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Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

D00268

United Nations Industrial Development Organization

Distr.
LIMITED

ID/WG.50/1
24 November 1969

ORIGINAL: ENGLISH

Expert Working Group Meeting on the
Manufacture of Chemicals by Fermentation

Vienna, 1 - 5 December 1969

PROBLEMS OF CULTURE IMPROVEMENT IN INDUSTRIAL MICROBIOLOGY

METHODS OF INDUSTRIAL STRAIN IMPROVEMENT 1/

by

C.T. Calam
Imperial Chemical Industries Limited
Pharmaceuticals Division
Macclesfield, Cheshire
United Kingdom

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id.69-6017

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ID/WG.50/1 SUMMARY
10 October 1969

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SUMMARY

PROBLEMS OF CULTURE IMPROVEMENT IN INDUSTRIAL MICROBIOLOGY 1/

by

C.T. Calam
Imperial Chemical Industries Limited
Pharmaceuticals Division
Macclesfield, Cheshire
United Kingdom

The methods used for the improvement of commercial cultures of microorganisms will be described and examples given of the results obtained.

Mutation and selection have been extensively used for this purpose. During recent years considerable interest has developed in the hybridisation of different microorganisms, bacteria, fungi and actinomysetes. Methods used, apart from sexual methods, have included the para-sexual process, transformation and transduction. This work has been mainly

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GENERAL DISCUSSION

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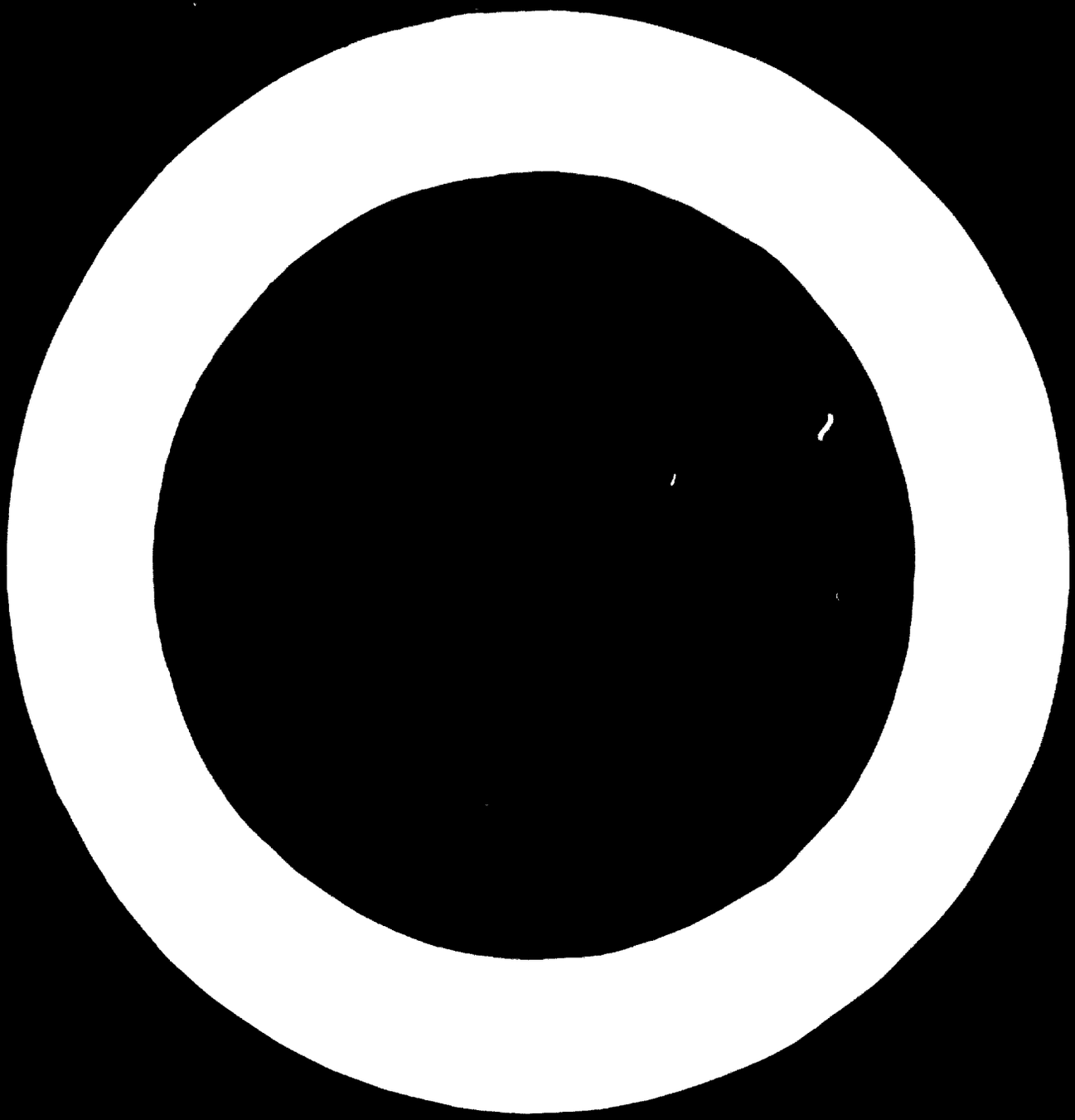
INTRODUCTION.

As a matter of convenience the present review has been divided into two parts. In the first is given a general account of the methods used in etrain improvement which it is hoped will be helpful to those who are relatively unfamiliar with the subject. The second part deals with a number of different aspects of the



academic, aimed at the elucidation of genetical processes, though these methods have also been applied in industrial strain improvement work with some degree of success.

Practical experience in the industrial field has shown that the application of breeding methods is often difficult and that it is not always very successful. This is partly because difficulties may be experienced in producing recombinations and also because in many cases multi-gene processes are involved, so that the chance of obtaining improved cultures becomes statistically very unlikely. Often a limited advance in productivity occurs, after which further progress becomes very difficult or impossible. As a rule straight forward mutation and selection is more reliable.

A consideration of the situation brings out two points. Firstly, that in an industrial organisation the number of organisms available for mutation or for crossing is often limited, so that the possibilities for developing new strains is restricted. Secondly many of the difficulties encountered are problems already recognised in plant breeding, and they could perhaps be better solved by an expert in breeding rather than by someone whose field was purely genetical.

If it is hoped to establish new microbiologically based industries in the developing countries, the production of new strains suitable for these purposes could be of great assistance. U.N.I.D.O. might be in a good position to initiate or sponsor developments of this kind. It might also be able to obtain a wide range of organisms to serve as a starting point and to bring into action experts and laboratories concerned with plant breeding who had special experience and skill in work of this type if not specifically in the field of microorganisms.

subject. These are arranged somewhat arbitrarily, and it has been found impossible to avoid some degree of repetition. The field is a wide one and the review concentrates on those aspects with which the author is most familiar. In particular the approach is practical rather than theoretical, and there is little or no reference to genetical theory.

Part 1. OUTLINE OF METHODS OF STRAIN IMPROVEMENT.

Botanically plants and micro-organisms are divided into genera and species. However not all members of a species are in every way identical. For instance in the course of work in Professor Raistrick's laboratory on the fungus Aspergillus terreus, it was found that out of a dozen cultures one produced the antibiotic geodin, another produced itaconic acid while the remainder gave nothing of interest. All these cultures were typical of Aspergillus terreus which is a light sandy brown fungus. The one providing geodin was called the geodin producing strain, the other the itaconic acid producing strain. The word "strain" corresponds to "variety" or "clone" which are more generally used for plants.

Another example will be quoted as it illustrates not only the meaning of "strains" but also the importance of strain selection in production work. This is the instance of penicillin. The original culture which Fleming picked up gave only a few millegrams of penicillin per litre, in surface culture. When the Americans came into the picture they immediately started a large scale search for new strains. In this they were undoubtedly guided by their experience with fungi, and this was to a large extent developed by the work done at Wisconsin University and the Regional Research Laboratory at Peoria which had devoted much energy to the moulds used in cheese manufacture and to problems of the utilization of agricultural products. The account is given by Backus and Stauffer (1955).

The fact that these laboratories were in existance stemmed from a very early realisation by the American Government of the importance of research of this kind and the establishment of the Land Grant Colleges and research institutions at a very early stage in the country's development.

The search produced results of the greatest value and interest. Fleming's strain belonged to the species Penicillin notatum-chrysogenum, and it was cultures of this type that were collected. The majority of strains produced no significant amount of penicillin, but several were obtained which were far more productive than was Flemming's, and which, with the aid of improved media containing corn-steep liquor and precursors, increased the yield thirty to fifty times.

A very important addition to this was a second objective in the mind of the Americans. This was to obtain a culture which produced penicillin in submerged culture. The Flemming strain did not do this, and the one or two strains which did were not very effective.

In these days screening is usually done in submerged culture in any case, and it is not always realised that strains exist which work in surface culture but not in submerged, and no one knows how to get over this difficulty if the mould proves obstinate. The search was in this case successful, and a culture named N.R.R.L. 1951 was discovered which has been the basis of commercial production throughout the world. Its productivity has of course been greatly increased by mutation programmes.

The events which have just been described illustrate the general sort of process which happens when a new fermentation process is brought into operation. They illustrate the need for a farsighted outlook, in this case the requirement of strains suitable for submerged production of penicillin, a need recognised at a time when the technical practicability of submerged fermentation was still a matter of debate.

They also bring out the value of experienced workers. Much of the work being done at Wisconsin University and at Peoria must have seemed of limited value, but from it arose the possibility of vast new progress in pharmaceuticals. The success of the Americans in this field has been attributed to their enormous financial and industrial resources. It was also due to the availability of skilled people who knew how to make this possible. At the present time expertise has become much more widely distributed and many research institutes are at work in the world. One should not forget the need for this expertise when new projects are being started lest only the financial and commercial aspects are considered.

Strain improvement methods.

