective pyrethrin insecticides are purely synthetic improvements on the natural products discovered in the flowers of a daisy that is lethal to insects. Similarly, synthetic rubbers are a good attempt to mimic the natural product of a tree.

Nevertheless, in spite of the great industrial importance of fossil hydrocarbons as industrial feedstocks, significant industrial production is still based on agricultural raw materials such as wood, cotton, natural rubber, natural oils and fats, starch, sugar and medicinal plant extracts.

A report (Anon 3, 1985) prepared recently by the European Council of Chemical Manufacturers' Associations (CEFIC) shows that 3 million tonnes of technical oils and fats, 1.26 million tonnes of starch and 0.1 million tonnes of sugar are being used each year by European industry despite the discouragement to use of the carbohydrates by current pricing policy.

The products made from these raw materials include the obvious ones of timber, paper, cotton cloth, rubber and many less obvious ones such as soaps, detergents, coatings, thickeners, lubricants, organic acids, amino acids, antibiotics and industrial enzymes.

In the last decade the incentive to use agricultural crops for industrial purposes has increased as fossil hydrocarbons have become relatively dearer. Thus the price of crude petroleum has increased during this period by an order of magnitude relative to that of US maize (Rexen and Munck, 1984).

In the chemical industry there is a considerable technical potential for use of agricultural feedstocks. The best opportunities for expansion exist for the manufacture of higher-priced products, the "specialty chemicals" to which the industry is already turning in the face of new competition in bulk chemicals from the Middle East and elsewhere. Some of these are already made in considerable measure from natural oils and fats, which in 1980 constituted 2% of the raw materials used for synthetic organic chemicals worldwide. This share is expected to rise rapidly if the price of vegetable oil relative to mineral oil continues to fall. (Table 1, from Thomas, 1982)

TABLE 1

Natural oils and fats as a raw material for chemicals (per cent)

Share in raw materials	1980	Expectations for 1990
Coatings	40	80
Detergents	45	70
Plasticides	15	30
Adhesives	1	5
Agrichemicals	10	25
Thermoplastics	2	4
Lubricants	20	30

The production of specialties by biological routes is a field in which Europe is particularly strong and is also one that a recent US report from the Office of Technology Assessment (Anon 4, 1984 p. 6) saw as offering more varied potential applications for biotechnology than any other industrial sector at the present time. Some obvious opportunities for substantial production are in the manufacture of polysaccharides, plastics and high quality oils and fats. All of these could offer important outlets for agricultural raw materials. Thus an optimum-sized plant for making the biodegradable specialty plastic, polyhydroxybutyrate (PHB) would require 120,000 tonnes of starch or sugar annually to produce 30,000 tonnes of PHB.

According to the US report just mentioned a number of oxygenated hydrocarbons used in the million tonne per year range, could become realistic targets for production from agricultural sources if costs could be reduced. It quotes a statement made in 1981 (p. 248):

"that the potential exists for a smooth introduction of four microbial products (ethanol, isopropanol, n-butanol and 2,3-butanol) into the US chemical industry, and that these products may foster other bioprocess development. In order for this transition to take place, however, either the costs of producing these products must be reduced (to about 20 to 40 percent of their existing costs) or the price of petroleum must rise. Reducing the costs of production of chemicals from biomass is a prerequisite to commercial success in all case studies thus far."

Among the oxygenated hydrocarbons the case of ethanol is worthy of special mention because its use in Europe has been suggested as an octane enhancer to replace lead alkyls in gasoline, or even as a motor fuel in its own right. There is certainly an immense potential market. However, in European conditions with agricultural raw materials more costly than in the US, very substantial technical improvements leading to large reductions in cost are essential to tap this market.

For forestry there is an immense opportunity to reduce the Comunity's imports of wood and wood products, which amount to about 50% of use and cost well over 10,000 million ECU each year, by substituting domestic production.

In the food industry, which takes almost all its raw materials from agriculture at present, the ingenuity being applied to adding value is increasing. Thus a recent European development is the successful test marketing in the UK by the supermarket chain, Sainsbury's, of savoury pies containing high quality mycoprotein made by fermentation. While in Japan the pharmaceutical giant, Mitsubishi's best-selling new biological is not a pharmaceutical, but soya milk.

4. A NEW RELATIONSHIP BETWEEN AGRICULTURE AND INDUSTRY

Biotechnology is now leading to a sharp intensification of the relationship between agriculture and industry as a result of the increased control that can be exerted over chemical processes in both sectors. It is becoming easier to dictate the chemical composition of the plants and animals produced by agriculture, and of the products made by industry. To an increasing extent a given desired change can be achieved in either sector. Inevitably agriculture and industry are being drawn closer together in a process driven by what can be sold on the market.

Plant agriculture is a system for using some of the energy that reaches us from the sun to convert carbon dioxide, a minor component of the air, into chemicals through the agency of plants. The primary products are the complex mixtures of chemicals represented by the plants themselves. Animal agriculture converts parts of plants rather wastefully into different mixtures of chemicals such as milk, eggs and meat.

Industry relates to both plant and animal agriculture by providing inputs: fertilisers, pesticides, machinery, vaccines ... and by taking outputs for downstream processing into commercial products such as those mentioned in section 3.

The push behind the revolutionary events that are bringing agriculture and industry together is coming from the results of R&D in many fields, and one can already point to probable changes whereby functions now performed by factories will be performed by plants and vice-versa (the examples of mycoprotein and soyamilk have already been given).

What is happening can be illustrated in a more general way by reference to recent developments in plant breeding. The new plant genetics of tissue culture, recombinant DNA and hybridisation technology have already added to the power of conventional plant breeding to such an extent that the first plants altered by them are now available to plant breeders for commercial production in the cases of corn, rice, oil palm, oilseed rape, alfalfa, tomato, potato, carrot, brassica and sugar cane according to a recent survey quoted by Kidd (1985).

The survey, which covered 400 agricultural, scientific and agribusiness experts from 20 countries concludes that the retail value of all seed improved by the new genetics worldwide will be 14.2 billion US dollars (1983 dollars) by 2005, i.e. nearly 30% of the value of all seed planted today. That represents a 57% annually compounded growth rate from a base of 8.5 million US dollars in 1985.

Significantly seed companies have for some time been subject to determined acquisition strategies by the manufacturers of herbicides, fertilisers, pharmaceuticals, energy products and processed foods. One could not expect these principals to reveal all their targets, but an inkling of what is in their sights may be gained from the first annual review of Calgene, a company that employs 60 scientists on commercial exploitation of the new plant genetics. Three of their aims are to engineer:

i) crops tolerant to specific herbicides (e.g., corn, soybean, cotton, tomato and tobacco tolerant to glyphosphate) thus expanding the range of crops in which these herbicides can be used and thereby reducing the need for the chemical industry to design new herbicides;

ii) commodity oilseed crops (soybean and rapeseed) to produce high-priced specialty oils thereby challenging coconut, palm kernel and petroleum derivatives as raw materials for detergents, lubricants and other specialty chemicals listed in table 3.;

iii) tomatoes to increase the solids content for savings in food manufacturing costs.

5. METAMORPHOSIS OF THE AGRO-INDUSTRIAL COMPLEX

The intensifying relationship between agriculture and industry requires that an overall view be taken of the agriculture, food, chemical, pharmaceutical and energy industries to guide decision-making as technical, political, economic, environmental and social influences ebb and flow.

Taking such an overall view of the energy balance could at some stage lead to the conclusion that greater emphasis should be placed on improving symbiotic nitrogen fixation in agriculture for the production of "chemical" crops at the expense of petrochemical inputs for fertiliser and chemicals production.

In the longer term the whole agro-industrial complex could undergo a profound metamorphosis of the type that has taken place in the past few decades in the electronics industries, as discussed by Pelissolo (1980) in his report to the French Prime Minister. Thus if the principles of photosynthesis came to be thoroughly understood this could permit direct and efficient use of solar energy for chemical synthesis without the need for using plants thereby making it possible to devise indefinitely sustainable abiotic systems for producing food, chemicals and liquid fuels that would make today's methods seem clumsy, in some cases, even barbaric.

6. THE WORLD CONTEXT

The United States probably occupies the world's most favoured position for participation in the agro-industrial revolution because it combines an abundance of unused or under-used land with strengths in all the relevant industrial fields and a large home market. The recent OTA report (Anon 4, 1985) affirms this position and predicts that already by 1990 35 million tonnes of maize, equivalent to 21 million tonnes of

starch, will be used in the US to produce chemicals. If this does happen substantial additional amounts of corn gluten products will be coproduced and add further to the pressure on world animal feed markets.

In contrast, Japan is short of land, must seek partners to complement its strong industrial and research capabilities if it is to compete effectively in the developing revolution, and has a smaller home market than Europe. Japan is already seeking agricultural partners in South East Asia where the often excellent growing conditions favour the development of plantation crops such as palm oil and rubber.

Europe has some big advantages. It has much more land than the Japanese, with a steady supply becoming available for new uses. It has a strong agricultural base, thanks to a successful agricultural policy that has encouraged the achievement of the highest yields of grain per hectare of any in the world. It has a powerful chemical industry, bigger than those of the United States and Japan, that is strongly innovative in the biological field, holding, for example, three quarters of the world market in industrial enzymes. It has a strong and innovative pharmaceutical industry.

These industrial strengths rest on an equally powerful and diverse pure and applied research base in the natural sciences that has produced many of the world's recent Nobel prize winners in these fields.

Europe is also the world's largest trading bloc based on a very large internal market.

Nevertheless Europe will need to use all its strengths if it is to play a leading role in the agro-industrial revolution. Ways must be found to link our agriculture to our industries as the only alternative to allowing productive land to fall into disuse while simultaneously becoming dependent on the efforts of others for a growing proportion of our biologically-based industrial products.

7. COMMISSION POLICY

The Commission identified six priorities to stimulate biotechnology in the Community and to increase competitiveness in Europe's bio-industries, including agriculture (Anon 5, 1983), namely:

I Research and Training
II Concertation of policies and actions
III New regimes on agricultural outputs for industrial use
IV Regulations affecting biotechnology
V Intellectual property rights in biotechnology
VI Demonstration projects.

A 5-year programme (Anon 6, 1984) embracing action on the first two priorities was approved by Council in March 1985 with a budget of 55 million ECU. The research and training elements of this programme relate to agriculture, food and industry. In so far as they relate to agriculture and food they continue and extend the 15 million ECU research and training programme in Biomolecular Engineering (1982-1986).

The concertation of policies and actions is being pursued through a specially created unit known as CUBE (Concertation Unit for Biotechnology in Europe). Its purpose is to monitor developments in biotechnology, to assess their significance for Europe and to promote coordination both within the Commission and between the Commission and Member States. A central activity of CUBE that clearly embraces agriculture is the "consideration of how safe and sustainable use of the renewable natural resource systems in Europe may be enhanced by the application of biotechnology". (Here as elsewhere, in collaboration with the services with direct responsibility for policies).

New price regimes for starch and sugar, designed to encourage increased use of these agricultural raw materials by making them available to industry at prices comparable to those enjoyed by competitors outside the Community, have been proposed to Council.

The promotion of a harmonised European approach to regulations and intellectual property rights in biotechnology should encourage new developments in agriculture, for example the creation of new crops for agricultural use, the testing of which - under practical farming conditions - could be the subject of demonstration projects.

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THE POTENTIAL OF RECOMBINANT DNA TECHNOLOGY IN AGRICULTURE

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In this presentation I will attempt to describe the current "state of the art" with regard to the application of Recombinant DNA technology in agriculture, with particular reference to plant breeding. In essence I will explain the principles of gene transfer in plants, and describe the currently available vector systems and their limitations. Simply put, the technology merely involves the isolation of a gene or genes of interest, and their insertion into a recipient plant, via the use of a suitable vector. In principle therefore, any gene, from any source can be introduced if it is thought worthwhile.

Plant breeding is possibly one of the oldest applications of science in history, but it was not really until this century that science was applied in a major way to improve crop plants. A combination therefore of trial and error and good science has, down the years, resulted in enormous improvements in crop yield. Why then is there a need for alternative methods of crop breeding? The reason is that there are inherent drawbacks to conventional plant breeding:

(1) **Limited available gene pool.** Restricted by the range of plants capable of sexual hybridisation with the crop plant.

(2) **Time.** Commonly it takes 10-15 years to get a new variety into the farmers field, where it may be quickly superceded.

(3) **Plant management.** The programmed application of agrichemicals necessary for maximum yield in today's crops, brings several penalties: cost; energy drain; and potential pollution.

(4) **Constraints of genetic framework.** Without radical changes in the anatomy and physiology of crop plants it is predicted that some of them may reach their yield plateaus in the near future.

Gene transfer technology depends upon the ability to cut and splice DNA, using restriction and ligation enzymes. Gene transfer to plants involves the insertion of a particular gene into a vector, and its transfer to a single cell of the recipient plant. Regeneration from this transformed cell, and expression of the transferred gene are then necessary. The essential requirements for the perfect plant gene vector system are: DNA transfer at detectable, and consistent frequency; transferred DNA of reasonable size; no rearrangement of transferred DNA during transfer; stable maintenance of transferred DNA; correct expression of transferred DNA; normal plant development not affected by transferred DNA;

The gene transfer systems currently under investigation are as follows:

(1) Ti-Plasmids of Agrobacterium tumefaciens;
(2) Plant Viruses: (a) Caulimoviruses; (b) Geminiviruses; (c) RNA viruses;
(3) Direct DNA systems: (a) Protoplast Transformation; (b) Microinjection;

Ti-plasmids

Agrobacterium tumefaciens causes Crown Gall tumour on plants, by virtue of the transfer of a small part of the Ti- (Tumour inducing) plasmid to the plant genome. This part, termed the T-DNA, carries genes which perturb the phytohormone balance, and result in the production of unusual metabolites, termed opines, which only the inciting bacterium can use.

Nature's genetic engineer has therefore been harnessed for artificial gene transfer. It is now known that the functions of DNA transfer and tumour formation, are separate. This has led to the production of disarmed vectors, in which the tumour genes are deleted, but which retain the ability to transfer DNA, and the resultant transformed cells are capable of regeneration to produce a fertile plant carrying the new gene.

The Ti-plasmids do have some drawbacks: their large size and complexity requires that much of the manipulation is done in E.coli; and despite recent reports of transformation in some monocots, at present there is no evidence to indicate that Agrobacterium can be used with the major monocot crops. However most of the current gene transfer experiments have been performed using the Ti-plasmids.

Virus Vectors

The most intensively investigated of these is Cauliflower Mosaic Virus (CaMV) which has already been used to introduce an antibiotic resistance gene into plants. The common drawbacks of plant viruses are their small carrying capacity, and the paucity of sites available for gene insertion, potentially alleviable by more research. Their common advantages are that they provide vectors capable of replicating to high copy number, rapid dissemination throughout the plant, and they remove the necessity to introduce desired genes into the germ line.

Direct DNA Systems

Using chimaeric genes developed through use of the Ti-plasmids, several groups have recently reported the successful transformation of protoplasts, of both dicots and monocots. This is clearly going to be a rapidly expanding area in the near future.

The availability of reproducible gene transfer systems has opened up enormous possibilities for plant molecular biology, allowing the analysis of problems previously not amenable. Physiological responses to plant growth regulators can be investigated, because the T-DNA carries single genes perturbing these systems. DNA recombination and transposition processes are also open to analysis. However, it is the area of gene expression and its regulation which is generating the most excitement.

It is only two years since expression of a foreign gene was first achieved in plants, by hooking up an antibiotic resistance gene to the nos promoter, derived from the T-DNA. My own group in Durham, has since produced a functional map of this promoter, the first demonstration at the DNA sequence level of the exact requirements for gene expression in plants. The regions of DNA responsible for light regulated gene expression are now known, and because transgenic plants can be produced we are beginning to analyse developmental regulation of gene expression. These analyses are allowing us to define DNA cassettes encoding different forms of gene regulation, which may simply be spliced to donor genes to achieve the desired expression patterns.

With the technology rapidly being developed for performing plant genetic engineering, it is worthwhile examining some of the possibilities for its implementation:

(1) **Seed Proteins.** The genes encoding the major seed storage proteins are available, so in principle the alteration of their coding sequences so as to increase the levels of certain amino acids, followed by their reintroduction into the plant is feasible. However, this will have to be achieved without altering the physico-chemical properties of the proteins, with correct seed expression, and against a background of endogenous, multigenic expression.

(2) **Pest and Disease Resistance.** A plant expressing the insecticidal toxin of Bacillus thuringiensis has already been developed by a group in Belgium, and the transfer of other antimetabolite protein genes is well underway.
(3) **Herbicide and Pesticide Resistance.** Calgene have recently announced the transfer of a gene conferring resistance to the widely used herbicide glyphosphate (Tumbleweed) from bacteria to plants. As with the previous case however, it remains to be seen whether the level of expression achieved is sufficient to produce the desired resistance. These two cases do represent one of the strongest possibilities for commercial application.
(4) **Nitrogen Availability.** Long touted as the answer to agriculture's nitrogen problems, the likelihood is that achievements in this area will most probably come from manipulation of soil and root bacteria.
(5) **Photosynthesis.** Protein engineering may be applied to improve the efficiency of the major rate limiting step, that catalysed by Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and is certainly aided by the availability of the genes for both subunits.
(6) **Drought and Stress Resistance.** As little is known of the molecular basis of such phenomena, these remain long term prospects.
(7) **Modification of biochemical pathways.** Transfer of enzymes capable of alteration of undesirable compounds, eg tannins, may extend the range of species acceptable for consumption. An example of this is a project recently begun in my group to genetically tailor the oil composition of oilseed rape. The alternative side of this approach is to design new secondary product pathways for the production of novel products in tissue culture or in the field. One of the more fanciful possibilities is the production of diesel oil in an oilseed crop.
(8) **Expression of "High-Value" Products.** The drift from bacteria to yeast and animal cells as expression factories in recent years, was necessitated by the special subtleties of gene regulation and protein processing in higher organisms. Plant cells are capable of most of the processes performed in animal cells, so they may be a viable alternative. However, except for the most expensive products, tissue culture is unlikely to be economic, but the plant in the field is a highly efficient bioreactor using free energy. Thus given the availability of the correct expression cassette, high value products could be produced in seeds, and encapsulated in protein bodies, which protect the merchandise, and minimise the problems of downstream processing. The current European agricultural surpluses will allow the diversion of land into alternative crop production.
(9) **Ornamentals.** The possibilities for producing unusual ornamental species eg. blue roses, or black tulips or even luminescent species, are quite staggering.