The object of the present paper is to discuss strain improvement, and this is the subject which immediately becomes important when a new fermentation process is introduced.

The first step is usually a search for new naturally occurring strains. This has already been described in the case of penicillin. Here the main object was increased productivity, but, as will be discussed later, other properties may also be sought, such as ease of working, improved quality of product and so on. The latter may be of particular importance in the preparation of food and beverages when taste is all important. Other steps may also be made to improve the process, as in the case with penicillin, by improving the culture medium or the fermentation plant. Important though these are they are usually more limited in scope when compared with strain improvement as the new strain can usually be used directly to take advantage of the new fermentation conditions.

When the best available strain has been obtained and fermentation conditions optimised, a mutation - selection programme is the next step on the road to increase production.

The culture is exposed to the action of a mutagen, which may take the form of ultra violet light or other radiation or a chemical and the surviving cells or spores isolated and tested on the small scale. The changes produced by the mutagen are genetical in nature. Originally mutation programmes involved testing thousands of cultures, but more recently a more limited scale of working has been found adequate to give useful results. Fuller details of these points will be given later. In practise a mutation programme normally takes six months or a year before improvements are achieved, and thereafter a considerable further degree of progress is possible. It is assumed here that a team of workers and suitable laboratory facilities are available. If this is not so there may be a considerable delay in starting.

Although it was known in 1944 that moulds could be improved by mutation, the introductory campaigns on penicillin were certainly very much of an adventure and in at least one case were quite unproductive. This type of work requires a good deal of skill but as well as this there is a considerable need for opportunism and the ability to spot, almost by intuition, where an advance has been made which can be followed up. In the penicillin case the N.R.R.L. 1951 strain was given a preliminary examination and an isolate N.R.R.L. 1951 B25 was selected. (Backus and Stauffer loc.cit). This was mutated and given a rough selection in Prof. Demerec's laboratory when a much improved mutant was obtained. This was in turn mutated at Wisconsin and another improve culture Q-176 was the result, giving about four times as much penicillin as the original strain N.R.R.L. 1951 B25 - in the course of 2-3 years. This brought yields to over 0.5 gm/litre. By 1949 strains two or three times as good and free from the undesirable yellow colour were produced, and since then yields have been slowly increased to 7 gm/litre or more.

The effect of the mutation work can easily be imagined. A plant with say twenty fermenters planned in 1946 in the expectation of obtaining 0.3 gm/litre would find that when it was in full working order three years later that the derived output could be achieved with only two or three fermenters and by much reduced effort on the extraction side.

Needless to say reduced prices and increased demand utilised spare plant capacity, but also stimulated the search for new products.

In addition to selection with and without mutation, hybridisation has also been used for strain development work. Of particular interest has been the discovery of conjugation processes in normally asexual micro-organisms. Although this is an interesting field it has not on the whole been as productive as mutation and selection .

Types of culture involved, and some examples.

Although in mutation work the greatest interest has been with fungi and actinomycetes, a considerable amount has been done with yeasts and bacteria. A great deal of work has been done with the latter types of micro-organism as part of the study of genetics. This has clearly shown that mutants can be readily obtained.

In the case of moulds and for industrial purposes, improved mutants have been used for the production of penicillin, griseofulvin and itaconic acid and probably in other cases as well. Less striking advances have been made in the production of gibberellic acid.

With actinomycetes mutation has been used to increase the yield of the tetracyclines as well as to obtain strains giving new types of antibiotic from tetracycline producing organisms. Other actinomycetes successfully mutated have been producers of streptomycin, erythromycin and others.

In an article published in 1962 Alikhanian refers to increase in penicillin yields in 1943 of around 200 u/ml increased to 8000 by 1955, fifteen to twenty fold increases in chlortetracycline and oxytetracycline during the early years, and a doubling of the yield of erythromycin between 1955 and 1961.

Important advances have also been made with bacteria. As a result of extensive selection work the Japanese established virtually a new industry to produce glutamic acid (c.f. Huang, 1964).

later glutamic acid producing strains were mutated by the Japanese to give lusine (c.f. Hueng; loc cit.). Lysine producing strains have been obtained in U.S.A., Czechoslovakia and in U.S.S.R., lysine being particularly important as a food-supplement for animals. Improvement in toxin production by Clostridium cultures has also been obtained using mutation with fast neutrons.

In connexion with the development of processes for the production of protein from hydrocarbons and waste products, extensive work has been done on the selection of strains, including the selection of strains which grow at higher temperatures.

Much has been written about strain improvement work. Backus and Stauffer (1955) have discussed penicillin work and reviews by Alikhanian (1962) and by Calam (1964) may be mentioned. A particularly interesting series of papers was given at a Congress on Mutation in Moscow in 1965, but the book published after the congress is hard to come by.

Mutation and selection methods

Organisms are usually mutated by making a suspension of the cells or preferably spores in water so as to give a viable count of 10^7 cells/ml., exposing the suspension to the mutagen until the viable cell concentration falls to 10^{4-5} cells/ml. and then diluting and plating. The degree of kill which gives the best results is a matter of argument, some preferring high, others low kills. When the diluted solution is plated to give 50-100 viable cells per plate, single colonies develop on incubation which can be picked of and tested.

The mutagen may be ultra-violet light, in which case the suspension is placed in a shallow petrie dish and exposed to the lamp for a suitable length of time, say 2-20 minutes at a distance of 15 centimetres. The dish is stirred or rocked during the exposure. If a chemical mutagen is used it is added at a low dilution, say 0.01M to the suspension and allowed to act for a time.

It is desirable to control the temperature to give reproducible results. Afterwards sodium thiosulphate is added to destroy the mutagen and the suspension is diluted and plated. Commonly used mutagens are nitrogen mustand, ethylene imine , diethyl sulphate, 6-mercapto-purine and others. Recently some nitroso compounds have become popular such as nitroso-methyl urea and N-methyl-N-nitroso-N-guanidine which gives very effective results. More than one mutagen may be used in succession. Ionising radiations such as X-rays or gamma-rays or fast neutrons are also used, usually applied to a colony of the organism.

The actual choice of the mutagen is usually based on experience or on trial and error. It is often necessary to test a number of methods until favourable results are obtained, and to build on experience. The methods of isolating mutants and preparing them for test depend in each case on the type of organism being used. Arrangements must also be made to store the mutants subsequently obtained, during and after testing. Slopes may be covered with oil and held in the refrigerator, or spores may be stored on soil, sand or silica or freeze dried or held in liquid nitrogen.

The colonies obtained from isolation plates are transferred to slopes and these slopes are used for testing. The methods used will be those appropriate to the subject being studied, but as a rule this involves culturing in small or large shaken flasks in an incubator, the product later being suitably assayed It is necessary to do this work with as high a degree of accuracy as is possible. The degree of accuracy and reproducibility to be aimed at depends on circumstances, but it can be dangerous to attempt mutation and selection if the methods are unreliable as they may make the detection of improved strains almost impossible. The selection method should be such that results obtained are reproducible on the manufacturing scale.

In selection work it is best to first test as many cultures as possible using a single flask per isolate. This test may be regarded as a rough screen which eliminates those cultures which are not worth a serious test.

One tenth to one quarter are then retested using 3-4 flasks so that the best ones can be retested. If conditions are good it will be found that the best five selected will include an improved strain, if such are present, and then mutation and reselection of these five strains forms the basis of the next mutation stage. It is better to run a series of mutations, testing a relatively small number of strains at each stage, than to screen a large number of strains at each step. The reason for this will be discussed later. When an obviously improved strain is produced it should be carefully examined and tested under different conditions to check its stability.

The recently published series of volumes "Methods in Microbiology" (Academic Press) will be found to contain much valuable information on the techniques used.

Factors in successful mutation work

Many factors contribute to the success of strain improvement work. One of these is a careful study of the organism being used and the fermentation process, so that all the isolation and testing work can be done in the most suitable way and with adequate reliability. The method used to estimate the product must also be carefully chosen, simplicity and ease of operation being balanced against reliability. For instance in the early stages of screening a simple almost yes-no test may be enough. Later when fermentation improvements are being sought an accurate and specific test must be used, as an unreliable method can be fatal to success.

There is general consensus of opinion that the nature of the strain being mutated plays an important part in the success of the programme. Some strains respond much more readily than others, while others are very difficult to budge. As Professor Alikhanian has pointed out, sometimes a mutation occurs which has little effect on productivity but which disposes the mutant to jumps in productivity when given a further treatment.

For these reasons it is better to include in a programme a succession of mutation steps (in the hope that one or two will succeed even if others do not) rather than screen on a large scale from a few mutations. The fact that frequent mutations are run, only a few mutants (e.g. 100-200) being screened, does not mean that overall only a few strains are tested. In a year as many strains will be tested as in a scheme with a few mutations and the screening of many mutants in each case (say 1000 or more), but there will be more opportunities for a mutation to occur.

As has been mentioned, mutation work is often highly successful, large advances in productivity being achieved in the course of a few years. After this the rate of advance may slow down or even appear to stop. The best thing to do under these circumstances is not easy to decide, but a slow advance may still be possible and as many variations as possible should be used and care taken to ensure accuracy of testing. Changes of medium and test-conditions may also be useful. At some point increases in the size of the fermentation plant may be required if a rapid (rise) in productivity is needed urgently.

An important factor is the careful choice of objectives. Careful consideration should be given to the actual requirements and the objective should be made as simple as possible. Ideally a single target should be sought, e.g. increased productivity. Circumstances may arise when several requirements have to be met and in this case due allowance must be made for increased effort. It may be best to consider a step by/approach which gradually brings together different needs.

If one is planning a mutation programme it is advisable to set limited targets to be achieved at intervals of say six or twelve months. These may be for instance so many isolates tested or a certain degree of improvement. Under circumstances where results may develop slowly this is helpful in keeping up an adequate rate of progress.

Hybridisation.

While the early work on mutation carried out in the late forties gave valuable results, it obviously depended on chance for success and it was natural that methods were sought which it was hoped would enable strain improvement to become a scientifically controlled process. For this purpose attempts were made to introduce the possibility of hybridisation so that chosen strains could be crossed. Since the most important strains of moulds and actinomycetes are asexual this seemed impossible at first. However in 1951 Pontecorvo, Roper and Sermonti discovered a method of hybridisation, the parasexual process, which has since been used extensively in genetical work and has also been applied to strain improvement.

The method involves preparing mutants of the two parental strains which will not grow on minimal media, that is on media free from amino acids and essential growth factors. If a mixed culture of such organisms is made, the hyphae interlink and nuclei of both varieties occur in the same cells. If, as sometimes happens, two different nuclei fuse, a new strain combining the properties arises. Being double it is called a diploid, and since it combines the properties of both strains it grows on minimal medium and can be isolated.

The properties of these diploids lie between those of the two parents, and in order to take advantage of the cross the diploids must be mutated to give strains with the nucleus further modified so as to give improved results. The mechanisms of this process are fascinating and for the study of genetics of the greatest importance. As a means of strain improvement hybridisation has certainly given rise to some increase growth and sporulation. For a general discussion of methods and results see "Methods in Microbiology", Alikhanian (1962), Sermonti (1954) and Macdonald et al (1963). Hybridisation has been carried out with moulds and actinomycetes parasexually, while Russian workers have used phages for crossing (Alikhanian and Iljina, 1957; Teteryatnik et al., 1962) Hybridisation is also possible with yeasts and bacteria.

A hybridisation programme (see "Methods in Microbiology" for details) involves the following steps:-

1. Mutation of parental strains and selection of mutants for crossing.
2. Hybridisation of the mutants and isolation of diploids.
3. Mutation of diploids to give new strains which are then screened for productivity. Probably at least two mutation steps will be needed.

Hybridisation is thus slower than mutation, as the first step may take a considerable time, and the second step at least a month, : neither of these steps is likely to lead to any increase in production in itself, all depending on the later mutation steps. The hybridisation system thus suffers from certain handicaps. None the less it is not to be discarded too quickly as it has a considerable potential under conditions when cultures grow poorly or when straight mutation is failing to produce results. Hybridisation is not the first choice when starting strain improvement work. A further discussion of this subject will be given later in the section on problems of strain improvement.

Laboratory facilities and apparatus required, outline of methods.

For mutation and strain improvement work the facilities required are relatively simple. A working team frequently comprises a skilled microbiologist with one or two assistants. For them a laboratory about 7 by 10 meters in size would be required, with a cubicle for aseptic working, incubators or a constant temperature room for growth of slope cultures and petrie dishes. A cold room or large refrigerator would also be required (+2°C). For culture tests a constant temperature room with shakers to take 200 to 500 flasks would be needed. The rotary type of shaker (2 5 cm. diameter circles, 250 cycles/min) is preferable. Details of these can be found in "Methods of Microbiology". Apart from this apparatus, centrifuges, filtration apparatus and so on are needed.

A considerable amount of washing up, medium preparation and sterilisation is involved. A separate kitchen is needed for this purpose. Facilities for the long term storage of cultures by one of the accepted methods is desirable. Space, apparatus and possibly an extra assistance may be needed if the productivity of the mutants has to be assayed by the mutation team. In some institutions many of the facilities described in these paragraphs are provided by central services.

In some cases stirred culture apparatus is necessary for a more complete assessment of new cultures, as shaken cultures may not sufficiently resemble plant conditions. Types of stirred culture apparatus and its usesare described in "Methods of Microbiology" Vol. I and in a number of articles.

The foregoing gives a general idea of the sort of facilities required. The actual details of the proposed programme will define laboratory and other requirements which will be needed, and it is impossible to cover in detail every possible need. It is also difficult to describe a general mutation programme that will meet every type of work, but a general outline of a programme with a fungus might consist of:

1. Selection from a previous mutation step of the five best cultures.

2. Mutation of the cultures as suspensions with radiations or with a chemical, followed by dilution and plating. This would require about 500 petrie dishes (Time, 1 day).

3. Incubation for 4-7 days while colonies develop.

4. Picking off forty to fifty colonies from each mutant and preparing 3-5 slopes for testing (1 day).

5. Incubation of slopes of 4-7 days to give spores, followed by examination of slopes.

6. Incubation of flask cultures from the slopes (200-250 isolates). Growth of cultures for production test, one flask each (first screen). Incubation for 5-9 days.

7. Assay of samples for production of desired material, choice of best 50 (1-2 days).

8. Retest of best fifty cultures using 3-4 flasks each. Incubation 5-10 days, possibly more. It may be desirable to use a two stage process.

9. Assay of samples, choice of the best five for re-mutation. One slope used to provide a master culture. (1-2 days).

10. Repeat of the process. Total length of cycle 24-43 days approximately. Number of cycles per year would be 8-15, though ten would be a good average for a small team.

With other organisms, e.g. bacteria, growth times would be much shorter and more cycles would be completed and a whole variety of modifications and short-cuts could be introduced. The figures and methods suggested are intended merely to outline the sort of work that is involved. In this example it is suggested that 500ml. Erlenmeyer flasks are used. In the writer's experience fairly large flasks holding 40-60 ml. of medium have proved best as they are reliable, give good results and give ample material for test.

Many workers use small flasks or large test tubes and for many purposes they give satisfactory results. However it is unwise to sacrifice accuracy for convenience if it introduces unreliability and thereby prejudices the success of the programme. Chemical methods of analysis are usually best but bio-assays are often found quite satisfactory.

A laboratory for strain improvement work usually forms a relatively small part of an organisation devoted to process development and shares in the facilities which are generally available.

Part 2. ASPECTS OF STRAIN IMPROVEMENT

1. OBJECTIVES IN STRAIN IMPROVEMENT PROGRAMMES

Up to the present time strain improvement has usually meant attempts to get better yields from existing cultures, using basically the original fermentation process. This has already been illustrated by the case of penicillin. As in the case of penicillin, strain improvement usually involves two phases. In the first phase productivity is substantially increased to a commercially feasible level. Thus with penicillin the original yields of a few milligrammes per litre were raised to around the 0.5 or 1gm/litre level. This would permit the production of considerable quantities of penicillin without excessive difficulty though very large plants would be required. Further development up to 2-3 gm/litre could probably be included in this phase. In the second phase of development the main factor would be a matter of commercial competition from the price aspect. The first-phase increases would make large scale production feasible and also bring prices down to moderate levels, i.e. from the levels of pounds to shillings or less per gram. (It is impossible to be precise over this). The second phase might reduce the price by a half or so but not much more, because at this stage isolation and purification costs per kilogram start to become dominant. Levels of 5-15 gm/litre seem to be the upper limit under plant conditions.

The foregoing relates to antibiotics for which relatively high retail prics are usually possible, since doses of a few grams are sufficient, while packing and distribution costs are usually also high for a variety of reasons, for instance because of the high standard of control involved. With more industrial types of material, sold in kilogramms rather than grams, sale prices / are much lower.

Here the cost of raw materials required for fermentation becomes dominant and much higher yields and conversion rates are needed. Instances of this type of fermentation are production of glutamic acid or lysine, or a food yeast.

All these points have to be taken into account when organising strain improvement work. It would seem that where biosynthesis is involved (e.g. of a complex antibiotic) yields of 5-15 grams/litre may be obtained, though sometimes maximum yields are lower. With conversions (e.g. glutamic acid, cell production) yields are higher. (20-50 gm/litre, with 60% or more conversion raw material.) In either case some degree of strain improvement is often necessary before a process becomes feasible.

The other possibility of strain improvement involves producing strains giving special products for special purposes. An outstanding example of this is the production of lysine by mutants which have been specially blocked biochemically. In a similar way, tetracycline producing actinomycetes have been mutated to give new substances. Another possibility is the production of mutants giving good yields but in a purer form or by an easier process. An example is the production of penicillin free from the orange colour which was a serious problem in the early days.

When a new industry is being established the discovery of suitable new strains is a matter of vital importance. While this is a problem of selection rather than strain improvement, many aspects are similar. More than one strain having suitable activity may be obtained, and the choice of the best one is important.

The first stage, as has already been mentioned, is to bring the best available strain to a level of yield which is technically and economically practicable. After this the main target is nearly always increased performance, this being valuable and usually experimentally straight forward. A second target is the selection of a culture which is stable and easy to use, e.g. with good sporulation and with a convenient form of growth.

A strain which is variable or unreliable in performance is always difficult to control and is probably more trouble than it is worth. In this part of the work biological knowledge, experience and know-how are of particular value.

Another possibility that would be important in developing countries, is the selection of strains that perform well under special local conditions, e.g. that are able to utilise cheap local raw materials. It has also been suggested that strains capable of working at increased temperatures might be useful, under conditions where cooling is difficult.

While it may seem that almost unlimited possibilities exist, in fact many objectives are difficult to achieve, and it is again emphasised that the simpler they are the better.

2. METHODS OF SELECTION

When industrial strains are being selected the method is usually to culture them in a way similar to that used for production. This usually means a shaken flask process, often with a medium somewhat weaker than that used in large fermenters. Every process sets its own conditions, but it is usually better to use a fair sized culture (e.g. 50-100ml.). When very small cultures are used it is difficult to get a satisfactory degree of agitation and there may be difficulties in getting sufficient solution for assay. The shaken cultures are conveniently done in 500ml. conical flasks on rotary shakers (5cm circles, 250 cycles/min.).

Any system which gives reliable results can of course be used, but the suggested method is usually found satisfactory.

Following one or two shaken flask tests, promising strains may be tested in stirred cultures. For this purpose fermenters of 3 to 50 litres working volume are used. Small fermenters are convenient for simple tests; where a complex process is being operated and where an extraction trial is needed, larger fermenters are more suitable. The object of the larger scale tests is to foresee and deal with any problems that might occur at plant level, but it is easy to do too much work at this level, while the crucial plant tests are delayed. As has been previously mentioned, the culture should be carefully studied for growth etc. at this time so as to avoid any unnecessary difficulties.