Gene transfer technology has definitely arrived, but has unfortunately outstripped the availability of genes worthy of its potential. The major advantage that plant systems have is their totipotency. However this has only been demonstrated in a limited number of cases, and this is another area that is inhibiting the development of gene transfer. It is all very well being able to transfer genes to single cells but if those cells will not regenerate, as is the case at present with the major crop plants, the monocots, the technology can not be applied for genetic engineering. Thus the true potential of this technology will only be realised through advances in tissue culture. However I believe that even at best this technology can never supplant conventional plant breeding, but merely to provide another tool in the breeder's armoury.

For a more detailed exposition of this subject, the reader is referred to:
Genetic engineering of crop plants: a strategy for the future, and the present. C.H. Shaw (1984) Chemistry and Industry, 23, 817-824.

FUTURE TRENDS IN PLANT BREEDING

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ABSTRACT

The application of plant molecular biology to crop plant breeding is subject to a number of constraints. These include the genetic complexity of the characters that breeders work with, the need for extensive field tests of new materials and present limits to our abilities in isolating and transforming genes of agronomic importance. Even so some applications have been possible and the prospects for many more to come are very promising. There is an urgent need to develop skills in the middle ground between breeding and molecular biology to facilitate technology transfer.

INTRODUCTION

Plant breeding has benefited in many ways from the application of discoveries in science and engineering (Day, 1985). Two that are exciting some interest at the present time are single seed descent and chemical hybridising agents. Single seed descent is used to hasten progress towards homozygosity in small grains like wheat and barley that are self-pollinating. The method requires a moderately large controlled environment facility that can run at low temperature and low light to vernalise seedlings of winter-sown crops. It must also support production of a single small ear on each plant grown under conditions of extreme crowding and dwarfing. By this means it is possible to complete three generations of selfing in a year. Chemical hybridising agents, or gametocides, facilitate large-scale production of F1 hybrid seed without the need for genetic male steriles or hand emasculation. These chemicals are applied before flowering and interfere with the production of viable pollen. The treated plants thus act as female parents and are wind pollinated by adjacent untreated plants. Provided the problems involved in producing large quantities of seed to meet appropriate purity standards can be overcome, F1 hybrids could lead to fairly rapid increases in yield. Small-scale production of hybrid seed is also useful as a tool for identifying the best parent combinations for pedigree selection breeding since the yield of F1 hybrids is a guide to the prospects of successful selection for high yield in later generations.

A BREEDER'S PERSPECTIVE

In this paper I shall limit myself to the application of molecular biology to crop plant breeding. Gunn & Day (1985) recently reviewed applications of tissue culture in plant breeding. Although it is clear that powerful new methods are emerging from work in molecular biology, like many other authors I will deal mostly with prospects rather than practical examples. It will disappoint some readers that there is so little to show in the way of practical applications at the present time. We cannot yet point to a newly-produced crop variety as a product of recombinant DNA technology. There are several reasons for this, and it is important to understand them.

Although potentially useful new traits such as herbicide resistance can now be introduced into plants by genetic transformation, the time taken

to use such variants in a breeding programme to produce useful new cultivars means that it is too early for this to have happened. A substantial part of the time taken in breeding and variety development involves tests and trials carried out over several seasons and at several different sites to evaluate new material. The extent of improvement has to be quantified by yield and quality data and by records of pest and disease resistance. The advantages that a newly-engineered trait may confer on a new crop variety must be clearly demonstrated. It must be shown also that no undesirable effects have been introduced.

To take winter wheat as an example, the first seven or eight generations of selfing and selection that follow the original cross take up to eight years before they result in a relatively true breeding line. During this time yield, quality and resistance data are accumulated that will form the basis of the technical description needed when the line is submitted for National List testing. Three years of national testing are necessary before a variety may find a place on the Recommended List, and a further year of multiplication may be necessary before it is on sale to the farmer some twelve years after the cross was originally made. The single-seed descent technique can reduce the twelve-year span by up to three years, but cannot be used in later generations when it is important to evaluate plant phenotypes in the field. The products of biotechnology require just as extensive testing by breeders before they are ready for national testing. This means, therefore, that even when we introduce a gene for herbicide resistance into an otherwise well adapted variety already on a Recommended List, it could still take up to six years before it is marketed. To shorten this time scale would mean taking risks. Farmers and seed merchants want assurances that a new variety is stable, has no hidden drawbacks, and lives up to the claims made for it. The rigorous testing and evaluation procedures already in use in the UK that apply to conventionally bred varieties are well suited to evaluate newly genetically engineered forms.

A second reason for the delay is the time taken to develop the technology to deliver new genetic information, to control its expression and to identify and isolate important crop plant genes that are amenable to manipulation in this way.

A third difficulty arises from the conceptual gap between plant breeders and molecular geneticists. They each appreciate something of the others' problems and motivations, but work at either end of a spectrum of activities. Progress will depend on the effectiveness of work in the middle ground between these two disciplines. This I believe is a challenging but neglected aspect of biotechnology. It will depend on the one hand on understanding the control of gene expression, the nature and organisation of genetic information in nuclei and organelles, and on the other on an appreciation of whole plant development and physiology in relation to breeding objectives.

BREEDING OBJECTIVES

The problems come into focus when we examine these objectives. They are to increase yield, improve quality and reduce costs. The last item includes resistance to pests, diseases and environmental extremes, and better utilisation of applied fertiliser. The pragmatic approach taken by plant breeders is to select parents that together have the other qualities that

are desired and look for segregants among the progeny that show increased yield.

The genetic engineer has to identify limiting factors in the many processes that contribute to yield. For example short straw has led to greatly increased harvest index in cereals by partitioning more fixed carbon into grains and less into stems and leaves. Increases in net rates of photosynthesis are a common objective, but in practice there have been few successful applications of selection for higher rates of photosynthesis that have resulted in improved varieties. The differences in net rates of photosynthesis between C3 and C4 species continue to be a spur to these efforts. A related objective is to manipulate the structure of the enzyme ribulose bisphosphate carboxylase (rubisco), which not only carries out carbon dioxide fixation, but is also responsible for a light-dependent form of respiration that converts fixed carbon into CO_2, which is carried out at the same time. C4 species are able to minimise the effects of photorespiration. The question that is still unanswered is, can the active site of rubisco be altered in such a way that the oxygenase activity in a C3 species is inhibited without impairing carboxylase activity?

Yield and quality are interrelated in the sense that the breeder wishes to maximise both in the harvested product. When it is possible to control the expression of genes that direct the synthesis of particular products,such as the endosperm storage proteins in a grain crop, the amount of these products could be increased either by increasing the copy number of the genes or by altering their control. The excitement of molecular biology is that it provides new tools and ways of exploring the black boxes represented by our crop plants. The size of the challenge however is such that it is small wonder that the progress may seem slow.

A number of characters are, like yield, controlled by many different genes of individually small effect. While it is possible that such genes may be manipulated in blocks, being transferred as chromosomes or parts of chromosomes, there is little or no practical application of such approaches because of the difficulty of analysing them and following their inheritance. Restriction fragment length polymorphisms (RFLPs) may well provide a more precise tool for the breeder to detect such gene blocks in segregating progenies (Burr *et al.* 1983). In this way selection could be based in part on a characterisation of the genotype without the need to allow it to develop a phenotype. One difficulty is coping with large numbers of DNA extractions and restriction digests and then carrying out electrophoresis. These operations are time consuming and labour intensive, however with automated equipment thousands of gel patterns could be compared, rather than tens or hundreds at the present time. For single major genes it will be useful to probe segregating families to detect individual plants which carry a particular gene (or genes). Although *in situ* hybridisation methods are very useful in detecting genes in high copy number, or in detecting alien chromosome arms, so far it has not proved possible to develop probes to detect single, or low copy number, genes sufficiently accurately to discriminate their presence from 'background' noise in the system.

The detection of nucleic acid sequences in plant material is also useful in identifying plants that carry virus or other infections (Baulcombe *et al.* 1984). cDNA probes are a cheaper and more easily produced means of detecting single-stranded RNA viruses than specific antisera used in the

enzyme-linked immunosorbent assay system. Nucleic acid probes are also useful in discriminating cytoplasmic male sterile phenotypes in maize breeding programmes (Flavell *et al.* 1983). In this instance the probe detects the presence of specific DNA sequences in the mitochondrial genome of the male sterile which are either different or absent in the normal plants. The role of chloroplasts and mitochondria in plant cells and the parts they play in photosynthesis and respiration of course make it essential that their molecular biology is fully explored in order to develop methods for manipulating their structure. Although there are still no reliable methods for transforming the DNA genomes of mitochondria and chloroplasts, at least in the latter we now know it is possible to effect a transformation of those genes whose products are exported from the nucleus to the chloroplast for assembly and function in that organelle. This knowledge will provide a basis for transformation affecting herbicide resistance (Goodman: this volume). Recent reports that T DNA can be found in the chloroplasts of some plants transformed with *Agrobacterium tumefaciens* suggests further possibilities of targeting transforming DNA to organelle genomes.

The catalogue of genes that are potentially useful in agriculture, that are being isolated and cloned, is steadily increasing. It includes genes for storage proteins in maize, wheat, peas and beans; genes for the large subunit and the small subunits of rubisco from a range of plants including algae, blue-green algae and several crop plants; genes for a variety of enzymes expresssed in plants, including alcohol dehydrogenase in maize, alpha-amylase in wheat, sucrose synthetase in maize, and genes involved in phenol propanoid synthesis in Antirrhinum and other plants.
Genes of bacterial origin that are of interest include those for nitrogenase and other processes involved in nitrogen fixation, and a gene for the insecticidal delta-endotoxin polypeptide produced by *Bacillus thuringiensis*. Genes for antibiotic resistance, such as chloramphenicol transacetylase and neomycin phosphotransferase are useful markers for selecting transformed cells on the basis of their resistance. A variety of methods are now in use for isolating new genes of interest, and a central issue is now their mode of regulation and in particular the control of their expression during development. It will clearly be extremely important for plant breeders of the future to be assured that newly introduced genes are only expressed at the right time and in the right place during the development of the crop. It would be a waste of energy and resources, for example, to synthesise a particular endosperm storage protein in crop plant roots or leaves. The recent finding that the bean storage protein gene carries sequences that limit its expression to when seeds are formed in tobacco is encouraging. It tends to confirm that the regulation of gene expression is subject to general controls that we may expect to find are common to a wide range of genera.

A SHOPPING LIST OF GENES

While RFLPs could one day greatly simplify the selection of polygenic characters, most breeders would probably agree that relatively simple probing methods to discriminate between different nuclear or cytoplasmic genotypes will be useful.

A good example of this is the demonstration by Bowman and Bingham (personal communication) who compared organellar DNA restriction digests to show that

the wheat variety Rendezvous, with resistance to eyespot (Pseudocercosporella herpotrichoides) derived from Aegilops ventricosa, has a chloroplast genome derived from Aegilops and not from Triticum. This information is very useful to the breeder concerned with cytoplasmic or organellar contributions to the phenotypes of his breeding lines. Probes that detect viral RNA in potato (Baulcombe et al. 1984), chromosome 1B with a short-arm from rye in European wheats (Hutchinson et al. 1985), and rapid methods for discriminating between mitochondrial DNAs to identify cytoplasmic male sterile lines of maize (Flavell et al. 1983) provide further examples.

I will not discuss herbicide resistance further except to note that it can be useful in other ways than extending the range of crop species on which a particular chemical may be used. One application is in the selection of hybrid from selfed seedlings when the male parent carries dominant herbicide resistance. Atrazine resistance has been suggested for this purpose in oilseed rape (Thompson et al., 1983).

Recent work by Nasrallah (1985) has shown that a cDNA clone prepared to stigma mRNA of Brassica oleracea can be used as a probe on DNA digests of segregating populations to recognise the S alleles that govern sporophytic self-incompatibility in Brassica. This work should soon lead to the transformation of Brassica with genomic DNA for specific S alleles and may better facilitate the production of hybrid seed in Brassica by using self-incompatibility.

Genes for disease resistance so far remain elusive. There is still insufficient information on the nature of their products and mode of action to design direct methods. For example a synthetic oligonucleotide based on part of the amino acid sequence of the B. thuringiensis delta-endotoxin was used to isolate the toxin gene by Monsanto scientists (Watrud, 1985). Transferred to Pseudomonas fluorescens, an epiphyte of corn roots, it may afford protection against root-eating larvae of sensitive Lepidopteran pests such as corn earworm. For the time being three approaches are being made to isolate genes for disease resistance (Day et al. 1983).

The first is to isolate a bacterial avirulence gene, and by characterising its product hypothesise its host target which is presumably either the resistance gene itself, its product, or a structure whose synthesis is directed by the gene product. Staskawicz et al. (1984) have made considerable progress in this direction by defining a small segment of chromosomal DNA in the soybean pathogen Pseudomonas syringae pv glycinea which determines avirulence on resistant soybean cultivars.

A second approach is to explore the co-ordinated control of protein biosynthesis that occurs either when resistance is evoked by an avirulent pathogen or when a similar reaction is evoked by a chemical or physical treatment. An example is recent work by Broglie et al. (1985) who have followed the induction of "defence" proteins such as chitinase that are synthesised by bean leaves following ethylene treatment. These authors have shown that the synthesis of many normal proteins, like rubisco, is shut down and that the leaves switch to the synthesis of a range of up to 40 proteins which were either not present or present in only small amounts. The synthesis of chitinase for example is increased 100-fold. Defining the DNA elements involved and understanding the transcriptional control of these genes may well provide important clues to the nature of

the genes that begin the cascade of syntheses culminating in resistance.

A third approach is to use the method of transposon tagging. In brief this makes use of a mobile DNA element called a transposon. When the transposon jumps into an otherwise functional gene it has the effect of blocking correct transcription and produces a mutation of that gene. If the DNA sequence of the transposon is known and it is present in the plant in low copy number, probing DNA restriction fragments of the mutant will recover those which carry the transposon. Some of these will be flanked by DNA sequences of the mutated gene. Experiments are in progress in several laboratories to use maize transposons in an attempt to find mutations from resistance to susceptibility to several maize pathogens. Spontaneous mutations resulting from other mechanisms can be eliminated by observing the restoration of the original phenotype in such mutants when the transposon 'jumps out' of the mutated gene. This can sometimes happen at high frequency to produce, in the case of pigment genes, characteristic patterns. The inheritance of these patterns enabled Barbara McClintock to describe the phenomenon of transposon mutagenesis in the 1950s for which she recently received a Nobel Prize.

The isolation of genes with products of direct interest such as seed storage proteins is of considerable interest. A recent paper by Day et al. (1985) reviews progress in this direction in breeding for quality improvement in wheat.

TRANSFORMATION

It is now common knowledge that the DNA delivery system of Agrobacterium tumefaciens can be harnessed to introduce foreign genetic information into those plants that are natural hosts of this bacterial pathogen. These plants are all dicotyledonous, but recent claims for transforming asparagus suggest that progress may be made with other monocots including the cereals. Although naked DNA can be prepared and used to transform protoplasts, either by natural uptake methods or by electroporation, our present inability to regenerate and recover whole plants from cereal protoplasts is, for the time being, an obstacle (Flavell and Mathias, 1984).

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2.
Potential Uses for Agricultural Crops

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STRAW AS A FERMENTATION RAW MATERIAL

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ABSTRACT

Straw is an abundant lignocellulosic plant residue which is generally regarded as a waste product of agriculture. The cellulose and hemicellulose fractions together account for about 80% wt/wt and the lignin content is about 14% wt/wt. The primary polysaccharide fractions (c. 50% of cellulose and hemicellulose combined) decompose more rapidly than a secondary fraction which appears to be bound to lignin. Even so, the primary decomposition is slow relative to other plant residues containing a greater proportion of soluble components. More rapid fermentation can sometimes be achieved with chemical or enzymic pretreatments. The primary products from cellulose are simple sugars which can be used for a secondary fermentation to produce alcohol. Using microbial consortia, primary and secondary fermentations may be combined. With such approaches it seems that proteins are the products which are likely to be most economically attractive. The protein products include single-cell protein, mushrooms, enzymes and microbial cells with crop protection value. Submerged fermentation with solid substrates is feasible but there is scope for the development of novel solid-substrate fermenters.