For testing strains a group of 6-12 or more fermenters is required. As a rule a simple type of system is best as it gives greater ease of operation and replication. In strain testing the fermentation system (medium, feed etc.) should also be as simple as possible, more elaborate conditions being used later on.

The general methods for culture work in shaken flasks and stirred fermenters are described in "Methods in Microbiology" Vol.I, as well as in other places, and it is not proposed to go into detail at this stage.

As has been mentioned above, it is recommended that selection is conducted in two or three stages, the first consisting of a test in a single flask, the second of about one third or one quarter of the cultures, giving the best results, using three or four flasks. A third test of the best 10% , also using four replicates, may be used if necessary. It is also suggested that instead of passing forward a single "best" strain for mutation, the best five or ten should be carried forward, all being mutated and then 20-40 of the isolates being screened. This contrasts with other methods in which, say, a thousand isolates are screened and the best carefully chosen for further work. It should be noted that using the proposed method as many cultures are screened as is the case where larger groups are used, the difference being that more mutations take place.

These proposals arose from an extensive mathematical study (Davies, 1964) based on the results of screening work in the penicillin field. A summary of this has been given by the writer (Calam, 1964). In this work the effect of random testing errors was eliminated mathematically so that the distribution of mutants in the treated population could be described with reasonable accuracy.

It was assumed that following a mutation it is likely that there will be present more mutants giving a small degree of improvement than there will be mutants giving a large improvement. It is therefore advantageous to screen a small group, hoping for a small increase, than a large group to get a larger increase.

The difficulty is the testing error which makes it hard to find the best strains; even so it is likely that the few apparently best strains will include those giving the highest yields.

Mutated populations considered by Davies were rated poor, standard and good. Some criteria were:-

Mutated Population	Frequency of improved mutants.	Mean improvement %	Frequency of improved strains for 5%	for 10%
Poor	1/40	2.5	1/2000	1/20 000
Standard	1/20	3.0	1/200	1/3000
Good	1/10	4.9	1/90	1/300

As explained, these figures were based on estimates made with penicillin cultures which had already been mutated many times and progress was slow.

Applying these data the possible rates of increase per ten months were calculated, assuming batches of 200 flasks were in continuous operation, with a standard error per flask for the screen of 10%. A ten-month period was used as a basis : with three-stage screens less steps could be completed than with two-stage screens. The calculations included a reduction in each step from 200 to 10, 5, 3 or 1 best culture before re-mutating and screening.

It was found that with a "good" population it was slightly better to reduce to one culture at each screening stage, but with a "poor" population reduction to 5 or 10 was better. The rate of improvement over 10 months with the good population would be around 100%, with the poor population about 15% with the standard population about 45%. As has been indicated it is proposed

to mutate several strains to take advantage of the possibility that one culture may respond better than another. These calculations are no more than estimates but they cover the general principles involved in screening work.

When this system is in operation an improved strain may not show up prominently at first. On being passed forward to the next screening stages, the groups of isolates descended from it will tend to stand out as better than the others, thus bringing a considerable weight of evidence to bear when the choice is made.

When selection has continued for a time a selection of the best strains can be given an extended test to clear up the position. An advantage of the multi-stream mutation system is that cultures from other programmes can be fitted in, or several types of mutagen can be used in a single step.

AUTOMATION IN SCREENING

Automation of various types has been applied to screening work for many years. The author has reviewed the situation fairly recently, (Calam, 1969) and this present section is a shortened version of the original paper.

Whether automation is introduced or not is a matter of economics, depending on labour costs, the elaboration of the assay methods required and the number of samples to be dealt with. Fully automatic apparatus is probably more expensive in capital but cheaper if in regular use. When, however, the automatic system is not needed every day or where it would be necessary to have a number of systems to do different types of analysis, they may not appear as attractive as would be thought at first. In many cases the answer to the question "what advantage would be gained if automation was used more extensively?" is probably "very little". In many cases manual systems will handle the number of samples required and the only advantage would be the possibility of a quicker turnover. In some situations, however, rather more expensive automated methods may be justified when they save labour and make programmes much easier to run.

In the writer's view, one possibility introduced by automation is an improved flow of information and statistical analysis, thus enabling a faster and more reliable assessment of the position. This should allow better direction of the programme with an improvement in results over the best that can be done by intuition alone.

A single mutation programme requires a large number of petri dishes, test tubes for slope cultures and flasks for screening procedures. If several programmes are in operation at the same time, the weekly turnover can be in the region of several thousands of flasks and other pieces of glassware including pipettes and other minor items. The washing up and handling of these materials can advantageously be mechanised, washing up machines capable of dealing with 3 - 15 thousand pieces per day (depending on shape) are available which reduce the problem to reasonable proportions. The handling of the material between the central washing up laboratory and the mutation laboratory can be rationalised to a considerable degree so as to reduce to a minimum the expenditure of the efforts of highly trained assistants on this type of work, as well as reducing delays. Washing up is not popular and there is every reason to introduce mechanisation to the widest extent. Glass flasks, test tubes and pipettes are used. Petri dishes are usually made of plastic and can be discarded, showing an economy on labour in handling. Flasks and pipettes still appear to be more economical when made of glass and used repeatedly.

In the writer's view successful screening work depends on accurate mechanical operations of the laboratory processes involved, such as plating, isolation and testing of mutants, accompanied by a critical and thorough scrutiny of both results and technique. These can hardly be replaced by automation as we know it as present. It is a feature of automation that it carries out literally the instructions put into it and makes those observations which it is instructed to make, but it is unable to exert the sort of scientific criticism which is necessary in screening work if success is to be obtained. It is felt that progress

is usually made because of the careful observation by the graduate in charge. His critical faoilities enable him to deteot false leads and spurious results which can so easily be misleading. It is therefore felt at present that this step cannot be automated.

The analytical stage is seen as involving two or three steps. In the first place there is the preparation of samples for analysis which may involve a considerable amount of work. The second step is the actual analyses of the solutions produced. This may involve a simple colorimetric analysis but in many cases chromatography may be required, often by means of a gas chromatogram to avoid the effeot of interfering substances. In screening work relatively crude analyses can often be used, but it is necessary to be extremely critical, especially as mutants may show differing proportions of products from the parental strain. This can be misleading when different proportions give different responses in the assay method.

The greatest developments in automation has been seen in connection with the assay of the samples from the test flasks. About 10 years ago Technicon introduced the Autoanalyser which was immediately adopted for many routine antibiotic assays, for instance for the assay of penicillin. Since then considerable technical advances have been made and some interesting apparatus is now available. Typical of the units which were seen at Technicon's Autumn Congresses in 1967, was a continuously operating filter which would enable samples to be put into the analytical system without previous filteration, thus saving a considerable amount of labour. Apparatus associated with this was a Beckmann ultra-violet absorption spectrophotometer with automatic readout which would present the results on a typewritten slip, making

them immediately available. The automatic punching of data on tape for computer calculation is being introduced. Large sample trays are being provided so that large numbers of samples can be put into the machine for automatic treatment, this being important when it is desired to operate for apparatus continuously outside the ordinary working day.

One of the most significant developments by the Technicon Company has been the introduction of computer control of the so called automatic laboratory. This is intended for the hospital service where a very large number of laborious and uninteresting analyses have to be carried out with great accuracy and reliability. In addition, in the hospital the collection and presentation of results is extremely laborious and the computerised system has great value in this respect. Much of the apparatus displayed was foreshadowed in papers given at a Congress in New York (cf. Ferrari, 1965). An automated laboratory would be capable of handling thousands of assays a day, but it is rather unlikely that such large numbers would be necessary for the ordinary type of screening programme. Attention is therefore drawn to the considerable number of types of apparatus now available which one might call semi-automated, for instance the old fashioned flow-through cell for a spectrophotometer which enables the rapid turnover of samples to be obtained.

A number of automatic metering and dilution units are now available which would enable 200 samples to be analysed per day using quite a simple colorimeter, and this would be sufficient for the ordinary type of screening programme. In contrast to this, in a number of cases gas chromatography is necessary and here the time taken for the sample to pass through the chromatogram limits the number which can be handled by a single instrument in a day and therefore reduces the effectiveness

of automation. Automatic integrators are now being used to measure the peaks and record the results. The automatic measurement of spots in paper chromatograms is also being developed, but involves greater difficulties for various technical reasons. An interesting apparatus described at the New York meeting (cf Ferrari 1965), was the Kline and French automatic system for handling microbiological diffusion assays. The samples were applied to cups on a large assay-plate by automatic pippettes, and the zones were ultimately read by a zone reading system which recorded the zone sizes as a digital print-out and on tape for computer calculation. A turbidimetric assay system was also described in which the results were punched on to I.B.M. cards. A system at present being developed involved the use of calipers giving an electronic readout, connected to a desk computer which averages circle sizes and types the results directly. The introduction of computer programmes which can accept the data directly from the punched tapes produced by automatic analyses and treat it without the necessity for it to be previously listed and classified by the screeners, and the incorporation of existing material should provide a full survey of the position. A rapid analysis of the results should be possible, which would give immediate guidance to the next stage of the screening work.

At the present time the difficulty of statistical analysis and the time taken prevents this approach being practically as useful as might be hoped. The application of computers should enable more information to be obtained from limited amounts of data and make it easier to select the best dose or type of mutagen and to select the best populations of irradiated cultures for screening. In this way screening could be concentrated on those lines which appear most profitable and the wastage of effort on unsuitable lines avoided.

The author's assessment of the position is that very considerable possibilities are available for development at the present time, but so far they remain possibilities. The introduction of new types of data reading, the collection of data and its study will require an intense effort on the practical side. A considerable degree of imagination will also be needed if the best use is to be made of these possibilities. As far as one can judge, the automated laboratory systems have not yet been applied purely for antibiotic screening. It is unlikely that the expense and trouble involved would produce any immediate effect which could be assessed economically. Screeners would have to spend considerable time gaining experience in the new methods of results presentation and it would be some time before any progress could be attributed to the improved system. It would be necessary to deal with all these aspects before fully automated systems can be introduced into screening procedures.