STRAW AS A SUBSTRATE

The annual straw production in England and Wales is between about 11 and 13. 5 million tonne of which about one half is burnt because decomposition in the field can lead to harmful effects on establishment of subsequent crops (Ellis & Lynch 1977). Generally there is less straw burning in other countries but there is tremendous scope for the use of this resource worldwide. One of the major obstacles to utilization is its large bulk which makes transport from the locations of production to utilization difficult and expensive (Ministry of Agriculture, Fisheries & Food 1984). There is, therefore, considerable scope to utilize it close to the site of production unless high value products make transport worthwhile.

The major components of straw are cellulose (a glucose polymer) and hemicellulose (a polymer of xylose and other sugars). Studies on cellulolysis have been extensive in recent years, the degradation taking place by the following route:

Native cellulose
↓ endo-1,4-β-D-glucanase
Reactive cellulose
↓ 1,4-β-D glucan cellobiohydrolase
Cellobiose
↓ β-glucosidase
Glucose

It is now generally considered that for efficient cellulolysis the components of the cellulase complex must act synergistically.

Many studies on lignocellulolysis have been with pure cellulose substrates. There has been less study of xylanases and other enzymes responsible for hemicellulolysis. Trichoderma spp. are probably the most actively studied cellulolytic organisms. Generally they possess a xylanase but it does not follow that all biotypes will be hemicellulolytic.

There have been very few studies on the degradation of straw as lignocellulolysis per se. The degradation of the lignin (polymer of phenols) component has been studied with natural grass lignin labelled by the uptake of ^{14}C-phenylalanine or with synthetic ^{14}C-labelled dehydrogenative polymerizate of coniferyl alcohol (Patterson et al. 1984). The white-rot and brown-rot fungi and actinomycetes have been used most extensively in ligninolytic studies. There appears to be a general assumption that cellulolytic and ligninolytic organisms will act co-operatively in bringing about an efficient degradation of straw. This however is pure speculation. Indeed the evidence from studies in soil is that a large proportion (c. 50% wt/wt) of the celluloses of straw is broken down before any lignin is degraded (Harper & Lynch 1981). It appears that the lignified layers of straw are spatially inaccessible and recalcitrant during early decay. Possibly early ligninolytic activity could make more of the carbohydrate fraction of straw immediately available but unless this happens it might be preferable in a fermentation process of such a cheap substrate to accept that there would be a substantial residual waste.

Little is known about the variation of straw type (plant species or cultivar) or the effect of crop husbandry practices on the biodegradative potential although there have been limited studies (Harper et al. 1985; Knapp 1983).

Whereas the primary focus of this paper is on the potential use of straw in fermentation per se, it should be recognized that one of the oldest natural cellulolytic fermenters is usually in a field and moves around, viz. the rumen! Even here, however, there is scope for biotechnological development because straw is not readily digestible and biological pretreatment can improve this.

PRETREATMENTS

One of the principle objectives of physical and chemical methods of pretreatment has been to disrupt the lignin-carbohydrate bonds in the substrate. However this is not the exclusive objective as some methods are mainly focussed on making more surfaces of the solid substrate available for attack. The processes available have been reviewed (Dunlop & Chiang 1980).

Grinding, and cutting are common practices in the chemical industry for size reduction and an increase in surface area of the substrate; with straw this is typically achieved by ball-milling. Irradiation has improved digestibility of cellulosics, probably by altering cellulose/lignin bonds or the degree of polymerization. Treatment with heat and pressure has met with mixed results. Treatment with sodium hydroxide is one of the oldest and best known methods of improving digestibility of straw in the rumen but the chemical mode of action is still uncertain.

Aqueous, gaseous or liquid ammonia is another alkali treatment which has been used with success. Sulphur dioxide is a more recent but unproven technique. There is also a wide range of nonaqueous solvent systems that will selectively dissolve cellulose from a lignocellulosic material while leaving the lignin intact.

MICROBIAL CONSORTIA

Quantitatively, the major products of straw breakdown are simple sugars. As a saccharification process this is most unlikely to be sound on economic grounds. The potential of the degradation can only satisfactorily be harnessed if the product is used in a secondary process, either by the same organism or, more likely by a secondary organism. Hence there has been considerable interest in mixed culture fermentations.

The biochemistry of cellulose breakdown by mixed cultures was reviewed by Veal & Lynch (1984a). Amongst the beneficial activities that can occur with mixed cultures are: (a) removal of the hydrolytic products of the cellulolysis, (b) removal of metabolite inhibition, (c) modification of metabolic products, (d) provision of growth factors and (e) enzyme synergism.

One of the major interests in mixed culture studies has been where anaerobic cellulolytic and non-cellulolytic clostridia have been paired to enhance ethanol formation from lignocellulose. Our interests have been in linking aerobic cellulolytic fungi with anaerobic non-cellulolytic nitrogen-fixing clostridia (Lynch & Harper 1983; Veal & Lynch 1984b). The nitrogen so fixed is channelled back to the fungus to enhance cellulolysis in a nitrogen-deficient environment.

GENETIC OPPORTUNITIES

The lignocellulolytic process of straw breakdown clearly involves many gene products. U.v.-light and nitrosoguanidine have been used by Montenecourt & Eveleigh (1977) and Wood et al. (1984) as mutagens and an agar plate screening method has been devised to select from strains of Penicillium pinophilum which hyperproduce cellulase, β-glucosidase and xylanase enzymes in the presence of a catabolite repressor and/or end product inhibitor. Eveleigh (1984) has outlined the opportunities of cloning the genes of carbohydrate utilization (in the first instance cellobiose) using the plasmid vector pULB113 and direct transformation of DNA into Zymomonas. Patterson et al. (1984) outlined the molecular biological approach to enhance lignin breakdown and the first report of a lignin-degrading enzyme has now appeared (Tien & Kirk 1983), which now opens the avenue to cloning. Such approaches could give new dimensions to the potential of straw as a substrate.

BIOMASS PRODUCTS

We reviewed this subject earlier (Lynch 1983, Lynch et al. 1984, Wood & Lynch 1984) and there has been a major interest in lignocellulolysis worldwide in recent years. The European initiative has particularly come from the CEC R&D programme on Recycling of Urban and Industrial Waste. In the United States there has been a similar initiative by the Solar Energy Research Institute supported by the United States Department of Energy. Many of the new initiatives have ignored the classic and well-proven example of biotechnological utilization of straw, that of mushroom production. However some recent publications have emphasized this process

as successful biotechnology (Bisaria & Madan 1983, Tautorus & Townsley 1984, Wood 1984). In 1984 in the UK there was an estimated 84,000 tonnes of Agaricus bisporus produced with a farm gate value in excess of £100M, which now exceeds that of any other horticultural crops. Even with such a scale the UK production is still small compared with the United States and France which are greater by factors of about 3.5 and 2.2 respectively. Whereas A. bisporus accounts for about 75% of the total production, other mushrooms (Volvariella volvacea) which also utilize straw as substrates are also important, particularly in Asia. The basic procedure of A. bisporus production on straw is as follows:

1. Growth of spawn on sterilised cereal grains.

2. Composting of straw in stacks 2 m wide to which manure and fertilisers are added with a series of wetting and turning procedures (2 weeks at 60-70°C in stack centre).

3. Aerobic 'pasteurization' or 'peak heating' in a heated room for 5-7 days.

4. Inoculation of spawn into compost at 25°C. Incubation for 2-3 weeks.

5. Casing layer of peat and chalk is applied to induce fruiting.

6. Picking of flushes of fruits over 4-6 weeks.

The procedure is a non-axenic system, analogous to wine and yoghurt manufacture, and an intermediate level technology. It provides a useful model for both simpler and more sophisticated culture of fungi on solid substrates.

Mushroom production is a source of protein from straw but there is also scope for the production of animal feed, both by increasing the protein content of straw and by improving its digestibility. The semi-solid fermentation of ryegrass straw has been achieved scientifically (Han & Anderson 1975) and been patented (Han & Anderson 1976). In the procedure microbial consortia were used with cellulase coming from Cellulomonas sp and Trichoderma viride and white rot fungi providing lignolytic activity on NaOH-treated straw. On acid-hydrolysed straw, the yeasts Candida utilis and Aureobasidium (Pullularia) pullulans have been used. There have been only a few studies on the microbial upgrading of straw for feed but considerable interest in chemical treatment. Clearly this scientific option is there but the limitation is likely to be an economic one. A sensible target would be to utilize the straw close to the site of production and to use low-technology fermentation systems. However, a greater scientific input is needed because elevated levels of enzyme activity could have a marked effect on the economics; this has been the objective of the group at the Rowett Research Institute (Wood et al. 1984).

A variety of exo- and cell-bound enzymes are produced on straw as a solid substrate and it is possible that these could be useful economic end-products (D.A. Wood, personal communication).

It has already been indicated that Trichoderma spp. are powerful cellulolytic organisms and they are good colonists of straw (Harper & Lynch 1985). We, like many other groups (e.g. Papavizas 1984) have found them to be effective as biocontrol agents against a range of plant diseases.

Generally they have been produced in submerged fermentation on soluble carbohydrates. However production on a solid substrate is particularly effective in producing spores and these could either be harvested by washing them from the substrate or by using them directly on the substrate base which might increase their chances of survival in nature. Again there is scope for mixed culture fermentation with nitrogen-fixing bacteria as outlined above.

CHEMICAL PRODUCTS

In producing simple sugars from cellulose, alcohol production is an obvious potential fermentative end-product. The major interest in this fermentation has been with starchy substrates such as sugar-cane. Even for this the economics of the process are somewhat dubious, Brazil being an exception because of its heavy dependence on oil imports. With dominantly cellulosic substrates such as straw, the economics of alcohol fermentation would be even more dubious unless very efficient cellulolytic systems could be developed. Similar considerations apply to methane as a fermentation end-product, starch being the preferred feedstock (Hobson et al. 1984). However, there is scope for introduction of straw into the simple biogas reactors of some less well-developed countries (DaSilva 1979).

Lignin, being polyphenolic, is a potential transformation substrate to produce high-value chemicals. However, a clear target product is not obvious at this stage.

A variety of polysaccharides have been demonstrated as product of pure and mixed culture microbial degradation of straw (Chapman & Lynch 1984). These polysaccharides can be used to stabilize soil structure and it is possible that a suitable fermentation could be developed for extracted or combined polysaccharides to be used on weakly structured soils which are common in many parts of the world. However, more likely, suitable inocula could be produced for application to straw in the field and the polysaccharide development could take place in situ. Again, the primary fermentation to produce inocula could be on straw.

REACTORS

The need for a technological input to the development of a solid-substrate fermenter has already been illuded to. In our laboratory (P. Hand & J.M. Lynch, unpublished) we have already had some success with a system based on a stainless steel rotating drum.

An alternative system is to develop a column system packed with the substrate (cellulose, holocellulose or straw) and with a trickle irrigation from the top a continuous source of cellulase and xylanase can be produced (D.M. Gaunt, A.P.J. Trinci & J.M. Lynch, unpublished). However, as this has only been investigated on a small scale in the laboratory, the economic feasibility of such an approach is unclear.

Eveleigh (1984) has shown that Trichoderma reesei can be immobilized onto celite. This can give a useful source of continuous cellulase production but also might be applied in projects that use massive spore inocula, such as biocontrol of plant pathogens.

CONCLUSION

In the past straw did not appear to be a very attractive fermentation raw material because of its slow degradation. A notable exception is the mushroom industry which is based on it. New dimensions are opening with genetic and physiological modification of the necessary degradative enzyme systems. Providing a clear sight is set on the economic limitations in comparison with other relatively cheap substrates there appears scope for further scientific evaluation for this agricultural waste which has led to environmental problems (burning) to be used in the fermentation industry. It is unlikely, however, to make a direct impact on the straw surplus except where low-grade technology can be used on-farm.

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3.
Advances in Applications to Crop and Animal Breeding

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HERBICIDE RESISTANT MAIZE THROUGH CELL CULTURE SELECTION

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ABSTRACT

The weed problems in crops are changing to species that are extremely tolerant of the currently available selective herbicides. An alternative approach to finding new, more selective herbicides is to develop new crop varieties that are resistant to very effective herbicides that would normally damage the crop. The imidazolinones are a new class of herbicides that control a broad spectrum of annual and perennial grasses and broadleaves. A project was initiated between American Cyanamid and Molecular Genetics, Inc. to find maize lines resistant to the imidazolinones via cell culture selection techniques. The selection protocol required that the concentration of the imidazolinone used to find resistant lines did not inhibit the formation of somatic embryoids which are necessary for plant regeneration from these cultures. A cell line, XA17, was identified which could grow on medium containing levels of an imidazolinone 30 times higher than would inhibit growth of unselected callus tissue. Plants regenerated from line XA17 and crossed with imidazolinone susceptible plants passed on their resistance to the next generation. The imidazolinones kill plants by inhibiting acetohydroxyacid synthase (AHAS) which prevents the plant from producing valine, leucine and isoleucine. Imidazolinone resistance is due to a change in AHAS so that the enzyme is no longer inhibited by the herbicide.

INTRODUCTION

Efficient crop production requires that weeds be adequately controlled in the crop. Weed control in recent years has relied very heavily on the use of selective herbicides which will kill the weeds while not harming the crop. There are many excellent herbicides available to the growers, but through the use of herbicides there have been shifts in the weed populations to species which are as tolerant of the herbicide as the crop (Haas & Streibig 1982). This has meant that new herbicides have to be even more selective in order to control these new weeds without harming the crop. In order to discover and register these new herbicides, agrichemical companies must make large investments in research and development, which results in increased cost to the grower and the consumer. There are extremely potent herbicides available which can effectively control a broad spectrum of weeds, but these same herbicides will also damage the crop. An alternative approach to discovering new, more potent herbicides through random screening and synthesis of new chemsitry is to select for crop varieties which are resistant to previously non-selective herbicides.

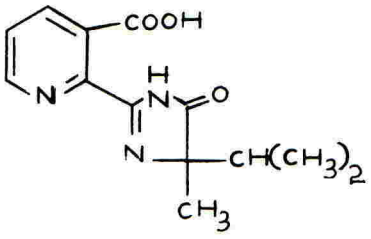
There are several approaches that can be used to select for herbicide-tolerant crop varieties. One of these is through classical plant breeding techniques in which either a great number of different genotypes are screened for herbicide resistance or a previously existant resistant trait from a closely related species is transferred into a crop. This latter technique has been successful in transferring triazine resistance from a resistant Brassica species into oilseed rape (Machado 1982). Classical breeding does require a large investment of time, labor and land in order to be successful.

A second approach is through genetic engineering in which a gene expressing herbicide resistance is transferred to a previously susceptible species. If the gene product is expressed in the recipient species then it should become resistant to the herbicide. The problem with this approach is that a gene must be identified that will confer herbicide resistance to a plant. For most herbicides the actual site of action of the herbicide is unknown, which makes it extremely difficult to isolate a gene coding for a resistant enzyme. An alternative approach is to transfer a gene into a crop that codes for an enzyme that detoxifies the herbicide. Unfortunately most of these enzymes are not known and many detoxification systems involve several different enzymes working in concert, making it even more difficult to transfer this trait into a crop. Another problem with the genetic engineering approach, besides the expression of a new gene in a plant, is that the techniques have to be available to transfer a gene into a plant. Currently the only system available works exclusively in dicots and not in monocots.

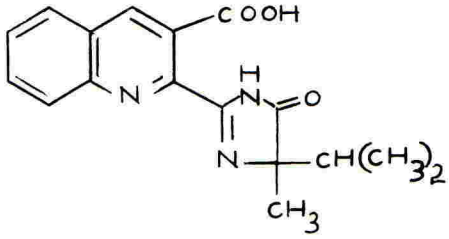
A third approach which has proved successful is to use plant tissue culture to select for cell lines that are resistant to the herbicide (Meredith & Carlson 1982). This technique takes advantage of the properties of plant tissue growing in culture, namely the ability to screen a large number of idividual cells for resistance in a fairly small area at a relatively low cost. Plant cells growing in culture have been shown to have varying genotypes due to growing under culture conditions (Meredith & Carlson 1982). The problems associated with this technique are that the herbicide has to have the same affect on plant tissue growing in culture as it does on tissue in the intact plant, and the culture has to be able to regenerate whole plants so that the resistance can be transferred to desirable commercial varieties of the crop. The examples in which herbicide resistance has been selected for in tissue culture has been confined to a small number of species (i.e. tomato, tobacco, and carrot) that have limited commercial potential. Until recently there have been no reported cases in which herbicide resistance has been found in a major monocotyledonous crop such as maize through cell culture selection. This paper will describe how imidazolinone resistance was selected for in maize cell culture and will characterize the mechanism of this resisitance.

SELECTION OF IMIDAZOLINONE TOLERANT MAIZE

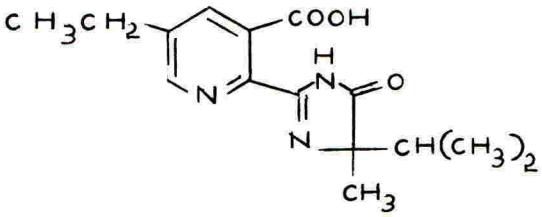
The imidazolinones are a new class of herbicides discovered and being developed by American Cyanamid Company, Princeton, NJ. Examples of the imidazolinones are shown in Figure 1. These herbicides control a broad spectrum of annual and perennial dicots and monocots. The imidazolinones are absorbed by the roots and foliage of plants and translocate in the xylem and phloem (Shaner & Robson 1985). They appear to kill plants slowly. The first symptoms are a cessation of growth followed by chlorosis and then necrosis of the growing points. Die back of the rest of the plant occurs from the growing points.



A-Imazapyr



B-Imazaquin



C-AC 263,499

Fig. 1: Structure of three imidazolinones: A-imazapyr; B-imazaquin; C-AC 263,499.