Another possible difficulty lies in the relative rigidity of an automated system, in which expensive apparatus and other facilities can be tied up in a procedure which can easily become redundant if circumstances or working methods change.

MUTAGENS AND MUTATION

The earliest mutagen to be used appears to have been X-rays. The early work on penicillin by Backus and Stauffer (1955), already referred to, describes the results obtained with P.chrysogenum, which was first mutated with X-rays, then ultra-violet light or nitrogen mustard. A series of selections was also made without the mutation step. The mutagens used represent the agents then being used, while the work without mutation arose from a feeling at that time that mutation might not have any effect on micro-organisms. Nitrogen mustard was one of the earliest cytotoxic agents and a precusor of extensive work on alkylating antitumour agents, many hundreds of which have been prepared and tested. Later a number of other chemicals were found to have mutagenic action, such as 8-ethoxy-caffein, 6-mercapto-purine riboside and others, and certain antibiotics. Most of the mutation work on antibiotic producing organisms was carried out in industrial laboratories and has not been published, but numerous papers have appeared from the All-Union Research Institute for Antibiotics and the Kurchatov Institute in Moscow (Prof. S. I. Alikhanian). Other work has been on plant cells and bacteria, and a book by Kihlman (1966) is of value here, while Prof. Charlotte Auerbach (e.g. 1966) has published much interesting work. The present review is largely based on these sources. Mutation work is abstracted in the journal "Plant Breeding Abstracts".

Some of the commonly used mutagens are:-

X-rays
Ultra-violet rays
Nitrogen mustard
Ethylene-imine
Ethyl methane sulphonate
Diethyl sulphate
8-Ethoxy caffein
Nitroso-methyl urea
1-Methyl-3-nitro-1 nitroso-guanidine
β-Propiolactone
Fast or thermal neutrons

Many other substances have also been brought into use. As has been already mentioned the mutagens are usually applied to the cells in pH 7 buffer or water at dilutions of ca. 1:1000, or 0.01 Molar for a few minutes up to an hour or more. Radiations are applied to suspensions or to sporulating colonies. Neutrons are obtained from an atomic pile. Details of concentrations, times of exposure etc. can be found in the literature but usually have to be adjusted to suit the organism being treated. Usually a kill of 99% or more is aimed at, though some of the chemicals are relatively non-toxic and low kills are used. Sometimes mutagens are used in pairs, such as treatment with ethylene imine followed by ultra-violet light (Alikhanian, 1962).

It was found that when X-rays were used there was an immediate effect on cells (chromosome breakage) and that mutagenic action was greatly stimulated by the presence of oxygen or air. The alkylating agents produced a delayed effect on the cells and were unaffected by the presence of oxygen. The abberations produced by chemicals tended to be localised, while with X-rays damage was distributed at random both within and between the chromosomes. It was later found that other chemicals, such as 8-ethoxy caffein resembled X-rays more closely; it was affected by oxygen and gave a non-delayed effect, but the effects were much more localised. It is typical of chemical mutagens that their effects are on specific loci rather than at random, as with radiations.

A considerable number of different types of action have been found to occur with chemicals, some being affected by anoxia, some not. Most give a delayed effect, others such as 8-ethoxy caffein and streptonigrin, not. The effect on chromosomes may involve stickiness sub-chromatid exchanges, chromosome exchanges or fragmentation. Others inhibit deoxyribonucleotide biosynthesis or remove DNA-bound metals; individual mutagens act during different stages of DNA synthesis.

While these facts are of little help in the choice of mutagens they indicate the wide range of effects that may be produced.

Auerbach (1966) has summarised the way in which the genetic background of the cells, the effect of preliminary storage or nutritional regime or the nature of the mutagen can affect the type of mutation produced. Thus with Neurospora a type of reversion increased many times when the dose of diepoxybutane was increased (decreasing survival from 49 to 12%) but the response/dose curve differed widely with other mutagenic agents. Alikhanian (1962) shows examples of how treatments giving morphological mutants did not produce changes in productivity. With Fusarium moniliforme cultures producing gibberellin, Erohina (1968) has shown that while nitroso-ethylurea and ethyl methane sulphonate gave more total and minus mutants than did ethyleneimine, alone or with ultra-violet light, the chemicals gave less plus mutants.

Zhdanov and Alikhanian (1964) described a long series of experiments on the mutagenic effect of fast neutrons, diethyl sulphate and combinations of diethyl sulphate and ultra violet light. Fast neutrons are considered to be most effective, especially when interspersed in a succession of mutations with the other agents (see page 36).

Sufficient has been written to show that the effects of mutagens are extremely varied and that is impossible to give any precise guidance in their choice. For what it is worth the writer would suggest ultra violet light or better ethyleneimine followed by ultra violet light as a powerful mutagen. Several chemicals are effective, such as 8-ethoxy caffein, 1-methyl-3-nitro-1 nitrosoguanidine, ethyl methane sulphonate, diethylsulphate and others. X-rays seem to be relatively little used at present, but an occasional treatment with fast or thermal neutrons can be helpful.

It is generally felt that a series of mutations using different mutagens is preferable to continuous treatment with the same one. Chemical mutagens, as expected, often seem to give large numbers of mutants of a single type.

The importance of the parental strain on the mutational effects produced has been stressed by a number of workers, e.g. Smith (1961). Alikhanian's group (Alikhanian, 1962) has also found, as Auerback (1966) also reports, that in a mutation programme some mutants respond better than others. For this reason mutational lines should be multiple rather than single. The occurrance of "great" mutations (Alikhanian 1962) has also been referred to, i.e. the occurrence of a mutation in a series which has no effect on yield but makes the culture able to respond to further treatments.

At the Moscow meeting in 1965 Dermikhovski suggested that after mutation the spore suspension should be heated to kill off the faster growing survivors. The others, which he referred to as "drowsy" spores, especially when treated with a further dose of mutagen gave improved yields. This is one of the methods which have been described to enhance mutation effects. In some cases it has been found helpful to make the culture resistant to the antibiotic it produces, or to another antibiotic before mutation. It is impossible to assess the possibility of the success of such methods except by experimental work. It is sometimes suggested that a culture might be given a series of mutations, interspersed with stages of re-growth, before selection is attempted. The writer feels that re-growth would tend to reinforce the population with the less desirable strains and that the procedure is not to be recommended.

While some cultures respond well to mutation, giving increases of yield of the order 5-10 times fairly quickly, others more move slowly.

Rapid increases in production were obtained with penicillin and the tetracyclines, but progress with erythromycin and the gibberellins seems to have been much slower, according to the Russian references cited.

A discussion of some of the problems which arise in mutation work will be given later.

5. MUTATION METHODS

Three typical experiments will be described, selected from the very many which are to be found in the literature.

Describing the production of mutants of Penicillium chrysogenum, Macdonald (1963a) writes as follows. "Ten ml. of a dispersed suspension of conidia (10^6 total conidia/ml.) were irradiated with a Hanovia lamp Type 11 with a 30 cm. tube. The suspension in distilled water was contained on a 9cm. diameter Petrie dish, with the lid removed, at a distance of 30cm. from the u.v. source and was mechanically rocked during treatment. The time of irradiation, usually 5-8 min., was adjusted so that the plating of the conidia on complete media showed a 1-5% survival".

In our laboratory, instead of rocking the dish agitation is provided by means of a magnetic stirrer. Bactericidal lamps are often used. The best distance and exposure are usually found by experience. The eyes must be protected by suitable dark glasses as u.v. rays can be dangerous.

The mutation of E. coli cells has been described by Adelberg, Mandel and Chen (1965) using N-methyl-N-nitro-N-nitrosoguanidine (M.N.N.G.). A suspension of cells in the logarthmic phase was prepared by growth at 37° with shaking. The cells were quickly filtered on a Millipore-filter, pore size 0.45µ. After washing on the filter with 5-10ml of Tris-maleic buffer (M/20), the cells were then resuspended by placing the membrane in the original volume of buffer at pH 6 in a 125ml Erlenmeger flask and agitated with the Junior Vortex Mixer. The membrane was removed and M.N.N.G. added directly to the suspension to give the desired concentration. The final concentration of cells was $5\text{-}8 \times 10^8$/ml. After incubation a 1ml. sample was filtered on a Millipore filter, washed with 5ml. of cold mimimal medium and resuspended in 10ml of minimal medium as described above. The suspension was then diluted and plated. Very high yields of auxotrophs were obtained with 50% survival of the cells. This method was

developed so as to obtain highly reproducible results. A considerable degree of simplification is possible for general mutation work, the principles remaining the same.

An example of an elaborate mutation programme is given in Figure 1 (Zhdanov and Alikhanian, 1964). It records work starting from Actinomyces erythreus ATCC 8594, which gave a four fold increase in erythromycin production. The programme was enlarged to allow a variety of mutagens to be compared, the authors being particularly interested in the effect of fast neutrons. Spore suspensions were placed in a Plexiglas container in a lead casing, and lowered into a reactor where the neutron flux reached 140-190 rad/sec, γ-rays being at a much lower level. The temperature in the reactor was 38.5° but the exposures (up to 30,000 rad) were sufficiently short to allow the sample to be withdrawn before the temperature rise became excessive.