One of the first parameters that has to be established before setting up a protocol to select for resistant cell lines in plant cell culture is that the herbicide is affecting the plant tissue in the cell culture in the same manner as it affects tissue in an intact plant. The concentraion of imazapyr needed to cause a 50% inhibition of growth of a maize seedling or of maize suspension culture is similar, occurring between 10 and 30 nM of imazapyr (Anderson & Hibberd 1985). Physiological studies showed that the imidazolinones affect amino acid levels in both maize seedlings and tissue culture causing a decrease in the levels of valine and leucine (Anderson & Hibberd 1985, Shaner & Reider 1986). When both intact maize plants and maize suspension cultures were exogenously supplied with valine, leucine, and isoleucine the phytotoxic effects of imazapyr could be prevented (Anderson & Hibberd 1985, Shaner & Reider 1986). Subsequent research showed that the imidazolinones are potent inhibitors of acetohydroxyacid synthase (acetolactate synthase) the first enzyme in the branched chain amino acid biosynthetic pathway (Shaner et al 1984). It was thus established that the mode of action of the imidazolinones was the same in both the intact plant and in cell culture.

One of the early observations of the effects of the imidazolinones on maize callus culture initiated from isolated embryos was that high concentrations of the herbicide did not kill the tissue immediately but did prevent somatic embryoid production. In order to regenerate plants from maize callus cultures it is essential that the cultures continue to produce embryoids. Therefore, the selection protocol had to use a concentration of the imidazolinone that would cause an inhibition of growth without preventing embryoid production. Therefore, vigorously growing maize callus initiated from isolated embryos was transferred from maintenance medium to petri plates containing 0.03 mg/l of an imidazolinone. This concentration caused about a 20% inhibition in growth. After 10 to 14 days calli which showed the most vigorous growth were subcultured to fresh medium with the same concentration of the herbicide. Each callus piece was labeled and became a line. Then at 15 to 30 day intervals each line that was still growing and maintaining embryoid production was subcultured to fresh medium. The consequence of this selection protocol was to decrease the number of lines at each transfer and to increase the number of callus pieces per line. At the end of the fifth selection cycle the concentration of the imidazolinone was raised to 0.1 mg/l to increase the selection pressure. In one of these selection procedures line XA17 was found that could grow vigorously in the presence of 0.1 mg/l of the imidazolinone. By the end of the seventh selection cycle XA17 contributed the majority of tissue still in culture in this particular selection series.

When line XA17 was cultured on media containing higher levels of one of the imidazolinones, imazaquin, it was found to have a 30 fold increase in tolerance over unselected tissue (Table 1). Line XA17 was placed on regeneration medium containing a level of imazaquin that would normally inhibit the ability of unselected tissue to regenerate. Plants were regenerated from line XA17 and pollen from these regenerated plants was used to pollinate a maize inbred line. Forty-one percent of the seedlings from these crosses were tolerant to the imidazolinones. Subsequent crosses have shown that the imidazolinone tolerant trait is controlled by one, stable, codominant gene. The imidazolinone resistance is expressed at the whole plant level as measured by spraying susceptible and resistant plants in the greenhouse with different levels of one of the imidazolinones (Table 2). Subsequent work has shown that the imidazolinone resistance is to all of the imidazolinones.

Table 1
Response of Two Maize Callus Culture Lines to Imazaquin

Imazaquin (mg/l)	Growth Rate[a] (% of Control)	
	Cell Line	
	Unselected	XA17
1	50	103
3	45	107
10	42	110
30	38	61

[a] Growth was measured as an increase in cell volume after 7 days of treatment

Table 2
Response of Two Maize Cultivars to AC 263,499

AC 263,499 (g/ha)	Growth (% of Control)[a]	
	Pioneer 3241	Imidazolinone-resistant cultivar
50	0	100
100	0	100
500	0	100
1000	0	100
2000	0	100

[a] Growth measured 5 weeks after treatment

The mechanism of tolerance appears to be due to a change in the acetohydroxyacid synthase enzyme so that the imidazolinones no longer inhibit the activity of the enzyme (Table 3). Furthermore, the enzyme is resistant to all the imidazolinones. When acetohydroxyacid synthase is extracted from large plants that are tolerant to the imidazolinones, the enzyme is insensitive to the herbicides.

Table 3
Response of Acetohydroxyacid Synthase Extracted from Two Maize Callus Cultures to Imazapyr

Imazapyr (uM)	Acetohydroxyacid Synthase Activity (% of Control)	
	Cell Line	
	Unselected	XA17
1.6	87.9	100.0
3.1	85.9	100.0
6.2	71.5	98.7
12.5	51.6	101.3
25.0	35.7	95.8
50.0	30.0	98.5
100.0	18.2	84.5

The success of this selection procedure demonstrates the utility of this method for selecting for herbicide tolerant plants. The imidazolinones were well suited for this type of selection protocol. They only appear to affect one enzymatic site that is essential for growth in maize tissue in either the intact plant or in cell culture. If the imidazolinones hit a multitude of sites, as some herbicides appear to do, then this procedure would not have succeeded as easily. The research to find an imidazolinone resistant line has occurred relatively early in the commercial development of these herbicides. This has not been the usual case in the past, although tolerance for the sulfonyl ureas, another new class of herbicides, has been selected for in tobacco callus tissue (Chaleff & Ray 1984). Future research will focus on transferring imidazolinone tolerance into agronomically significant maize inbreds using classical plant breeding techniques.

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RECENT ADVANCES IN COMMERCIAL MICROPROPAGATION

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INTRODUCTION

Although Man has been vegetatively propagating superior plant material because of its beauty, growth form or yield for centuries, it is only during the past 20 years that micropropagation has been available to increase the levels of efficiency and control over these systems to previously undreamt of heights.

WHAT IS MICROPROPAGATION?

What then are the practical "dos and don'ts" of micropropagation? It is clearly important if one is to produce many plants from a single individual that the starting material be of the right clonal selection. It should also be realized that the plant must be in an active, vigorous, healthy state for micropropagation to be most effective. Micropropagation cannot be regarded as a rehabilitation phase for poor, sickly material: if it is poor and sick on its own roots, it is going to be equally substandard in culture.

The normal starting material for a micropropagation program is a shoot tip, not necessarily as small as the meristematic apical dome, but something a little larger with leaf primordia already developing around it. This material must initially be surface sterilized. This is usually achieved by washing it with hypochlorite solution at concentrations which do not harm the plant tissue, but which will satisfactorily kill bacterial or fungal contaminants. The plant tissue has to be carefully washed with sterile water, after this treatment, since hypochlorite can damage the plant if prolonged exposures are allowed. Any tissue affected by exposure to hypochlorite is trimmed and the remaining material placed on one of a variety of different media. Since it is initially unknown which medium will be suitable for a specific variety or species involved, a number have to be tried based on literature reports or the experience of the operator. Particularly the growth regulator concentrations have to be adjusted in order to allow the plant tissue to initiate into culture. This phase is really an acclimatization of the tissue to the *in vitro* conditions and can take, in some species, as long as 3 or 4 months, but more usually can be completed within a few weeks. Under the right conditions the small shoot tip will start to grow in a normal fashion, giving rise to a small, unrooted shoot in culture.

The initiated shoot can, after a suitable period on the initiation medium, be subcultured such that nodal sections of the shoot are placed onto a multiplication medium. This medium differs from the initiation medium in that it contains higher levels of cytokinins which are likely to give rise to precocious shooting of the axillary buds. The normal period between subcultures is 3 weeks and during this time the number of shoot tips available for subculture will multiply as a result of breaking of nodal axillary buds anywhere from 2 to 12 times. Thus, after the 3 weeks on multiplication medium, somewhere on an average of about 5 shoot tips can be placed onto fresh medium, and these in turn will multiply 5 times in the subsequent 3 weeks, and thus micropropagation has led to a 25-fold increase in the plant material available over a total of 6 weeks.

As might be expected, there are a number of obstacles which "Mother

Nature" delights in putting in the way of achieving this 25-fold increase. The environmental conditions under which cultures are maintained can have a profound effect on the performance of the tissue. Temperature, light and even the aeration of cultures can all influence the growth and multiplication rate of the tissue. Since the medium on which the plant tissue is growing contains sucrose, it is an ideal habitat for contaminating organisms and the threat of infection from contaminants is always present. All materials must, therefore, be handled under carefully controlled, sterile conditions. This is normally achieved using laminar flow cabinets which provide a flood of filtered, sterile air over the working area. Because the plants grow and multiply over long periods of time, the sensitivity of the plant tissue to the applied growth regulators frequently appears to change, and thus the medium which was initially ideal for multiplication can, in fact, become suboptimal and even harmful to the tissue. When this happens, the tissue distorts and frequently becomes dark, translucent green, giving it a waterlogged appearance. This tissue is sometimes referred to as vitreous tissue with the leaves looking almost like a succulent rather than a normal papery or leathery leaf. Under these conditions, multiplication slows down or can even stop, and it is necessary that the hormone levels and agar levels in the medium be altered before multiplication can resume.

Up to now we have spoken only of the micropropagation of shoot material. Rooting of these micro-shoots can occur in two ways. One can transfer the shoot to media rich in auxins which will allow for the induction of in vitro roots. This is necessary in some species since they need to be actively induced to root. The roots formed in vitro are frequently somewhat different morphologically from those normally formed. They can be slightly swollen and brittle, and frequently upon transfer to the soil new roots have to be formed by the plant before any extensive growth of the shoot can occur and the initial in vitro roots die back. This lag in the establishment of the plants can be critical and certainly costly to a grower. It can be avoided by the direct rooting of the micropropagated shoots into a natural or artificial potting mix. If the micropropagated shoots are treated as micro-cuttings, and simply placed in artificial potting mix and maintained at high humidity such that they do not dry out, roots will form naturally within 2-3 weeks. These are normal physiological roots and not like the in vitro roots mentioned above. They cause no lagphase when the material is potted on and represent an important part in the overall strategy of plant establishment. Some species require that their bases be dipped or treated in auxin powders or pastes in order to induce these roots, but because the handling can be done outside the strictly sterile environment required for in vitro rooting, the process is very much more simple and cost effective.

Once the micropropagated plants are satisfactorily rooted in soil or soil substitutes, they must be weaned to a normal humidity level. During the rooting process they are maintained at close to 100% humidity, and this does not allow for the normal development of cuticle and stomatal activity associated with drier conditions. The rooting normally takes place under conditions of reduced lighting, and therefore the plants must now be exposed for the first time to full sunlight. This is done slowly over a period of perhaps a week to 10 days, during which time they are initially heavily shaded and ultimately weaned in terms of both humidity and increasing light. For the first time the small plants are asked to be autonomous, photosynthesizing for their own fixed carbon. During the rooting phase under the reduced lighting conditions, they are really living on carbohydrate reserves built up during the micropropagation period in vitro. Once this weaning process is completed, the hardened plants can be incorporated into normal

growing systems for their further development.

Using the techniques described briefly above, it is possible to develop hundreds of thousands or even millions of clonal plants. Clearly this raises some questions of monoclonal planting and the problems that might be associated with the epidemic spread of diseases should the clone be planted en masse. It requires that the grower appreciates these problems, and mixes clones appropriately in order that disease and stress resistances can be mixed yet maintained in high yielding, superior varieties.

SOME ADVANTAGES OF MICROPROPAGATION

Numbers and Speed

These comments have covered the generic aspects of plant micropropagation common to almost all systems in almost all species. The system for each particular variety will vary from another in detail. But what are the overall advantages of embarking on this somewhat sophisticated scheme for plant propagation rather than relying on traditional vegetative methods?

Because of the nature of the process, the numbers of plant propagules that can be developed in a short time are extremely large. Currently, worldwide use of micropropagated plants is about 50 million per annum. It is likely that the market itself could absorb more like 250 million per annum, if sufficient facilities were available. The significance of the system lies in the fact that if one sport or hybrid plant becomes available from a grower or breeder, it is possible, using micropropagation, to bulk up this individual plant into many hundreds of thousands of plants within a relatively short time - possibly 18 months to 2 years. This allows the rapid introduction of new lines and varieties into the marketplace. It is estimated that this system shortens the average length of time taken to introduce new lily varieties by about 50% from 15-16 years down to 7-8 years. The same is true of many other plant species.

The constant demand for new varieties and novelty within the ornamental industry means that the micropropagation process can answer an industry requirement by allowing for the introduction of new varieties on a rapid basis. Another factor with regard to micropropagation is that this large scale-up can be done in a relatively small space without having to commit larger areas of valuable production space to this bulking-up process. It means that the plant tissue can be maintained in a sterile environment. Therefore, it is not to be subjected to the risks of pathogens and infections which might otherwise form hazards to crop development.

Plant Health

Good health is a major advantage in the development of elite mother stock material for both the herbaceous ornamental producer and the woody plant grower. Before micropropagation, plant material can be put through stringent tests to bacterially index and virus index the material. The presence of fungal contaminants or pathogens can also be detected and eliminated prior to micropropagation. Thus, the plants produced by the system can be certified as being disease indexed, and they represent clean, elite mother stock material for future conventional propagation methods.

It must be pointed out that there is a distinct difference between being disease indexed and being disease resistant. The growers must accept this disease indexed material into appropriate facilities, and treat it in a manner likely to maintain hygiene and cleanliness. It must be viewed as a propagation resource for the grower and treated with a great deal of care and

respect.

Virus elimination, as mentioned above, can be achieved using micropropagation techniques. The growth of most viruses is slowed or to some degree absent from the meristem or meristematic dome of the growing shoot. This is a small area, highly active with respect to cell division, measuring perhaps 0.1mm by 0.2mm - if this is removed from the growing shoot, it can be cultured so that it grows and starts propagating just as the growing shoot would have done. However, as there were no viruses present in the meristematic dome, the tissue produced is free of viruses. If maintained in a satisfactorily healthy environment, it will be free from risk of infection.

There are other methods of virus elimination which can be used alongside the micropropagation systems. Various chemicals can be added to the medium which slow down or eliminate the development of viruses in the younger portions of the plant tissue, and these can be used in conjunction with meristemming. Heat treatment of many species of plant prior to meristemming can also successfully reduce the virus population in the rapidly growing regions.

The ability to multiply up elite individuals rapidly and the potential for developing disease indexing programs for such material permits easier acceptance of such in vitro propagated material by quarantine authorities. This gives rise to a potential for much more rapid international exchange of new varieties and lines, yet it maintains a high standard of plant health and minimizes the risk of disease spread.

Germplasm Storage

The concept of a tissue bank (a collection of valuable breeding or growing material) in a relatively confined space under controlled environment conditions is particularly attractive for plant breeders. Many plant breeders wish to maintain specific breeding lines or heterozygous individuals in the field or greenhouse for subsequent years' testing or seed production. Micropropagation techniques applied to these materials allow them to overwinter the material in the safety of a controlled environment and also give rise to the potential for multiplying up individuals for specific crossing or testing.

This elimination of the seasonal nature of much breeding and growing work has been another advantage often suggested with respect to micropropagation. However, a word of caution is necessary here - although it is possible to micropropagate plant species throughout the year, in the case of biennial species requiring vernalization, it is necessary to do the micropropagation at a suitable time of year to allow the tissue to become vernalized for flowering the next year. In the case of plants which have a dormant period (such as most deciduous trees), shipment to the southern hemisphere will occasion dormancy problems due to the change in photoperiod and temperature experienced upon arrival. These changes can throw the plant propagation and subsequent growth of propagules into some confusion; if they arrive at the wrong time of year, it may take up to three years for the trees to behave normally. Therefore, the seasons cannot be ignored completely by micropropagators.

Although it is not the concern of this paper to consider the disadvantages of micropropagation, they should be mentioned briefly. As the techniques are relatively sophisticated, considerable capital expenditure is involved in purchasing equipment and the necessary facilities for micropropagation practices. The labour force must be relatively well educated and

flexible in terms of its response to the individual plant species that it will be handling. Considerable expertise needs to be applied to the weaning and establishment of plants from culture into greenhouse conditions, and this expertise can be gained only through experience.

APPLICATION OF MICROPROPAGATION

Let us consider the end product of a micropropagation program. Provided the systems have been properly developed and controlled, one is left with a very large number of clonal individuals. These individuals are genetically similar and under ideally controlled conditions would behave similarly, showing the same disease resistances, tolerances, environmental stresses, fruiting times and yields. In dioecious species, it is clear that this is not enough. If fruit is to be the final product, clones must be mixed such that there is a composite population of male and female individuals in order to bring about fertilization and crop development. There may be other reasons for mixing clones. Because the plants will contain similar disease tolerance and resistance, the spread of epidemic disease through a micropropagated monoculture is an ever present threat. In these circumstances, multi-line planting is necessary in order to circumvent some of the problems of monoculture. This requires that several similar clones are selected prior to the micropropagation phase. Multi-line planting has been shown not only to be extremely effective in epidemic disease control in cereals, but it is also important in terms of harvest. Uniform harvest date is of paramount importance when one utilizes machine harvesting methods. However, uneven harvest dates can have their advantages as well. A spread harvest, lasting several weeks, can be handled manually and provides for an even supply to a limited market size, rather than one-time flooding of the market. This latter point has implications for a number of third world countries and markets less developed than those of North America and Europe. Where machine harvesting is an advantage, because of labour shortage or market size, it must be remembered that even genetically identical material will not yield in a completely similar fashion, particularly if environmental conditions are sufficiently different to influence the growth of plants.