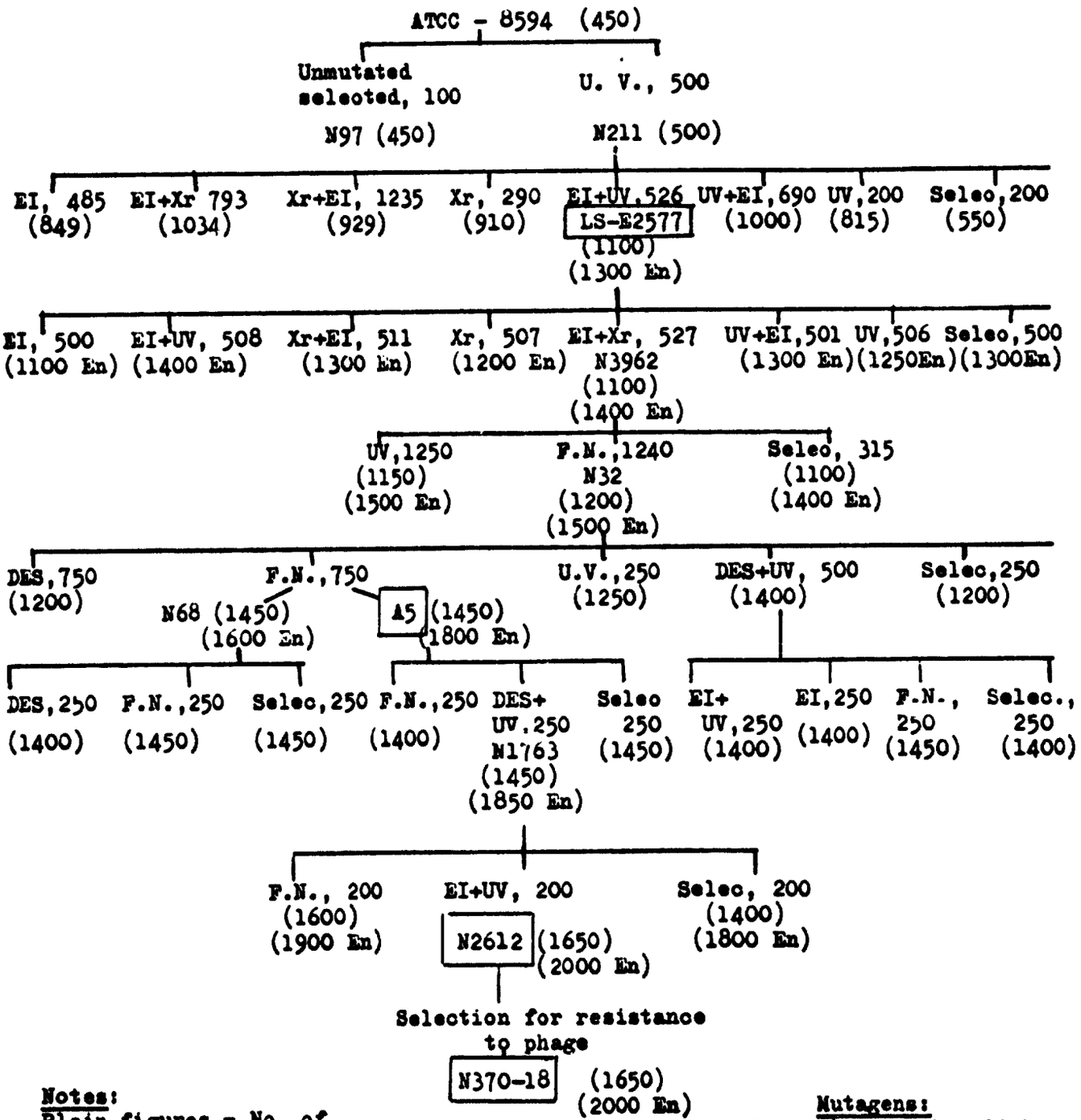
The authors draw attention to the jumps in titre which occurred when mutants such as LS-E 2577 and A5 were produced, interspersed with apparently unsuccessful mutation steps, and later the production of N2612. A final step to give phage resistance was also involved. The apparently random occurrence of these mutations will be observed, but the authors draw attention to the value of the fast neutron steps, the valuable effect of which is emphasised by more detailed studies.

The programme involved more mutation and screening work than would be necessary for an industrial programme. This was done to allow a number of comparisons to be made. For details of these it is necessary to refer to the original paper.

A description of the laboratory facilities required and the daily work pattern of a typical programme have been given above (page 13).

Fig. 1. Mutation scheme for erythromycin producing strains.

Zhdanov and Alikhanian, 1964.



Notes:
Plain figures = No. of variants tested.
In brackets, units of erythromycin,
En = enriched medium.

Mutagens:
UV = ultra violet light
EI = ethyleneimine
Xr = X-rays
Seleo = selection unmutate
F.N. = fast neutrons
DES = diethyl sulphate
\+ = two mutagens used in succession

6. HYBRIDISATION METHODS.

Diploidisation.

Macdonald et al. (1963b,c,) have described the method used for crossing fungal strains, in their work with Penicillium chrygrogenum. For this purpose biochemical deficients were obtained from strains giving different yields of penicillin and also of different colours (white, brown etc.). The media used were minimal medium (M.M) and complete medium (CM). The former consisted of Czapek-Dox medium (glucose + nitrate and salts), the latter a sucrose + corn-steep liquor medium with supplements of growth factors.

One ml. of saline was pipetted onto 10ml. of C.M. agar in a McCartney bottle (cylindical, screw cap, capacity 30ml.). To this was added a mixture of the spores of the two strains. After 4-5 days at 25° the felt was removed and halved. The halves were either (a) quartered, the pieces being plated on MM agar, or (b) teased out, mixed with molten MM agar and plated. Heterokaryotic colonies grew and hyphae, taken from the extreme edges, were subcultured on MM and allowed to sporulate. When spores from the heterokaryons were plated on CM, both parental types grew, easily identified by their spore colour.

Diploids were obtained by plating large numbers of heterokaryotic spores on MM. E.g. in one case 10^7 spores gave 500 diploid colonies recognisable by their green spores. Diploids were also isolated from green sectors in heterokaryotic colonies. The diploids also usually showed increased spore size.

Hybridisation of actinomycetes has been described by Sermonti and Spada-Sermonti (1956). Minimal and Complete Media were similar to those described above. When spores of two biochemically deficient strains of Streptomyces coelicolor were plated together on mixtures of MM + CM, often 1:1, and incubated for 4 days at 30°, tufts of exuberaut growth occurred at the junction of the colonies. These sectors sporulated and the spores when plated

on MM proved to be recombinants from the parental strains. Recombinant spores were also obtained when thick spore suspensions of two kinds were spread over the surface of mixed MM + CM agar medium in slopes. In the case of the actinomycetes fusion of nuclei and recombination occurs within the hyphae and diploid spores are not obtained.

In later work Mindlin et al. (1961) crossed pairs of biochemical deficients obtained from Russian and Hungarian strains of S. rimosus, producing oxytetracycline. Results were similar to those obtained by the Sermontis. Later (Vladimirov & Mindlin, 1967) further crosses were made between deficients from these two strains and a considerable account of information obtained about the possibilities of hybridisation in this field.

Methods for the hybridisation of yeasts have been reviewed by Powell (1966). Yeast cells are normally diploid and multiply by budding. Hybridisation occurs via the haploid spores produced naturally within the cells (in asci) under suitable conditions. Spores are usually obtained by growth on solid media. Gypsum blocks have been used, though at present agar slopes containing acetate media are more commonly employed.

A variety of methods have been used for the recovery of the spores. This was originally done by micromanipulation. Snail enzyme is frequently used to dissolve the ascal walls and release the spores. In recent years a number of methods have been devised for mass separation of the haploid spores. It is possible by careful heating to destroy the growing diploid mother cells, leaving the haploids which can be recovered as haploid colonies on plating.

Mating can be brought about by pairing spores, using a microscope. Pairing by haploid cultures is also possible. Industrially mass hybridisations are required and for this it is usual to grow the haploids together, then subculture several times under conditions giving vigorous growth. The diploids outgrow the haploids and can be isolated and purified in the usual way, being

recognisable by the larger size of the cells. Increased mating can be obtained if the mixed haploid culture is shaken during growth. Some types of industrial yeasts are difficult to cross but the newer methods are facilitating the use of hybrids for a variety of purposes.

Recombination

In general the diploids show behaviour intermediate between that of the parents, and to obtain an advantage it is necessary for recombination to occur, i.e.a rearrangement of the double nucleus, preferably a breakdown into two haploid nuclei each containing components of one or other of the parental nuclei. In early work with Aspergillus nidulans this occurred spontaneously. With P.chrysogenum the diploids often proved rather stable and a mutation treatment is necessary. This is of course undesirable as effects due to recombination are mixed with effects due to mutation. With actinomycetes and bacteria the diploid stage is short lived and haploid cells are produced almost immediately.