Where, then, has micropropagation had a significant impact on world horticulture and agriculture? To date, its main influence has been in the area of ornamental horticulture where enormous advances have been made both in the plant health status and in variety selection of a number of species. An example of this would be the aforementioned decrease in lily breeding time, and also the time taken for breeding new Narcissus varieties has been decreased by a factor of 3. The development and maintenance of virus and viroid-clean mother stock material of Chrysanthemum, carnations and potatoes is well established and demonstrated. This sort of technology is rapidly being accepted by other field crop users, notably in the area of sugar cane production, and the supply of virus-free banana plants by micropropagation has made a significant impact in Central American countries such as Honduras. The technology is currently approaching a watershed, having been tried and proven under limited greenhouse conditions (albeit on an extensive scale) and is now spilling over into field crops in all latitudes from the Tropics to to the Temperate Zone.

The opportunities brought about by the large-scale, rapid micropropagation of potato obviously has significant potential worldwide. Because potato suffers from significant virus and viroid infections as a result of aphid depradation, the production of clean seed stock potatoes has effectively become an almost separate industry from the production of retail potatoes. The crop itself is rapidly growing in importance in some of the tropical

and subtropical countries, particularly India and South East Asia. In these climates, the collection and long-term storage of potato tubers is a difficult and capital intensive problem. The system of seed potato importation is expensive both in terms of labour and overseas exchange. A viable alternative to this system is the use of micropropagated plants, which can be safely stored away from virus and viroid infections, to produce micro-tubers, i.e. stem tubers formed on micropropagated plants. In the United States, micro-tubers or second generation mini-tubers are gaining acceptance as an alternative source of cleaned plant material for potato growers. Although considerable effort must still be put into understanding the germination and dormancy of the micro-tuber, it offers a potentially valuable and exciting option to traditional seed potato production worldwide.

There are those who say that micropropagated material will never be able to compete with seed in terms of its price. Certainly in the extensively planted crops such as the cereals, this is probably true. However, there are examples in the vegetable and ornamental crop areas where micropropagation methods may be applicable. The technique of so-called "artificial seed" production, using somatic embryos rather than conventionally micropropagated propagules, may have sufficient advantages to warrant its commercial use. It must be remembered that when using somatically produced embryos, one again is handling genetically similar individuals. There is some concern that somatic embryogenesis may lead to genetic changes during culture. Despite this possible complication, there are a large number of crops in which variability within seedlots is already so great that the element of uniformity, which a crop derived from somatic embryogenesis could give, would be of enormous advantage. The complications arise in terms of the delivery systems used to get the in vitro tissue to the field. Modifications of existing fluid seed drilling techniques have been suggested, as have the potentials of encapsulating the somatic embryo in some rigescent material for field sowing. Further complications arise from a lack of dormancy exhibited by somatic embryos and the necessity for an induced period of dormancy which can be reliably released to give even germination in situ. Because of the elements of sophistication for the control of embryo development and field delivery of such propagules both at the chemical and biochemical levels, this technology must be regarded as second generation micropropagation and might well be five to ten years away from its large-scale application.

Micropropagation, albeit on a somewhat more limited and specialist scale, also holds centre stage in the development of cellular and protoplast technologies for crop development and engineering. During the potential transformation or genetic manipulation of crop species, the end result will be a single individual plant of elite characteristics. In order to first of all test this individual genotype with respect to different environments and different physiological advantages, it must be cloned up by micropropagation and planted under various environmental conditions. Subsequent to this testing, large numbers of plants will be required for trial plantings and eventually for final yield and variety trialling. The only direct way in which this may be done without genetically modifying this elite germplasm is by micropropagation. Simply breeding the plant, unless it is entirely apomictic, will not compete with micropropagation in building up the mass of material needed for selection and trialling work. Even in the less than light speed technologies of plant breeding, the development of selected parental material in sufficient numbers for seed production trials can be done by micropropagation more rapidly than by conventional vegetative propagation.

In conclusion then, micropropagation has already a proven record of increased yield and vigor through high health programs in ornamental horticulture, and a big role to play in the rapid introduction of new varieties of both ornamental and vegetable crops into the market. Micropropagation also holds centre stage in many of the novel genetic manipulation and genetic engineering techniques that are being developed, for it alone holds the key to the rapid bulking up of elite germplasm. The technology also has important roles in the potential hastening of plant breeding, both in ornamental and crop plants. It is clear that if the correct strategies for the use of micropropagation are developed and used in the appropriate circumstances, the technology will have a major effect in the next decades on both the nature of the crops that are grown internationally and also on the whole structure of horticulture and agronomy worldwide.

ADVANCES IN PROTEIN ENGINEERING

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ABSTRACT

Biotechnology is entering a new phase; protein engineering has demonstrated how to dissect the structure, and modify the function and selectivity of such diverse proteins as a neuro-receptor, peptide hormone, hydrolase, protease, anti-protease, transmembrane "pump", and other enzymes involved in antibiotic resistance and basic metabolism. Its proven capacity to alter the biological specificity of proteins, their stability in vitro and catalytic characteristics is discussed in the context of potential markets and future implications for agriculture. Protein engineering may be young, but it is certainly precocious.

INTRODUCTION

Recently, a question in the Cambridge University tripos examinations asked students to discuss an extension to the "central dogma"; that DNA makes RNA makes Protein makes Money. This variant dogma has been the driving-force behind the phenomenal growth in biotechnology where, to date its success is largely attributable to advances in obtaining expression of foreign proteins in novel host organisms. Protein engineering now takes this a leap forward by achieving designed alterations in proteins and their expression in the same range of host organisms. Biotechnology is growing on the claim that it can do things better, and protein engineering is now illustrating a further route to substantiating that claim.

Catalysis and transduction are processes that are fundamental to life. The central role played by proteins in these two processes is increasingly falling under industrial scrutiny, both to harness the catalytic potential of enzymes and to exploit the specificity of protein-ligand interactions when designing novel pharmaceutical and agrochemical products.

Traditionally when industry has commercialised processes based on enzymic catalysis, process improvements have involved either the identification of novel sources of enzymes, or mutagenesis/selection programmes to generate advantageous enzyme characteristics

(Rastetter, 1983; Winter and Fersht, 1984). Recent developments in biotechnology have the potential to subordinate these indirect, non-specific routes; advances in molecular biology now permit the extensive manipulation of gene templates, so that the amino acid sequence of proteins can be altered in a predetermined fashion. This applicaton has been dubbed protein engineering, the creation or alteration of functionality of a protein by rational redesign of its structure.

POTENTIAL MARKETS FOR PROTEIN ENGINEERING

Any commercial venture in protein engineering must ultimately be justified in terms of a market need. Protein engineering has the potential to access numerous markets, either by generating the immediate product, or by providing alternative routes to improved products. Potential market sectors for engineered proteins include industrial enzymes, food technology, the pharmaceutical and agrochemical industries, and the developing biosensors field. Over the next 10 to 15 years protein engineering may capture a percentage of these markets, and may even dominate select sub-sectors; but in order to offer a return on the heavy capital and personnel investment required, there must be the incentive to defend an existing market position. Alternatively, protein engineering must provide proteins which are totally innovative in function (no current market) or radically superior in performance by comparison with existing proteins. Naturally the regulatory issues which prevent widespread application of recombinant organisms and their protein products must be resolved in the first instance.

The world market for industrial enzymes is currently worth some $US400m p.a., representing between 80-100,000 tonnes annual production. This market size is primarily attributable to 3 classes of enzymes (proteases, 59%; carbohydrases, 28%; lipases, 3%; others 10%) (Maugh, 1984; Godfrey and Reichelt, 1983), and sales of a mere 12 enzymes account for more than 90% of the total enzyme market (Ulmer, 1983). Single major markets are for rennin (chymosin) for cheese making ($US40-110m p.a.), proteases, for use in detergents ($US100-160m) and glucose isomerase for production of high-fructose corn syrup as sweeteners ($US40m p.a.).

Food technology has need of improved or novel proteins for numerous roles such as sweetening, gelling and viscosity modification, meat tenderizing, and preservation against spoilage. This total market exceeds $US80bn p.a., and is potentially addressable by protein engineering.

Revenues from the pharmaceutical and agrochemical industries currently exceed $US80bn p.a. each, and both industries are already reliant upon some of the component skills of protein engineering to generate sales (e.g. molecular graphics for drug and pesticide design; genetic engineering for the production of improved organisms and therapeutics). Several market leaders in these industrial sectors have already initiated in-house programmes for protein engineering.

The development of biosensors for a wide range of applications is attracting considerable commercial attention at present. Predictions on the market for biosensors to the Year 2000 exceed the $US1bn mark. Realisation of these predictions is heavily dependent upon advances in protein engineering. Initial target features may be the provision of external scaffolding for signal transduction systems, and stability (resistance to desiccation, amenability to immobilization, retention of full activity after immobilization, etc.). Ultimately, however, it is anticipated that the surge in the biosensor market will hinge on the incorporation of reporter groups within the protein structure; read-out devices which harness developments such as interstitial fluorochromes will spawn a new generation of biosensors.

Catalysts of a non-biological origin are extensively used by the petroleum and chemical industries. Their market value at this time exceeds $US0.5bn p.a. However, considering the established technology, capital investment in fixed plant, product throughput and the relative absence of enzymes from the spectrum of catalysts in these industries, penetration of improved enzymes generated by protein engineering into these sectors is likely to be slow.

This clear market potential for engineered proteins with advantageous and innovative features should in no way encourage a blythe decision on which protein(s) should be the subject of investment. As will be seen from the examples of engineered proteins discussed subsequently, all have been the focus of intense biochemical research throughout the world for at least a decade.

However, this gestation period will undoubtedly be shortened to 2-3 years as the technology expands and matures. In a commercial context the decision-making process should include consideration of criteria such as the industrial importance of the protein, whether there is potential financial backing from an interested party, whether the protein has wider potential beyond its prima facie role, whether it has been well characterized (enzymology, chemistry, crystallography, etc) and whether there is substantial competition from other groups or technologies.

ENZYME CHARACTERISTICS AS TARGETS FOR PROTEIN ENGINEERING

Numerous properties of enzymes have been discussed as candidates for manipulation by protein engineering (Robson, 1980; Rastetter, 1983; Ulmer, 1983; Maugh, 1984; Winter and Fersht, 1984; Perutz, 1985). Often, the modifications envisaged for engineered proteins segregate according to in vitro or in vivo applications. Thus, stability characteristics such as enhanced thermal stability, activity over a broad pH range, resilience in organic media, prolonged activity after immobilization onto various process substrata, resistance to proteases and broad range solubility characteristics are advantageous in industrial enzymology.

Enzymes with modified activity characteristics will prove equally beneficial for in vitro and in vivo applications. Target features could be Michaelis constants (K_m), substrate specificity, susceptibility to product inhibition, regulation by allosteric factors, and co-factor requirements. Engineering to enhance catalytic activity towards an atypical substrate is of considerable industrial relevance. Glucose isomerase, used in the production of high-fructose corn syrups is actually a xylose isomerase in vivo (Streptomyces and Bacillus spp.; Bucke, 1983). Similarly, the alteration of substrate range for enzymes of wider industrial and biological application could provide a stable of catalysts derived from a well characterized stud; Winter and Fersht (1984) have discussed the possible redesigning of subtilisin to confer esterase activity, or modifying function so as to favour oligopeptide semi-synthesis under appropriate reaction conditions.

CURRENT ACHIEVEMENTS IN PROTEIN ENGINEERING

Without any doubt, the successes achieved by protein engineering in the rational modification of protein function have been remarkable both from the point of view of the diversity of systems already investigated and the unambiguous specificity of information generated. In some instances, output has been prosaic and confirmatory, rationalization of consequences sometimes coming with hindsight; but this is a pattern of events not unfamiliar to the experimental sciences, nor is it alien to the commercial environment (viz prototypes). Yet, indicative of the power and pace of this technology is the recent comment (Winter and Fersht, 1984) that "it is virtually impossible at present to (computer) model the hydrogen bonding, electrostatic and hydrophobic interactions made with the substrate, let alone predict the effect of altering the active site". Within 5 months a subsequent publication (Fersht et al, 1985) has applied protein engineering of an active site to quantify the role of hydrogen bonding in biological specificity.

Zoller and Smith (1983) have summarized many of the early examples of site-directed mutagenesis for protein engineering; candidate proteins in more recent investigations are collated in Table 1. Another powerful aspect of the technology, the rational design of fusion proteins to facilitate downstream processing of biotechnology products, has been reviewed (Brewer and Sassenfeld, 1985).

In vivo, beta-lactamase functions in the periplasmic space of bacteria to hydrolyse and deactivate the beta-lactam ring of penicillin derivatives. During transport across the inner membrane, the signal sequence peptide of beta-lactamase (comprising 23 amino acids) is cleaved off. Critical features of the signal sequence for transport and processing have been probed to reveal that determinants for these activities do not reside in the signal sequence alone (Kadonaga et al, 1984). The essential nature of an active site serine in beta-lactamase has been confirmed (Dalbadie-McFarland et al, 1982) and a serine to cysteine substitution not only reduces activity towards penicillin but confers susceptibility of residual activity to a sulphydryl reagent (Sigal et al, 1982).

Similarly, the catalytic activity of dihydrofolate reductase has been analysed by rational changes to the active site. A single Asp to Asn modification was associated with a thousand-fold decrease in specific activity, supporting the thesis that the Asp is involved in stabilizing a protonated transition state. A Gly to Ala mutation in a region of the protein predicted to be sensitive to steric perturbation totally abolished activity (Villafranca et al, 1983).

Analysis of the X-ray crystallographic structure of insulin has suggested that the full length of the native C-peptide (35 amino acids) might not be essential for linking the relevant termini of the insulin A and B chains. Wetzel et al (1981) designed and synthesised an engineered pro-insulin molecule with an abbreviated primary sequence for the C-peptide (hexapeptide) which had chromatographic and immunological properties consistent with the native pro-insulin.

Kaback's group has investigated the molecular action of inhibitory alkylating agents such as N-ethylmaleimide (NEM) on bacterial transport proteins (Fox and Kennedy, 1965; Kaethner and Horne, 1980). In the lactose permease of E. coli (M-protein, lac y gene product) a cysteine to glycine substitution suggested that this active site sulphydryl was not essential for transport activity, but that its alkylation with NEM inhibits binding of the transport substrate (Trumble et al, 1984). The molecular basis for the coupling of lactose transport activity with proton gradients has also been investigated (Padan et al, 1985). Hydropathy plots and antibody interactions have been employed to generate a model for transmembrane folding of the lactose permease, and chemical modification of the protein has implicated histidine residues as being associated with proton/lactose symport activity.

Site-directed mutagenesis (His to Arg) has revealed that of the four histidine residues present in lactose permease, two (His 35, 39) play no essential role in transport. Mutagenesis of His 205 is associated with total loss of transport function, whereas mutation of His 322 destroys proton-coupled lactose uptake while leaving the facilitated diffusion of lactose operational.

Stability against thermal inactivation has been engineered into phage T4 lysozyme (Perry and Wetzel, 1984). On the basis of theoretical calculations and a visual scan of the modelled crystal structure for T4 lysozyme, a single mutation was introduced (Ile to Cys) to permit the formation of a solitary disulphide bridge via mild oxidation with tetrathionate or glutathione buffer. Residual activity of the mutant enzyme was about 200-fold greater than the wild-type after 3 hours incubation at 67°C.

In contrast, improved stability has been engineered into human fibroblast interferon (INF-beta) by removal of one of its three cysteine residues (Mark et al, 1984). Antiviral INF-beta forms inactive dimers and oligomers, and a Cys 17 to Ser mutation was engineered to reduce the

possibilities for potentially damaging inter- and intra-molecular disulphide bridge formation. The resulting mutant protein retains wild-type antiviral activity but displays improved storage characteristics under the conditions described.

Construction of a hybrid repressor protein, differing from its parent by five amino acids in the putative alpha-helix specificity-determining region, was used to demonstrate the molecular basis of the DNA sequence-specificity of these DNA-binding proteins (Wharton et al, 1984). Sequence specificity of the bacteriophage lambda 434 repressor protein switched to that for the cro protein following directed mutagenesis of the former to give a hybrid bearing putative cro recognition features.

Extensive studies on the biochemistry, physiology and molecular biology of the acetylcholine receptor have provided a detailed understanding of this ligand-gated channel protein. Site-directed mutagenesis in the absence of crystallographic data has now enabled a functional analysis of those specific regions of the receptor considered to be involved in acetylcholine binding and transmembrane channel formation (Mishina et al, 1985).

Site-directed mutagenesis has been applied to probe the catalytic activity and role of conformational changes in aspartate transcarbamoylase (Robey and Schachman, 1984). Similarly a phylogenetically conserved Phe of cytochrome c has been found to be non-essential for electron transfer reactions, but does participate in determining the cytochrome c reduction potential (Pielak et al, 1985).

A possible solution to the incidence of cigarette smoking-related emphysema (and hereditary emphysema) arises from studies on the consequence of directed mutagenesis of a Met residue at the elastase binding site of $alpha_1$ - antitrypsin (Courtney et al, 1985). The normal functioning of this antiprotease is to inhibit the degradative action of elastase on connective tissue. A Met 358 to Val mutation generated a fully active elastase inhibitor which was resistant to oxidative inactivation, while a Met 358 to Arg mutation produced an efficient antithrombotic protein. Of a complementary nature is work on the redesign of the substrate specificity of trypsin (Craik et al, 1985).