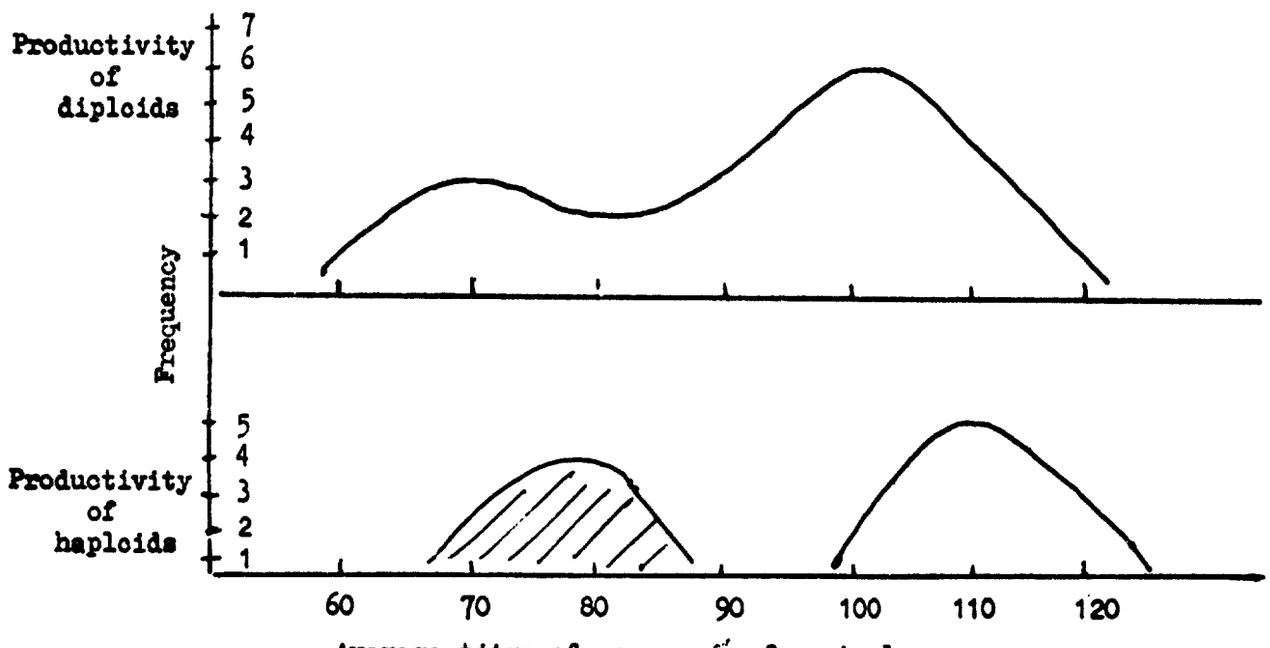


Fig 2. Distributions of productivities of segregants of diploid No. XXXI (49-133 x 50-1247). Shaded areas requires nicotinamide (50-1247 mutants), unshaded area (haploids) require pyridoxine (49-133 mutant). From Sermonti (1957).

A considereble amount of work has been done with fungi, and a number of references to this are given by Calam (1964). In the case of Penicillium a considerable number of the recombinants produced have turned out to be diploid. Commenting on the situation in 1961 Sermonti thought that improvements in yield due to successive mutations cannot be considered as a purely additive effect, but rather the building up of a complex of a mutant alleles, which gives adverse results if altered. The simple idea of combining them in a single strain by crossing is inadequate. The general effect obtained is illustrated in Figure 2, based on Sermonti's work. The crossing of two strains followed by recombination leads mostly to the production of diploid recombinants having a range of productivity, the best producers possibly rather better than the best original parent, plus a small number of haploid or diploid segregants marked with the deficiencies of one or other parent, with corresponding titres. The fact that productivity is often reduced during the formation of the biochemically deficient parents, implies that these strains have their nuclear apparatus seriously disturbed in the process, and that on crossing this will have a deleterious effect rather than be merely cancelled out when the other haploid nucleus is added.

Macdonald has published several papers indicating the complexity of the situation. In his 1966 paper he describes the crossing of biochemical deficients from the same parent. These showed differences in productivity, possibly due to the instability of the nucleus of one of the deficients.

Daglish and Calam (1967) have described difficulty in obtaining crosses with Penicillium patulum, and other work in our laboratory, with a Fusarium culture, has also shown unexpected effects. It would appear the many possibilities arise in hybridisation and that not all of them have as yet been adequately described.

Work with actinomycetes has already been mentioned above.

7. THEORETICAL PROBLEMS IN HYBRIDISATION AND MUTATION.

Fermentation processes are of course under genetical control, and the object of hybridisation and mutation is to change the genetic balance and thus increase production.

Biochemically a fermentation involves two main stages. These are: (1) a growth stage, with high metabolic activity and protein synthesis (during this stage cells are formed and the product-forming enzyme system is laid down), (2) a maintenance and biosynthetic stage when the quantity of cells remains about the same and product formation occurs; this stage ends when the system breaks down. The genetic effects involved in the two stages differ. In the first stage the important factor is maximal formation of enzyme. In the second stage the requirements are (a) successful adaptation to the new conditions, (b) a reasonably stable system and (c) a favourable balance between the reactions which maintain the cells and which form the product. In both stages, more in the second, energy formation (coupled to biosynthesis) uses up a large amount of material. Raw materials are likely to be used up in the proportions 60-75% for energy formation (via A.T.P.), 10-25% for cells, remainder for formation of main product and byproducts.

When a wild strain is isolated, giving a small yield of product, the amount of enzyme is probably the limiting factor in product formation. Hybridisation and mutation will increase the amount of enzyme present, thus increasing yield. When the product level increases, the need to balance the distribution of raw material between the different usages becomes much more important. Cell growth and maintenance may have to be reduced to make way for product formation. Product formation may also require a great deal of A.T.P., which must also be taken into account.

The production of an improved strain is thus a complex problem involving a considerable number of genes. It will be realised that all the processes mentioned, e.g. growth, A.T.P. formation, biosynthesis, are multistage and involve many genes. All these processes must work in harmony and must be changed harmoniously. The type of change required will also differ as the productivity

of the strain increases. At first the formation of a single enzyme is critical, later the whole metabolic pattern is involved.

This brief introduction is given to show that not only is the genetic problem in itself a complex one, but that it is controlling a biochemical mechanism which is in itself complicated and difficult to describe in detail.

Since most of the work done so far on hybridisation in micro-organisms has been devoted to genetical research, it is natural that very little effort has been made to consider it from the point of view of breeding, as has been done in the case of plants and animals. To obtain some ideas of the difficulties involved it is necessary to turn to writings on plant breeding (e.g. Allard (1960), Hutchinson (1959). The discussion which follows is mainly based on Allard's book "The Principles of Plant Breeding" (loc. cit.).

The transfer of properties from one plant to another by breeding involves the transfer of genes from one nucleus to the other. This occurs in the course of ordinary cross-breeding. During the cross the nuclei of the two plants fuse and then recombine giving rise to new nuclei, some of which resemble the parents and some of which are mixtures. In the simple case where two strains differ in two genes only ($\frac{a}{a}\ \frac{B}{B}$ and $\frac{A}{A}\ \frac{b}{b}$), the double homozygous recessive class $\frac{a}{a}\ \frac{b}{b}$ constitutes 1 : 16 of the F.2. progeny provided the two genes recombine freely. When three gene differences are segregating the frequency of the triple homozygous recessive is 1 :64. The general formula for the frequency of the class, homozygous for n freely recombining recessive (or dominants) in the F.2., is 1 in 4^n. Thus in plants the proportion of descendants in which there will have been transfered 4 and 10 genes are 1 : 256 and 1 : 1,084,576.

In the case of the parasexual cycle, using haploidisation, recombination is obtained only between and not within linkage groups. From a diploid of genotype $\frac{a}{A}\ \frac{B}{b}$ haploidisation yields equal numbers of the four possible haploids, aB, AB, ab, Ab. The general formula for the frequency of any genotypic class is $1 : 2^n$ where n unlinked genes are segregating. This gives ratios of 1 : 16 for segregation with 4 genes and 1,024 for 10 genes. Thus when the desired property depends on the transfer of a group of genes, with the parasexual cycles the frequency is higher but the possible types of combination are more limited. Screening still remains a major operation.

Although in some important cases such as the transfer of resistance to a parasite, or of fruit colour, a single gene transfer may be involved, breeding is still complex because in fact the resistant parent may differ considerably in other genetically determined respects from the one it is desired to improve. Thus the non-resistant plant may be of the ideal type with say short growth, large leaves, numerous branches with a well-developed root system and early fruiting. The best resistant strain available could be tall with small leaves and other undesirable properties. The resistant hybrids would thus include very few plants which had the original ideal properties plus resistance, the majority would be quite unsuitable and would have to be discarded.

Two further important factors in breeding would be expected to intervene at this point. These are (a) response to environment, and (b) pleiotrophy. The first is obvious enough; the behaviour of the plant depends on its genetic constitution (genotype) and the response of that constitution to its environment. Thus although the new hybrid may have the desired new genetic constitution it may be unsatisfactory if it does not respond as well to its environment as the old one did.

This factor of environmental response is heavily stressed by writers on plant breeding. The other factor is pleiotrophy. This refers to the side effects produced by a gene change. It occurs in plants and is very evident in moulds (cf. Macdonald et al., 1963c). The introduction of a growth requirement in P.chrysogenum, due to the loss of a single gene, often produced a more or less severe drop in productivity which is not corrected by addition of the factor which the organisms now required for growth. The cause of pleiotrophy is not well understood but reflects the fact that genetic effects are highly complex and individual genes considerably affect others, even others situated on other chromosomes.

The outcome is that in the hybridisation referred to above the best plant that could be obtained would probably be resistant but in other features would be only fairly similar to the ideal parent instead of identical. A further cross between the new form and the ideal parent could be carried out in the hope of improving the plant while maintaining resistance. This re-crossing could be repeated several times. The breeder, however, might be able to get no further than to obtain a resistant plant which was equal to the ideal only in certain ways and to a certain degree. A breeding programme such as the one described could be relatively simple. After crossing the two plants, large numbers (tens of thousands) of the hybrid seeds would be planted and the young plants sprayed with the spores of the organism against which resistance was required. After further growth the survivors would be re-sprayed. On complete growth the survivors would be inspected for obvious growth characteristics and the few most-promising plants retained as parents for further trials with suitable replication.

Two other important factors which must be recognised as important in plant breeding are (a) preliminary selection of parents before crossing and (b) the availability of highly diverse populations as a source of materials for breeding.

Preliminary selection of parents is regarded as the point at which major improvements are made (Hutchinson, 1959). Extensive selections are normally made for this purpose. At the same time it is important to have available highly diverse collections of plants, each possessing important cultural features so that breeding can be directed in the desired manner. Large stocks of varieties are maintained and searches are made for new wild forms.