The very basis of active site binding and catalytic activity is being systematically analysed in tyrosyl-tRNA synthetase. A range of mutant enzymes has been generated which are active but have altered kinetic parameters (Winter et al, 1982; Wilkinson et al, 1983). The molecular explanation offered for an engineered 130-fold reduction in K_m (ATP) in one mutant (Wilkinson et al, 1984) was confirmed in a series of experiments in which enzymes bearing double modifications in the active site were constructed (Carter et al, 1984). In a momentous step Fersht and colleagues (Fersht et al, 1985) have now offered the progenitor of future tours around enzyme active sites. Their quantitation of active site hydrogen bonding as a biological specificity determinant encapsulates

the future potential of protein engineering. We now have not only an analytical tool to dissect the processes of catalysis and transduction, but a rudder with which to steer them.

FUTURE PROSPECTS IN AGRICULTURE

Commercially speaking, it now boils down to a question of what and when. Three broad areas for potential short- to medium-term impact can be illustrated by way of examples from plant agriculture, i.e. pest control, disease resistance, and metabolite partitioning.

The Bacillus thuringiensis insecticidal protein is a potential candidate for protein engineering. It has already been cloned into the rhizosphere bacterium Pseudomonas fluorescens (Sun, 1985) and tobacco (Anon, 1985). Analysis of its mechanism of action could well lead to the design and production of a family of proteins with species specificity, high toxicity and resistance to denaturation or protease degradation.

Expression of a viral coat protein in transformed tobacco plants (Bevan et al, 1985) illustrates novel potential for virus-resistant breeding programmes, using clones in which the natural infection cycle would be subverted by host-expressed mutant virus coat proteins.

Bioregulators are used to enhance the productivity of selected crops (Ory and Rittig, 1984). Commercial interest in compounds such as glyphosate ^{R} (N-phosphonomethylglycine), which is not only a herbicide but also a ripening agent in sugar cane will accelerate research into their biochemical action. In sugar cane, glyphosate increases sugar content per stalk by increasing the partitioning of dry matter away from fibre production towards sucrose accumulation. If this response is attributable to glyphosate action at one or a few sites, protein engineering of these sites could provide a rational, selective approach to breeding for enhanced partitioning of photosynthate into commercial product.

On the animal agriculture side we can expect early advances in growth rate enhancement, rapid disease diagnosis, improved vaccines, manipulation of lean meat/fat ratios and alleviation of some nutritional requirements. Advances in protein engineering for the human healthcare sector will accelerate these innovations ahead of radical developments in crop agriculture. But who knows, in twenty years time we may well be looking at crop species with ribulose bisphosphate carboxylase/oxygenase activity engineered for greater fixation efficiency, interior design herbicide resistance and pest resistance, oxygen-stable nitrogenase activity in leaves to reduce fertilizer requirements, and enhanced phosphate and mineral uptake systems in roots to out-compete weeds.

The fine detail may be unclear at present, but it is undeniable that protein engineering will ring in some fundamental advances in agriculture.

TABLE 1

Examples of proteins with engineered functions

Protein	Function manipulated	Reference
beta-lactamase	secretion	Kadonaga et al, 1984
	activity, susceptibility to inhibition	Sigal et al, 1982
	ablation of catalytic activity	Dalbadie-McFarland et al, 1982
dihydrofolate reductase	ablation of catalytic activity, conformational and catalytic modification	Villafranca et al, 1983
pro-insulin	abbreviated primary sequence	Wetzel et al, 1981
lactose permease	transport activity	Trumble et al, 1984
	chemiosmotic coupling	Padan et al, 1985
phage lambda T_4 lysozyme	thermal stability	Perry and Wetzel, 1984
interferon	stability	Mark et al, 1984
repressor proteins	DNA-binding specificity	Wharton et al, 1984
acetylcholine receptor	structure/function	Mishina et al, 1985
aspartate transcarb-amoylase	catalytic activity	Robey and Schachman, 1984
cytochrome c	electron transfer, reduction potential	Pielak et al, 1985
$alpha_1$ -antitrypsin	oxidation susceptibility, biological specificity	Courtney et al, 1985
trypsin	biological specificity	Craik et al, 1985
tyrosyl - tRNA synthetase	reduced affinity for ATP	Winter et al, 1982

TABLE 1 continued

Protein	Function manipulated	Reference
	reduced affinity for ATP	Wilkinson et al, 1983
	affinity for substrate increased about 100-fold	Wilkinson et al, 1984
	double mutant to confirm explanation of above	Carter et al, 1984
	hydrogen bonding and specificity	Fersht et al, 1985

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IMPROVING THE AMINO ACID CONTENT OF CEREAL GRAINS

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ABSTRACT

The poor nutritional quality of cereal grain for feeding to non-ruminant animals is due to the small proportion of lysine, and to a lesser extent threonine, in the major endosperm storage proteins (called hordeins in barley). Four strategies for improving the nutritional quality of barley grain are described and evaluated. These are: (1) decreasing the total content of hordein; (2) manipulating the amino acid composition of hordein; (3) increasing the amounts of other more lysine-rich seed proteins, and (4) selecting for mutants with increased pools of free lysine and threonine resulting from relaxed feedback inhibition of biosynthetic enzymes. It is concluded that the greatest chance of success lies in a combination of approaches.

INTRODUCTION

Wheat and barley are the major cereal grain crops in Europe. The majority is used in feeding domestic animals, chiefly pigs and poultry. These simple-stomached animals rely upon their diet to supply the amino acids needed for growth, including the nutritionally essential amino acids. The grain of wheat and barley contains insufficient lysine and threonine (and marginally histidine) for optimal growth. However, if

TABLE 1

Characteristics of barley diets and the performance of pigs fed upon them

Characteristics of the diet	Barley + minerals basic diet (1)	Supplemented diet (2)	% Change in diet (2)
% N	1.70	1.78	+ 5
% Thr (g/100 g)	3.0	4.8	+ 60
% Lys (g/100 g)	3.6	7.5	+ 98
Biological value	0.51	0.86	+ 69
Effective % protein N	0.87	1.33	+ 35
Growth characterisitcs of the pigs			
Liveweight gain (kg/day)	0.32	0.64	+100
Food conversion ratio (g food/g wt. increase)	5.00	3.00	-40

This table is drawn up from data in Fuller *et al.* (1979a,b). The pigs were gilts with an initial live weight of 20 kg and a final weight of 60 kg.

supplemented with these amino acids barley becomes a highly satisfactory food (e.g. for pigs Table 1). Since the supplements used in the U.K., whether synthetic amino acids or soybean meal, are imported whilst cereals are overall in surplus, there is some interest in improving the amino acid quality of cereals. This paper will discuss ways in which this might be achieved for barley. Similar approaches have been used by others with maize but will not be discussed here.

The distribution of amino acids in different fractions of the barley grain is shown in Fig. 1. The largest proportion of the grain nitrogen is present in the hordein (prolamin) fraction. The prolamins are the major storage proteins of barley and wheat. They are polymorphic series of proteins which are soluble in aqueous alcohol solutions. They can be classified into three groups, the sulphur-rich, the sulphur-poor and the high molecular weight prolamins. In barley these groups are termed the B, C and D hordeins respectively. The B hordeins are the quantitatively most important comprising about 80% of the total. (For a more detailed account of our knowledge of these proteins consult Shewry & Miflin 1983, 1985, and Kreis et al. 1985). Fig. 1 clearly indicates that the imbalance of lysine occurs because of the small proportions of lysine present in the prolamins. On the basis of this improvement in lysine content could be achieved by either changing the proportion of the different groups, particularly decreasing the amount of prolamins, or by increasing the lysine content of one or more of the different fractions or a combination of both approaches. In this brief article we will review some of the attempts that have been or are being made to achieve these aims. These approaches have been reviewed in more detail elsewhere (Bright & Shewry 1983, Shewry et al. 1981).

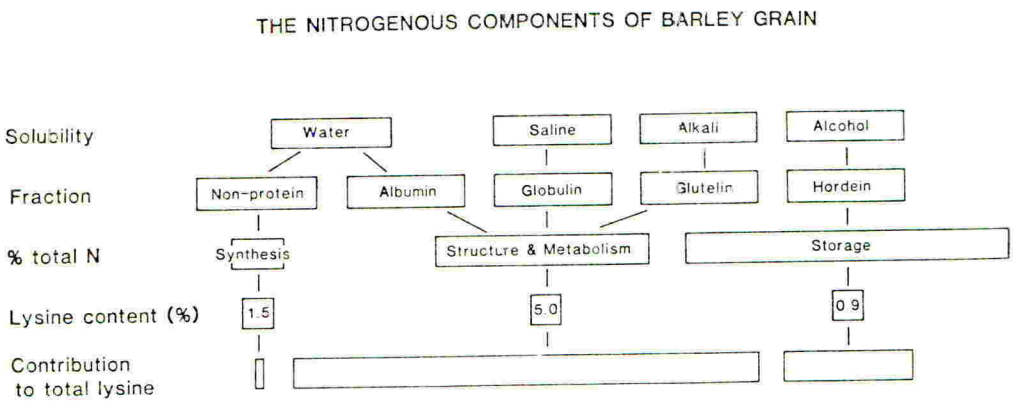

Fig. 1. The distribution of nitrogenous components in the barley grain, their lysine content and their contribution to the total lysine content of the grain. The size of the blocks are to scale for '% total N' and 'contribution to total lysine', the words across the former blocks indicate the major function of the fraction.

DECREASING THE PROPORTION OF PROLAMINS IN THE GRAIN

Munck et al. (1970) surveyed the world barley collection and identified one line named Hiproly which was high in protein and lysine content. This has been shown subsequently to be due to the presence of a gene termed lys located on chromosome 7. This gene has a number of pleiotropic effects, one of which is to decrease the total content of prolamins by about 20% (Miflin & Shewry 1979). Subsequently Doll and colleagues (Doll et al. 1974) at Risø screened large numbers of mutant populations of barley grains and identified several with improved lysine content. Of particular interest are the mutants Risø 56 and 1508. Risø 1508 has the most pronounced decrease in prolamin content and the correspondingly greatest increase in lysine content (Miflin & Shewry 1979, Doll 1983, 1984). This is due to a recessive mutation at the lys 3a locus also on chromsome 7 (Jensen 1979). This gene has a large number of pleiotropic effects (see Miflin & Shewry 1979 and Doll 1984 for discussions) but despite detailed analysis at a number of levels (e.g. see Kreis et al 1984) the nature of the locus is unknown. Risø 56 contains a mutation which maps to the structural locus for B-hordein (Hor-2), and causes a large decrease in the amount of B-hordein with partially-compensating increases in the amounts of C and D hordeins (Doll 1980, Kreis et al. 1983). Molecular analysis using cDNA probes has shown that the mutation is due to a deletion of at least 90 kb of DNA containing Hor 2 genes (Kreis et al 1983).

Despite the fact that all of the mutants have much enhanced nutritional quality, none of the mutations has yet come on the market after introduction into a cultivar. This is due to the depressing effect of all the mutations on grain size and yield (see Doll 1983, 1985). Even though the mutant genes have been used in a number of breeding programmes, it has so far proved impossible to separate the high-lysine low prolamin character from yield depression.

CHANGING THE AMINO ACID COMPOSITION OF PROLAMINS

In concept, and given a means of cereal transformation, it is possible to envisage changing the amino acid composition of the prolamins. The role of these proteins as stores of nitrogen, carbon and sulphur probably only places limited constraints on their amino acid sequences which vary considerably between different members of any sub-group (Kreis et al. 1985). From analysis of their structure regions of the molecules have been identified in which charged residues are concentrated.

Analysis of the structure of B hordeins based upon sequencing cDNA and genomic clones shows that the protein is made up of two domains (Forde et al. 1985a,b, Miflin et al 1984, Kreis et al. 1985). Domain 1 consists of repeated sequences based on variations of a consensus repeat of PQQPFPQ and few or no changed residues. In contrast, domain 2 contains several basic residues, chiefly arginine of which there are seven in the one complete B hordein sequence known. In contrast C hordein lacks almost all of domain 2, and is almost solely comprised of repeats. In order to bring hordein up to a nutritionally acceptable level about 15-20 lysine residues would have to be added. Based upon present knowledge it is difficult to predict whether or not a storage protein with a synthetic high-lysine domain 2 would function as a storage protein. However, replacing the seven arginine residues with lysine could make a substantial improvement in quality and not markedly change the charge properties of the protein.

A potentially greater difficulty in increasing the nutritional quality of prolamins by transformation is the complexity of the genetic system specifying these proteins. In barley three complex loci specify the three different groups of hordeins. Each of the two major loci probably contains 10-30 genes (Kreis et al. 1983, Shewry et al. 1985). Thus transformation with a few improved genes would have little effect unless the recipient lacked a majority of the endogenous genes. A possible recipient is mutant Risø 56 which has been shown to lack a large proportion of the genes specifying the B hordeins. It is estimated that this mutation is a result of a deletion of about 90 kilobases of DNA containing eleven hordein genes (Kreis et al. 1983). However, this mutant has a depressed yield and it is not clear whether this might be due to the deletion of other important genes interspersed between those for hordeins. If this is so, and there are parallels from other species to support the suggestion, then repairing the deletion solely with "improved" hordein genes would not restore the normal yield potential. There is also the problem that as yet there is no method for producing transformed cereal plants although encouraging progress is being made (Lorz et al. 1985, Potrykus et al. 1985).

INCREASING THE CONTENT OF "HIGH LYSINE" PROTEINS

A number of proteins have been identified in barley which have a disproportionately greater content of lysine (19, 20, 21). Some of these proteins have the characteristics of storage proteins (see Shewry & Miflin 1985 for a discussion). The relative amounts of these proteins in the grain are altered by different 'high-lysine' mutations. The more important of these proteins, their lysine contents and the effects of different mutations on their amounts is given in Table 2.

TABLE 2

Amounts of different 'high-lysine' proteins in various lines of barley. Further information can be found in Shewry & Miflin (1985)

Amounts in different barley lines	High lysine proteins (mg protein/g grain)			
	CI-1	CI-2	β-Amylase	Protein Z
Mona	0.24	0.08	0.98	2.2
Hiproly	4.2			4.7
Hiproly cross	1.5	0.4		4.1
Risø 1508	1.5		4.9	0.5
Risø 56				4.0
Pirkka				0.2
		(mol %)		
Lysine content	6.1	9.1	4.0	5.3

The reasons for the different effects of the mutant genes are not known, but in all instances they would appear to be due to pleiotropic rather than direct effects. In order to understand the system better we have cloned DNA encoding the chymotrypsin inhibitors. Preliminary results suggest that the changes in amount of the protein are due to increased

amounts of mRNA present during development (M. Williamson & M. Kreis unpublished). Future work will be aimed at identifying factors important in increasing the expression of these genes in the hope that they may be subject to manipulation by plant breeding or transformation.

INCREASING THE AMOUNTS OF NUTRITIONALLY-ESSENTIAL FREE AMINO ACIDS

Lysine, threonine and methionine are synthesized by a branched pathway starting from aspartate (Fig. 2). This pathway is subject to a complex series of regulatory loops which limit the production of the end-products (Bryan 1980). Based on our knowledge it has been possible to devise methods to select mutations which lead to the loss of some of the regulatory loops and the overproduction of threonine and lysine.

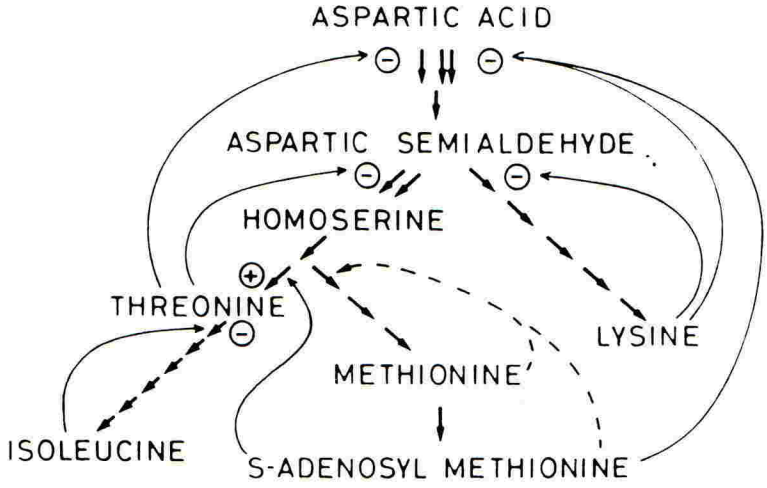

Fig. 2. The pathway for the biosynthesis of lysine, threonine and methionine. Biosynthetic steps are shown with short heavy arrows and feedback loops with thin long arrows - indicates negative feedback and + positive feedback regulation of enzyme activity.

The first step in the pathway is catalysed by the enzyme aspartate kinase. Two classes of the enzyme exist, one inhibited by threonine and one by lysine. When barley embryos are germinated on a medium containing these two amino acids their growth is inhibited due to the complete inhibition of this step and the consequent inability of the plants to synthesize methionine (Bright et al. 1978). Any mutants containing an aspartate kinase not inhibited by either lysine or threonine would be expected to be able to make methionine and overcome the inhibition. We have selected such mutants in barley and analysed both the amino acid contents of their seeds and the regulatory characteristics of their aspartate kinase enzymes (Bright et al. 1982a,b, 1984).

TABLE 3

The threonine, lysine and methionine content of the free amino acid pools from mature grains of barley mutants resistant to lysine plus threonine, and the regulatory characteristics of aspartate kinase (AK) enzymes isolated from the mutants.

Plant	Amino acid content ($\mu mol.g^{-1}$ d.wt.)				Characteristics of		
	Thr	Lys	Met	Total	AKI	AKII	AKIII
Bomi	0.12	0.08	0.02	10.4	s	s	s
R2501	6.13	1.27	0.15	28.2	s	r	s
R2506	9.03	1.62	0.07	20.4	s	s	r
Bomi	0.13	0.08	0.02	6.9	s	s	s
R3005	2.37	0.11	0.02	12.4	s	s	r
R3202	0.12	0.11	0.02	11.7	s	r	s
R3004 x R3202	0.69	0.12	0.02	10.1	s	r	r

Pure breeding lines of Bomi, the parent cultivar, the single mutants R2501, R2506, R3004 and R3202 and of the double mutant containing the homozygous resistant alleles originally isolated in R3004 and R3202 were analysed. The sensitivities of the aspartate kinase isoenzymes to inhibition by threonine (AKI) and lysine (AKII) were determined on extracts of shoots; 's' denotes the isoenzyme was as sensitive to feedback control as the parent, 'r' indicates that the isoenzyme was partially or fully resistant to feedback inhibition. (Data from Bright *et al.* 1985 and references quoted therein).

We have found that barley has at least three genes for aspartate kinase, one specifying a threonine-sensitive aspartate kinase (AKI) and two specifying lysine-sensitive aspartate kinases (AKII and AKIII). We have identified mutants with desensitized forms of AKII and AKIII and have constructed a double mutant. Amino acid analysis of the grain shows that certain of these mutants have considerably enhanced amounts of free threonine and/or lysine (Table 3). Somewhat surprisingly the double mutant has no greater amounts than the single mutants. Although these increases are significant in terms of the free pool of amino acids, they are not yet sufficient to completely satisfy the nutritional requirements of simple-stomached animals.

CONCLUSION

A number of approaches have been and are being used to improve the nutritional quality of barley. As yet none of them has resulted in a cultivar with an acceptable nutritional quality for feeding non-ruminants and a normal yield. The research has, however, considerably enhanced our knowledge of the genetics of barley, particularly of the factors involved in determining the final amino acid composition of the grain. The hope is that this knowledge will provide a better basis for reaching the above

goal. The greatest chance of success may lie in a combination of all the approaches discussed above, rather than in attempts to find a single dramatic change.

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POTENTIAL BENEFITS OF IMPROVED BREEDING PROGRAMMES

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ABSTRACT

To date, genetic progress in livestock breeding programmes has relied either on breed substitution or upon the traditional quantitative genetic approach of testing and selection. The rate of progress possible is dependent upon selection intensity and generation turnover. Actual progress is usually lower than that theoretically possible. Biotechnology provides means of increasing selection intensity - the MOET could double the rate of progress in dairying. Cloning provides a method of obtaining carcase information without the delay of progeny testing. At present, the only option available for DNA transfer is that of direct injection into culture cells or embryos. Transgenic rabbits, sheep and pigs have been produced. The limitation at present is the knowledge of major single gene effects in farm animals and considerable effort will be required to identify such effects. There is only limited effort in this area on farm animals. It is essential that any gene transfer is considered in terms of the overall effect on production efficiency - correlated effects could have important consequences. Examples of potential transfers are discussed. The importance of considering the implications of genotype environmental interactions is raised. Genetic engineering can be used to complement traditional methodology of quantitative genetics - in order to be of real significance biotechnology should provide an average improvement equal to that of traditional methodology.

INTRODUCTION

The definition of biotechnology can be extremely broad and could be considered to include the general manipulation of genes by selective breeding and techniques such as artificial insemination and, more recently, embryo transfer. Skjervold (1984) produced an interesting table of important discoveries within biotechnology starting with artificial insemination in 1782 and finishing with initiation of artificial chromosome work (Table 1). Whilst some of these techniques will be referred to, the term biotechnology, in general, has been kept in a more restricted sense to the actual manipulation of genes.

TABLE 1

Important discoveries within biotechnology

1782	Artificial Insemination	Spallanzani Used in great scale in USSR during 20's and 30's.
1890	Transplantation of fertilized ova (rabbits)	W. Heape Used in reproduction of beef-cattle from the beginning of the 70's

1949	Deep frozen semen	Polge et al. Used in practice since the last part of the 50's.
1972	Low temperature storage of embryos	Whittingham et al. Used in cattle ova transplantation since 1974 (sheep since 1976).
1973	Transplantation of single genes from animals to microbes	S N Cohen and H Boyer This technique is the basis for the great expanding recombinant DNA-technology.
1977	Parthenogenesis	Kaufman et al. Activated occytes without participation of sperm.
1978	Splitting of embryos	Moustafa and Hahn Dividing embryos in identic genotypes by microsurgery.
1979	Transplantation of synthesized genes	Gilbert et al. Some of the genes which are used in bio-industry are in fact synthesized.
1981	In vitro fertilization of eggs from cattle	Brackett et al.
1981	In vitro ovulation	Kobayashi The ovary is held ("grown") on sbustrat.
1981	The marketing of gene synthesizer	Hapwood
1982	Transplantation of single genes from one species to another	Palmiter et al.
1982	Sexing of embryos at an early stage	T E Wagner The sexing is based on immune reaction and does not harm the embryo.
1983	Toward artificial chromosomes	Murray and Szostak (So far only in yeast).

SELECTIVE BREEDING

Genetic change can occur in only four ways - mutation, chance, migration and selection. Organised, directed changes can only happen when migration and/or selection occur. To date, progress in animal breeding has used both methods - migration usually involving material from other breeds and/or countries and selection taking place within a breed. This latter approach is based on the quantitative theories which suggest that traits are influenced by many genes, each with relatively small effects. In general, the specific genes of interest are not known, but the measured performance of the trait (which is either the breeding objective or is related to it) is

known and can be recorded. Selection has been based on this "phenotypic" performance. Statistical analysis provides estimates of the genetic parameters - the heritability and genetic correlations - which together with the phenotypic correlations and economic values are used to combine the information to optimise the estimate of the breeding value of an animal. In this respect, the knowledge of the biology involved can be extremely limited and the significant progress has been achieved without such information. It is interesting to note that the theoretical estimates of progress in using such techniques would suggest improvement in the order of 2-2½% of the mean for each trait, whereas in general, rates of progress have been more in the order of ½-1% (Smith, 1984a) although there are one or two exceptions to this. For example, the pig breeding scheme in Britain reported by Jones (pers. comm.), indicates significant progress for many of the objectives over a ten year period and this work was subsequently evaluated by Mitchell et al (1982). For some traits, the progress was around 2% of the mean per year and the value of such progress accumulated over time means that the present day value of this progress is well over one hundred million pounds per annum. Most dairy schemes for improving milk production have estimates of progress in the order of half to one per cent - quite considerably different from the theoretical estimates. Essentially, there are practical reasons for the difference between estimated and actual rates of progress and one has to consider how much effort could be justified in improving techniques so that the "slippage", which is indicated by the difference in rates of progress, does not continue. In the dairy industry the "slippage" is usually accounted for by considerable selection effort being put on non-production traits of the cow, e.g. physical appearance. However, improvements in application, even when achieved, are not necessarily immediately obvious - it takes around ten years for a young dairy bull nominated as a result of breeding work to be brought into the stud and used as a proven sire.

To put improvement into a financial context, perhaps the most useful summary is the "farm gate" value of the products which are summarised in Table 2.

TABLE 2

Value of output (£ million) (1984 forecast)	
Cattle	1922
Milk and Milk products	2293
Pigs	1000
Sheep	579
Wool	37

Source : M.A.F.F. Annual Review 1985.

Much effort has been exerted in obtaining estimates for the parameters required to estimate breeding values more precisely. However, rates of genetic progress not only depend upon the accuracy of the traits used in estimating the breeding value for the objective, but also involve the selection intensity which can be achieved and the generation turnover.

INTENSITY OF SELECTION

Under normal farming conditions, the reproductive rate in cattle is just under one calf per year, in sheep around 1.5 progeny per year, in pigs approximately 20 per year and in poultry over 200 progeny per year - in all cases, only one sire and one dam are required as replacements. The implications for potential progress are obvious. It is quite feasible that the selection intensity of animals could be increased, both by improving reproductive rates and also by ensuring that all animals do have adequate contemporary comparisons and breeding values can, therefore, be estimated. (This is a considerable problem in herds/species, where the numbers kept together are very small, e.g. pedigree beef herds in Great Britain). The other factor which affects progress significantly is the rate of turnover of the generations. There is a considerable difference between the practice of a breeder and the theoretical calculations of the geneticist, but certainly increasing the turnover of generations would have a major effect on the rate of genetic change which can be achieved. It is, therefore, quite feasible to expect, with proper and reasonable control, rates of progress in the order of 2% per annum.

BIOTECHNOLOGY

It would appear that biotechnology is considered (by some) as the solution to all problems in animal breeding. However, one has to consider how these improvements would take place and what the relative likelihood of increasing rates of progress really is. Certainly, the mechanical techniques have provided some gains. The use of biotechnology has already had a major effect on the intensities possible - the ability to collect, freeze and store bull semen to use by artificial insemination (AI) has meant that the intensity of selection of bulls has increased considerably, and, more recently, the ability to freeze and store cattle and sheep embryos can have similar but less dramatic effects on female selection. Indeed, the whole structure of selection programmes can be changed by the use of such techniques (in as dramatic a manner as that of AI development in cattle). Land and Hill (1975) indicated that beef progress could be 50% greater using embryo transfers and, more recently, Nicholas and Smith (1983) show that the use of Multiple Ovulation and Embryo Transfer (MOET) can double rates of progress in dairy cattle. More recently, the ability to clone by the micromanipulation of embryos (Willadsen 1982) increases the selective capacity available to animal breeders.

ACCURACY OF BREEDING VALUE ESTIMATION

These techniques have an additional advantage in that they often provide an opportunity to increase the accuracy with which a breeding value can be estimated. The use of AI provides the means by which many progeny can be produced in several different environments at the same time and the ability to do this with many different sires provides better contemporaneity and, hence, better accuracy. The result can be much better progeny tests for those traits which cannot be measured directly on the live animal, e.g. milk production ; carcase measurements.

The use of MOET techniques in dairy cows could easily result in nucleus herds being the basis for national progress rather than reliance on national recording schemes based on many herds. Such a dramatic change in structure

would provide an opportunity to measure traits not possible in a national recording scheme, e.g. feed efficiency (Steane and Swanson 1985).

The use of clones could provide a measure by which carcase measurements could be taken on one animal while a twin is kept alive (or stored as a frozen embryo - dependent upon costs etc.). Selection can then be exerted without resorting to costly, generation lengthening progeny tests. The total testing facilities required would also be less, providing either a cheaper means of progress or greater selection intensity within a given facility. Everett (1985) suggests dairy response could increase by 20% by the use of cloning.

MOLECULAR BIOLOGY

Over the past ten to fifteen years there has been considerable changes in the understanding and the ability to manipulate the genome at the level of the DNA. A review by Gillings and Frankham (1982) adequately deals with the roles and techniques of detecting, isolating, sequencing, mapping and manipulating DNA from original genes. The important question for animal breeders is how to use and to benefit from these new technologies. Smith (1984) produced an interesting list of potential techniques (table 3). This progression in molecular biology technologies should enable much more precise approaches to animal breeding to be made, although that situation is not yet achieved. At the cellular level, it may be possible to transfer or substitute chromosomes, but of more likelihood, it is the manipulation or transfer within or between species which could be of greatest value. The basis is the ability to transfer DNA but, at present, the techniques are still developing. Franklin (1984) discussed the development of reliable delivery methods and points out that, at present, only one option is immediately available in livestock - that of the direct injection of DNA either into cells in culture or into embryos. Several genes have been transferred in the mouse (Brinster et al 1981) and of more recent note, has been the achievement of Palmiter et al (1982), transferring rat growth hormone genes to mice resulting in insertion at many sites, in expression (of growth hormone), phenotypically in larger body size and, indeed, in the transmission of this to progeny. In the last few months, Hammer et (1985) have reported the production of transgenic rabbits, sheep and pigs using microinjection. Within this country, the Animal Breeding Research Organisation has one transgenic sheep (Smith, pers. comm.). However, this achievement is still a considerable way off being applied in animal breeding improvement. The level of success was not high and certainly unpredictable and one would wish for more predictable results and better control of the situation. Nevertheless, only one successful transfer is required in order to obtain the benefit desired.

GENETIC ENGINEERING - SINGLE GENES

However, one should consider what use can be made by animal breeders of these new technologies. For example, it may well be possible to identify single genes which have major performance effects and to transfer from some species to those to whom the benefit would be greatest, without taking with the attribute the accompanying undesirable genes. It may well allow us to eradicate certain genetic problems and to establish gene libraries. The animal breeder is to consider this gene transfer as a special case of migration and initially has to ask what are the potential genes for transfer

for use in this particular way. There are few examples which are known and indeed animal breeders are almost certainly in a situation where the enabling technology is ahead of the potential application, simply because of the lack of knowledge of specific gene effects. The problem of lack of knowledge of single gene effects in domestic animals was pointed out by Robertson in 1982. A good discussion of the problems of applying genetic manipulation of major genes is given by Bulfield (1983) and reiterates the problem suggested by Robertson. It should be pointed out that the major genes that are known, for example, the "B Locus" disease resistance genes in chickens, the Halothane (stress) gene in pigs and the Booroola prolificacy gene in sheep, together with the double-muscling gene in cattle, can already be handled reasonably adequately by traditional breeding methods. However, gene transfer could be quicker and could enable the benefits to be disassociated from any linked harmful effects (if gene transfer were used).

However, this general lack of knowledge regarding the basic biology involved in the desired performance traits does create problems in the application of molecular genetics - a real difference from quantitative genetics. It may well take considerable effort to obtain the information which puts animal breeders into a position where the benefits of molecular biology/genetic engineering can be properly exploited. There are, indeed, only a limited number of projects going on in domestic animal genes, for example, the keratin families of wool fibres in Australia (Ward, 1982) and work in pigs (Pond 1983). In 1982, Davies reviewed reports concerning the isolation and transplanting of genes to E Coli and found altogether some thirty-nine reports of human genes, thirty-one from rats, forty-two from mice, seven from rabbits, forty-three from poultry and only six from cattle.

The detailed comprehensive knowledge of biochemistry may be essential to make proper use of the technology. The control of an individual biochemical pathway may be of limited value and Kacser and Burns (1979) modelled a biochemical network and suggested that if a particular limitation (of an enzyme) is removed, another pathway may become limiting so that response in output may be limited.

This emphasises the point that in dealing with animal production, it is essential to deal with the whole system and the whole animal, rather than simply with the constituent processes and parts involved in production. It could well be, as the investigations with mice by Palmiter et al (1983), Brinster et al (1983) suggest that the effect might well depend on the number of genes expressions and there may be an optimal number. Similarly it is clearly not yet known what the "related/correlated" response to some gene transfers might be. A likely example could be additional maintenance that could well be incurred if mature size of the breeding females was increased considerably by the response to increase growth hormone. It must be remembered that, for example, in cattle, half the feed cost of the slaughter animal is in fact the feed consumed by the mother during the year taken to produce the off-spring - any increase in such costs would have a serious effect on efficiency, however dramatic the growth rate. However, the possibility of using the growth hormone gene to create faster growth in young animals for slaughter and turning off the effect at a suitable stage in females so that dam size is not increased, would be attractive. Also the clear benefits in terms of growth, could create serious management problems - for example, the slaughter problems created by the additional size (the costs

of developing the correct facilities would then have to be borne by the benefits of that particular gene transfer system).

The pleiotropic effects of genes cannot be ignored in any of the assessments of production systems and the evaluation of any genetic engineering must be in terms of the total influence of the system, not on a specially dramatic change in one particular character. The important overall consideration must be the production efficiency of a high quality product.

GENE MAPPING

The identification of Restriction Fragment Length Polymorphisms (RFLP) will provide detailed genetic maps and these can assist in the identification of loci affecting performance traits. Similarly, two dimensional gel electrophoresis can help by identifying large numbers of proteins. However, Soller, Brady and Genizi (1976) calculated that with about 200 polymorphic loci some 2000 F_2 individuals should be sufficient to pick up around 95% of the loci contributing at least 1% to the phenotypic variance in the F_2. They go on to show how this can be used in conventional selection programmes to increase the rate of genetic change.

POTENTIAL

Potential gene transfers are quite clearly a field of guess-work but likely examples might well be the genes to increase rate of synthesis of, for example, milk or egg protein and possibly muscle protein. There needs to be very considerable investment in research to identify what genes might be useful for transplantation, i.e. which genes might have major effects on production efficiency.

As more single genes with major effects are identified the opportunities extend - the limits may only be with regard to the overall effect of adding a specific gene but such assessment may well be very costly. With regard to prolificacy in pigs, some Chinese breeds appear to be outstanding and identification of the genes involved could be of major importance. Similarly, copies of genes coding for various hormones and, indeed, antibody genes aiming to improve the defence mechanism of animals could be of great value. A useful example could be the resistance to Trypanosomiasis as exemplified by certain African cattle breeds which are otherwise of relatively low productivity. One can envisage improvement of milk quantity and quality as well as changing milk constituents to suit national requirements.

The ability to test the status of an animal for a specific single gene can be valuable - the so called Halothane gene in pigs which provides additional leanness but couples to this advantage problems of meat quality and stress susceptibility. The identification of the heterozygote would be most useful in attempting to provide homozygous lines (either clear or reactor).

An obvious example for real dramatic change would be the ability to determine the sex of semen and hence predetermine sex of progeny (obviously poultry differ since the female is the heterogametic sex). However, the

following quote from Rutledge and Seidel (1983) may serve as a salutary reminder: Professor Jay L. Lush's dissertation topic was the centrifugal separation of semen. He wrote: "In short, this investigation has not strenghthened the belief that there is any method of controlling sex among the higher animals which is practical, simple and of wide application" (Lush 1925). Lush's 1925 comment applies today. Nevertheless, search for separation methods will continue because rewards could be great.

As well as using gene transfers to improve the normal traits in farm livestock, there is also scope for introducing novel gene constructs for other products. It is not now beyond belief to consider the use of mammary glands to collect milk which includes other valuable products which can be extracted. In addition, one basic advantage of livestock production when compared to the investments in genetic engineering research in plants, is that there could well be major spin-off information from medical research and there clearly needs to be close liaison between agriculturalists and the medical profession in this context.

One aspect not yet considered is the multiplicity of environments in which commercial production takes place. It is important to assess any genotype environment interactions and again the manipulation of genomes could result in populations which perform well in specific environments but less well in others - it would be important to identify this before progressing too far down the road of genetic engineering, since it would depend on the context of the benefits and the frequency of the environment concerned in assessing the overall advantages/disadvantages of engineering this particular gene.

CONCLUSION

Certainly, the developments over the last decade have given us potential for improving livestock at dramatically improved rates - increases in reproductive rates and in potential selection intensity, will themselves, be of great potential value. The opportunity to engineer specific genes giving advantage has considerable potential and should not be underestimated. Certainly, the technology is the most exciting development in recent times. The potential for improvement in animal production is enormous. However, it must be pointed out that there is a need for considerable investment to get animal production into a situation where present techniques may be used properly, since the basic biological knowledge and gene identification is not yet known. It behoves the animal industry to improve its present practices using normal, apparently slow methods of improvement until these new bio-techniques can be used properly. It would be irresponsible to stop existing programmes operating even at relatively low levels of efficiency on the basis of hope that we might get progress from the new technology. The different technologies should be used to complement one another so that there is a synergistic reaction - perhaps similar to that experienced after cattle AI was developed.

Given present rates of progress from the quantitative approach, it is essential that we insist that, if genetic engineering is to contribute significantly, it needs either to generate frequent and major improvements which produce an annual average rate of progress of at least 2% or to provide progress at much lower costs than other methods - certainly, present evidence gives one no confidence that this is achievable in the near future.

TABLE 3

Genetic engineering techniques

Chromosomal substitution
- Specified chromosomes
- Specific sections

Mitrochondrial substitution

Gene transfer
- Intra species
- Inter species

Modify expression of existing genome
- Regulation of gene expression
- Time of activity
- Level of activity
- Tissue expressed

DNA Library
- DNA sequencing
- Gene mapping, e.g. Restriction fragment length polymorphisms, two dimensional electrophoresis
- Linkages, Multi gene complexes, Homology among species
- Identify loci for economic traits

In vitro mutagenesis

Indirect manipulation
- Genetic engineering of rumen bacteria
- Genetic engineering of micro-organisms to:
  1) Modify animal feeds
  2) Replace animals in protein production.

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ARTIFICIAL BREEDING

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ABSTRACT

Artificial insemination and embryo transplantation provide powerful tools for the genetic improvement of livestock. Embryo transplantation in particular has enabled the development of new technologies associated with the manipulation of eggs and embryos. The preservation of embryos by deep-freezing makes possible the international exchange and conservation of valuable genetic material. Immunological methods are being developed for determination of the sex of embryos. Identical twins and quadruplets have been produced by micromanipulation of embryonic cells and possibilities for further clonal reproduction through chimaerism or nuclear transplantation are discussed. Techniques have also been developed for the maturation and fertilization in vitro of farm animal oocytes and these extend the possibilities for experimentation and genetic manipulation. Development and application of techniques for the introduction of foreign cloned genes into eggs and the production of transgenic animals could have a major impact on animal production in the future, but much remains to be learned about the methods and the type of gene constructs which might produce a desired physiological response.

INTRODUCTION

Many important advances in artificial breeding and the control of animal reproduction have been made during the last few decades. Artificial insemination, superovulation and embryo transplantation, methods for the control of oestrous cycles, techniques for increasing prolificacy, induced parturition and early pregnancy diagnosis are but some examples and all of these provide new opportunities for the improvement of livestock production. There is no doubt that the development and wide-scale application of artificial insemination has had the greatest impact on animal breeding, particularly in cattle and, when coupled with effective systems for progeny or performance testing, has led to marked genetic improvements. The ability to store spermatozoa for prolonged periods of time by deep-freezing has also enabled the transfer of valuable genetic material between countries and provided a unique method for genetic conservation. In some species there is still a need to improve methods for low temperature preservation of semen in order to enhance fertilizing capacity, but perhaps the ultimate goal for artificial insemination technology will be to develop methods for 'sexing' semen by the separation of spermatozoa into populations of those bearing either an X or Y chromosome thus permitting the preselection of the sex of animals at the time of fertilization. Many attempts to do this have been made, but unfortunately the several claims to success in this area have not generally stood up to critical testing or analysis. It is hard to say how long such

technology might remain a 'pipe dream', but perhaps the most promising basis upon which separations might be made is the difference in DNA content of X and Y spermatozoa and the advances that have been made in techniques for cell sorting (Keeler et al. 1983).

Embryo transplantation is a technique that has been developed more recently than artificial insemination but it provides a means for exploiting the genetic potential of the female rather than the male. The basic procedures are now well worked out and they can be applied effectively in most of the large domestic species thus adding another dimension to the opportunities for artificial breeding and livestock improvement. In some countries commercial companies for embryo transplantation are already well established and it is to some potential new developments associated with embryo transplantation that the theme of this paper will be directed.

EMBRYO TRANSFER AND PRESERVATION

The success of embryo transplantation depends to a large extent upon the ability to obtain an increased number of embryos from animals of superior genetic merit. This is achieved by the use of exogenous gonadotrophic hormones such as purified follicle stimulating hormone or pregnant mares' serum gonadotrophin administered at an appropriate time for the induction of superovulation. In some species such as cattle in which non-surgical methods for embryo collection can now be performed, the procedures for superovulation and embryo recovery can be carried out repeatedly in individual animals (Christie et al. 1979), thus providing a relatively large number of embryos over a short period of time. It is obviously important, however, also to be able to store embryos so that they can be transplanted into recipient animals at a different location to that of the donors. Embryos of most species can be kept alive in suitable culture media for one or two days, but the only effective method for really long-term storage is by deep-freezing and preservation in liquid nitrogen. It is now established that cattle embryos at the late-morula to early-blastocyst stage of development (6-7 days after fertilization) can be frozen satisfactorily and the proportion that survives after thawing is only slightly reduced as compared with fresh embryos. The techniques first developed involved the use of dimethylsulphoxide as a cryoprotective agent and the embryos were cooled very slowly at a rate of 0.3 to 0.5°C per minute to a temperature of about -60°C before immersion in liquid nitrogen. They then had to be thawed quite slowly for maximum recovery (Willadsen et al. 1978). More recently the technique has been simplified to the extent that slow cooling can be terminated at a temperature of around -35°C. After transfer to liquid nitrogen the rate of thawing must then be rapid in order to avoid recrystallization of residual intracellular water, but the survival rate of the embryos is equally high. One of the chief advantages of being able to freeze embryos is that it is not necessary to maintain a large herd of recipient animals in order to provide a sufficient number at an appropriate stage of the oestrous cycle to receive fresh embryos.

The frozen embryos can simply be thawed and transplanted to recipients as they become available. The long-term preservation which is afforded by deep-freezing also means that embryos can be kept 'in quarantine' before export and this enables health requirements to be met which in many circumstances restrict the importation of live animals into some countries.

Opportunities are also provided for genetic 'banking' for the conservation of rare or endangered breeds. It is a paradox that the techniques of artificial insemination and embryo transplantation may help to hasten the dilution or elimination of certain breeds with as yet unevaluated characteristics. Embryo banks should therefore be established to safeguard possible future needs.

The survival of sheep embryos after freezing and thawing is equivalent to that obtained for cattle and indeed the stage of embryonic development at which freezing can be done is probably less critical, but so far a satisfactory technique has not been described for the deep-freezing of pig embryos.

EMBRYO SEXING

Better opportunities for 'sexing' embryos are provided than for spermatozoa and, although this does not enable the preselection of sex, it at least means that a choice could be made of the sex of an embryo to be transplanted and this could increase the efficiency of some breeding schemes. One of the methods is to remove a few cells from an embryo at an early stage of development and to make a preparation from these cells in which the chromosomes can be examined (King 1984). The removal of a few cells from an embryo does not necessarily restrict its further developmental capacity, but the cytological methods have some drawbacks in that it is not always possible to obtain analysable chromosome spreads. By contrast, a more promising line of approach is by the application of immunological techniques. These are based on the detection of H-Y antigen which is a male specific antigen in mammals. There is evidence that this antigen is expressed quite early in embryonic development and there have been some encouraging experiments in mice in which a preponderance of females were born following the transplantation of embryo exposed to H-Y antibody and complement (White et al. 1982). Similar experiments with cattle embryos are already being carried out and these include attempts to develop monoclonal antibodies to H-Y antigen and to detect sex by means of an enzyme linked immunoassay system (Wachtel 1984). It remains to be shown how effective these methods may be, but they appear to offer considerable opportunities for the future.

MICROMANIPULATION OF EMBRYOS

Major advances have been made in recent years in the development of microsurgical techniques for the manipulation of embryos of the large domestic species. These involve studies on the developmental and regulatory capacity of individual cells or groups of cells from embryos at early cleavage stages. With the aid of a micromanipulator and very fine instruments it is possible to remove the cells from the outer membrane of the embryo, the zona pellucida, and to separate them. Early attempts to produce identical twins or quandruplets by this method were not very successful because it was found that the cells did not survive after the zona pellucida had been severely damaged and when they were returned to the female reproductive tract. This problem was overcome by Willadsen (1979) who devised a method for embedding embryos in agar following radical microsurgery. The agar coating effectively sealed any holes or defects in the zona pellucida and allowed the manipulated embryos to develop normally

when returned to the oviduct of a recipient animal. At a later stage when they had developed to blastocysts and protection from the zona pellucida was no longer required, they could be collected from the first recipient, the agar coating removed, and returned to the genital tract of other recipients in order to develop to term.

By this means monozygotic twins have been produced in sheep, cattle, pigs and horses and these have been derived by separating the blastomeres from either two-, four- or eight-celled embryos into two equal groups (Willadsen 1982). It is interesting that the capacity of these embryos to survive and form a normal animal appears to be unimpaired even though they contain only half the normal number of cells during early stages of development and differentiation. Indeed, a more radical reduction in cell number can also be tolerated and several sets of identical quadruplets or triplets have been produced from 'quarter' embryos (Willadsen 1981). It appears unlikely, however, that a larger number of identical animals can be obtained from a single embryo by the simple technique of separating blastomeres. Single blastomeres from eight-celled embryos or groups of two blastomeres from sixteen-celled embryos seldom produce a viable fetus (Willadsen 1981) because when the cell number at the time of blastulation is so drastically reduced, the embryos generally fail to form a functional inner cell mass.

Nevertheless a set of identical quintuplet lambs derived from a single eight-celled embryo has been produced (Fehilly et al. 1984). This was achieved through the technique of chimaerism. It is possible to aggregate the cells from one embryo with those of another to form a composite embryo. If the total number of cells at the time of aggregation is about equal to the normal cell number, the composite embryo generally gives rise to a chimaeric fetus in which both cell types are represented. By contrast, if chimaeric embryos are composed of less than the normal cell number, such as 'quarter' embryos made from single blastomeres from each of two separate eight-celled embryos, it frequently occurs that the inner cell mass is derived from parent cells of only one of the donor embryos. Hence there is a potential to produce identical octuplets but in the normal course of events it is unlikely that all the embryos will survive to term.

These techniques for the manipulation of cells from early cleaving embryos provide many exciting possibilities for the multiplication of animals of selected genotype. Because they are rather complicated, however, and require agar embedding in order to protect the embryos during early development, they have not yet been applied except in experimental circumstances. By contrast, a very simple method of splitting embryos into two halves at the late morula or early blastocyst stage of development has recently been devised. If blastocysts are divided in such a way that each half contains inner cell mass and trophectoderm cells, both halves are capable of development to term. Since protection from the zona pellucida is no longer required at this stage, the divided embryos can be returned directly to the reproductive tracts of recipients (Willadsen & Godke 1984). This simple method is already being applied commercially in cattle embryo transplantation. It not only provides an opportunity for obtaining some identical twins, but it also increases the supply of embryos available from any one donor.

The groups of genetically identical animals that have been produced from manipulated embryos represent small clones, but is there a possibility of producing much larger clones through techniques of nuclear transplantation? The ultimate objective, of course, would be to transplant a nucleus from a cell of an adult animal into an egg and see if it could produce a fetus. In mammals, this is still in the realms of science fiction since it appears that nuclei in differentiated adult cells become irreversibly restricted in their potency and they are unlikely to initiate full embryonic development. However, there may be greater possibilities for cloning with the nuclei derived from embryonic cells in which totipotency is still retained. Successful methods for nuclear transfer which inflict little damage to the eggs have been developed. These involve sucking out a nucleus with a micropipette from a donor egg surrounded by a small piece of the egg plasma membrane. The fragment containing the nucleus is then inserted beneath the zona pellucida of the recipient egg into which it can be fused with the aid of a fusogenic agent such as Sendai virus (McGrath & Solter 1983). Experiments in mice involving the transplantation of pronuclei from recently fertilized eggs into enucleated fertilized or artificially activated eggs has resulted in the birth of live young (Surani et al 1984). Successful full-term development following transplantation of more advanced embryonic nuclei into enucleated mouse eggs have not been confirmed, but experiments in other species are also in progress.

Certainly such techniques would appear to offer one of the best approaches to cloning. Other potential sources of nuclei can be considered. It has recently been shown to be possible to establish progressively growing cultures of pluripotential embryonic cells which have been isolated directly from cultures of mouse blastocysts (Evans & Kaufman 1981). These cells can participate in the formation of chimaeras when mixed with normal embryonic cells. If it becomes possible to establish cultures of normal pluripotential cells from a wide variety of species, then these could represent an important source of material for nucleus donation or for other forms of genetic manipulation.

SOURCES OF EGGS AND EMBRYOS

Many of the manipulative procedures described above require a plentiful supply of eggs or embyros. The number of eggs that can be obtained from donor animals by means of superovulation is limited and in the case of farm animals it is also relatively expensive to keep large numbers of donors. There has for long been, therefore, a considerable interest in an alternative method for 'tapping' the very large pool of immature oocytes which is present within the ovaries of all mammals. Many experiments have been carried out on the maturation *in vitro* of oocytes collected directly from immature ovarian follicles. The problem has been to establish a system whereby oocytes matured *in vitro* are capable of normal embryonic development following fertilization. Recently, however, a simple and reliable method has been developed for producing large numbers of fully matured sheep oocytes in an *in vitro* system (Staigmiller & Moor 1984). The oocytes collected from small follicles are cultured with their cumulus and coronal cells intact in a medium containing hormones and supplementary follicle cells. A large proportion have then been shown to be fully competent to undergo fertilization and normal embryonic development leading

to the birth of live young. Reliable methods have also been developed for the fertilization of oocytes in vitro. An almost unlimited supply of ovaries from which oocytes can be obtained is available from slaughterhouse material, thus opportunities for experimentation are greatly extended.

GENE INJECTION

An area of active experimentation at the present time concerns the introduction of foreign cloned genes into mammalian eggs and the production of transgenic animals. In mice it has been found that one of the most effective methods for the introduction of DNA into the genome is by microinjection of cloned genes directly into the male pronucleus of an egg shortly after fertilization. The foreign genes may then become incorporated into the chromosomes during early development and following transplantation of the eggs produce transgenic animals. In some animals the genes may become stably integrated and expressed and be transmitted through the germ line (Constantini & Lacy 1981; Wagner et al. 1981; Gordon & Ruddle 1981; Palmiter et al. 1982). Some transgenic mice derived from eggs that had been injected with a construct in which a growth hormone gene had been linked with a metallothionein promotor sequence expressed considerably enhanced growth (Palmiter et al. 1982) and such experiments raised great interest in the possibility of producing transgenic farm animals. In the eggs of most farm animals, however, the cytoplasm is too dense to be able to visualise the pronuclei for the purpose of microinjection. For pig eggs, however, this problem has recently been overcome by centrifuging them so that the opaque cytoplasm is concentrated to one pole and the pronuclei then become readily visible under the microscope. Several transgenic pigs have recently been produced containing the growth hormone - metallothionein gene construct (Hammer et al. 1985), but although evidence of the expression of the foreign genes in some of the animals was obtained, their body weight was not increased dramatically.

At the present time the efficiency of gene transfer is very low and large numbers of eggs must be injected to obtain a positive result. Much remains to be learned about the types of gene construct which will enable optimal integration into the genome, result in tissue specific expression and produce animals with a desired phenotypic response. The identification of genes for characteristics of special economic importance is also a major area of experimentation. Nevertheless, the possibilities for genetic engineering by recombinant DNA technology open new vistas for the future.

CONCLUSION

The new developments which have been described in the control of early embryonic development in animals open up exciting possibilities for large scale production of improved livestock. Technical opportunities for sexing, cloning, banking and distribution of embryos and genetic manipulation provide new methods for improved efficiency of animal production.

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