A difficulty which develops as breeding proceeds, which is referred to by Allard, is an increase in closeness of linkage. In effect the improved strains become so highly specialised that when crossed most of the new forms are inferior and the only ones which stand a chance of selection, or even of survival, are those which closely resemble one or the other of the parents. This phenomenon is referred to as "parental genome segregation" and it can put a severe brake on progress. Closeness of linkage can imply that the genes involved in making the strain so highly productive are situated so close together on one of the chromosomes that they can only move together

during crossing, or that althoughtthe genes are separated, good growth and performance can only be obtained when they all move together. The loss of a single member of the group of genes is sufficient to neutralise the beneficial effect of the whole group. It is recognised that the most difficult tasks are those involving quantitative changes in yield, not only because strain improvements will involve the transfer of several genes but also because such changes are difficult to detect (Allard, loc.cit.). Another factor of importance is the careful choice of a well-defined target so that the programme can be clearly laid down.

The use of mutation instead of genetic recombination, does not appear to change the principles involved in strain improvement. After the preliminary selection of the best cultures from the original source, diversification is produced by mutation instead of hybridisation. The cycle of selection and mutation is then repeated.

Since with mutation strain improvement occurs by chance, the possibility of directing strain improvement in some predetermined direction is not available, though screening can be designed to favour the selection of strains, of a particular type. Changes in titre are accompanied by other effects, possibly due to pliotrophy. These changes particularly affect growth and sporulation which tend to decline as mutation proceeds. As has been indicated above reduction in growth rate may be needed to allow product formation to compete more favourably for raw materials.

The advantage claimed for genetical breeding is that it introduces the possibility of producing new cultures which could not be obtained by mutation and allows some direction to be given to the course of the work. It should be possible to select the parental strains in such a way that progeny will be obtained which have desirable qualities which can be chosen beforehand. An obvious case with P.chrysogenum would be improved titre combined with improved sporulation, by crossing a highly productive strain with a high sporer. Very little information is available which can be used to apply genetical theory to strain improvement. Thus in a recent work on microbial genetics (Pinchem and Day, 1963) the parasexual process is dealt with in one and a half pages and there is no discussion on strain improvement. It is made clear that fungi are being used to help in the study of genetics and not vice versa.

The view has been expressed that strain improvement in P.chrysogenum should be based on a thorough knowledge of the genetics of the organism, including chromosome mapping etc. Such a programme would take at least several years, and although Sermonti (1961) and Macdonald et al. (1963, 1966 etc.) have done a large amount of work in this field, it cannot be said that the back of this problem has been broken. One cannot but doubt whether this method is really viable, Allard indeed (loc.cit p.67) has given reasons why "plant breeding has not become the exercise in assembling favourable genes that was

optimistically predicted by early genetics" and gene-by-gene analyses between parents have not been carried out. It would seems that any work done in an industrial laboratory will have to proceed on empirical lines and the main purpose of theoretical genetics will be to suggest ideas and give possible explanations for phenomena observed in the course of the work.

It is recognised in breeding (cf. Allard loc.cit, p.25-28) that it is desirable to have available large stocks of strains suitable for crossing, and large sums are paid for plants and animals for breeding. In the case of penicillin (other antibiotic producing strains are analogous) since practically all the high yielding strains used thoughout the world come from the same source. it would be difficult to obtain new high yielding cultures from outside sources. An alternative would be to develop new mutants for crossing, making a fresh start. In the case of penicillin this would take a long time and is not at present regarded as a practical proposition, though with other moulds the situation may be different. Another approach would be to cross high yielding strains with strains from other lines and then back cross the best decendants.

The barrier that exists in the way of using bybridisation to increase production has been discussed by Mindlin and Vladimirov (1967) who crossed oxytetracycline producing mutants of Russian and Hungarian origin. Prototrophs having a wide range of productivity were obtained. Although some of the recombinants equalled the original, none were better than the best of the parents. It was considered that this was due to the original strains being genetically ...ed. Macdonald (1968) has discussed the problem of parental genome seg-...on in some detail. He considers that when using mutagens to bring about ...gation from diploids, compounds giving minimal chromosomal damage should b... ...ed. It is also desirable to cross olosely related strains if free recombi...ion is to be obtained.

His work with nitrogen mustard mutants showed that parental genome segregation is not due to preferential selection of parental segregants, but to differences in chromosomal homology between parents.

An interesting mutational device has been used by Russian workers in connexion with lysine-producing bacteria. In order to obtain lysine a glutamic acid producing strain is blocked by mutation so that it requires homo-serine when the biosynthetic route to glutamic acid is disturbed resulting in the formation of lysine. Unfortunately the blocked strains are not completely stable. After a time during the lysine fermentation revertants begin to grow in the medium so that lysine production slows down and the product is mixed with other amino acids. Various methods have been used to deal with this, for instance Legchilina and Shishkina (1966) found that if the blocked lysine producing strain is given a second requirement (e.g. for methionine) reversion is greatly slowed down and lysine production increased. It was also found that many of the best lysine producers are dwarf strains with damaged cytochrome metabolism. These strains are less sensitive to deficiencies in aeration.

8. TECHNICAL PROBLEMS IN STRAIN IMPROVEMENT.

In this section will be considered some of the more practical or tactical problems.

On the whole, the techniques involved in strain improvement are not particularly difficult in themselves. At the same time it may prove difficult to get results. In the first place it may take some time before satisfactory mutations are achieved, quite often mutagenic action is too strong or too weak with the result that the isolation plates are completely overcrowded or completely blank. This can be overcome by experience. Diluting and plating can be very laborious but as experience grows methods can be found to reduce the work involved. Testing of cultures involves a variety of practical problems, but again a sound routine can be established.

Assuming adequate routines have been set up, several difficulties may now arise. One group may be the appearance of inaccuracy in the selection test, as shown by a high degree of flask to flask variation or by variation in the results obtained from day to day. Often the problem is related to differing degrees of growth in the flasks. Alternatively assay methods, especially in the early stages of a programme and also when biological assay are involved, may show changes in value from day to day. This can be a great nuisance in assessing and averaging results. With experience and a degree of experimentation it is usually possible to reduce day-to-day variation to reasonable limits. The more assay errors can be reduced the better, as they make strain improvement work much harder and discouraging. To decide whether to push on with the mutation programme or to stop and try to improve techniques, can be a quite difficult managerial problem.

One of the most difficult problems that is continuously experienced is the failure of mutants of the desired type to appear. Sometimes morphological mutants appear and this is at least evidence that mutation is occurring. Studies of distributions of activity of populations of the original and mutated cultures may differ and these also indicate that mutations are occurring. Quite often the problem arises because the worker is not able at first to recognise the occurrence of improved strains among the isolates. This may be because the test procedure gives misleading results, especially at first when the worker is least experienced. A considerable degree of optimism and an ability to seize a chance are needed. Once again this is a position where experience is helpful. As a rule, after a few month's work many of the difficulties experienced at first seem to pass away.

One serious obstacle to progress is the initial absence of an improved strain which makes it impossible to test the screening procedure. There is no doubt that the isolation of an improved strain is a big step forward and makes a serious contribution to progress. While waiting for this to arise the selection procedure can be checked by using such naturally isolated strains as may be possible.

It is well worth while when starting strain improvement work to obtain more than one initial culture. It is often found that one of them will be distinctly better in yield and other characteristics and therefore makes a good starting point. On the other hand strains differ in their ability to mutate and if progress does not seem to occur an alternative strain may prove a better starting point.

As screening continues the assay of the mutants may prove troublesome. This can arise in a number of ways. When biological assays are used a considerable degree of variation is possible until complete standardisation is achieved. Methods of bio-assay have become more or less routine and conditions are far different from the early days of antibiotic work, not only as regards accuracy but also as regards convenience. In screening it is

a matter of balancing the effort available against maximum number of samples. When sales products are being evaluated it is necessary to use large numbers of inhibition zones in order to reach a specified degree of accuracy. In screening these are cut to a minimum with a reduction in the degree of accuracy that may not be fully appreciated. In fact the result of a single flask test in the first screen, with a minimum level of assay, can easily be misunderstood. Instead of appreciating that the main purpose of the test is the elimination of the worst strains, attention is concentrated on the most impressive working results.

Chemical assays are usually preferred, wherever possible. Care must be taken to make sure that the method truly measures the substance in question and that interference is not taking place. When a new substance is detected it may easily become associated with a simple chemical test, e.g. the production of a colour with ferric chloride. It may be found that the most active cultures give the strongest colour with ferric chloride so that the reaction becomes the basis of an assay. Later it will be found that the colour is caused not by the active substance but by accompanying metabolic products. At a higher degree of refinement an ultra violet absorption band can be misleading, owing to interference. In any serious selection programme the desired product must be investigated as carefully as possible and any side effects and other misleading details cleared up. In some cases thin-layer chromatography or gas chromatography should be used to separate the product which can then be measured directly. New apparatus is constantly being produced to facilitate this type of analytical work.

Troubles of this kind occurred frequently in the early days of penicillin development. Thus the early British work was done with pentenyl-penicillin, the American with benzyl-penicillin. In America penicillin 'K' appeared at one time

as an impurity. These penicillins have different molecular weights and different relative activities towards the common test organisms (Staph.aureus and Bacillus subtilis). Fortunately the introduction of paper chromatography combined with collaborative experiments did much to sort out the situation that arose. In addition to this it became possible to isolate and crystallise the penicillin and weigh it to check the yield. Although the isolation and weighing procedure is laborious it is often a well worth while method by which to check discordant analyses.

When the product is soluble a representative sample is easily obtained. When it is insoluble the obtaining of a representative sample can be difficult and requires special care. The homogenisation of the whole contents of a culture flask may be advisable.

A problem that often causes trouble is the provision of a suitable standard for checking assay results. As a rule a pure crystalline preparation can be obtained. However this apparent purity is sometimes misleading. Crystals may easily contain water of crystallisation or retain solvent, or be mixtures. Again penicillin presented a problem, since the sodium salt proved capable of crystallising even when quite impure, and at one time there were considerable differences of opinion over the activity of the pure sodium salt. Especially at first, when only small quantities of material are available, laboratories in the same organisation may be in possession of supposedly identical standards which are in fact different. When irreconcilable results start to appear, careful attention to standards is often helpful. This should involve checks for purity and collaborative assay work.

As strain improvement work proceeds and new cultures start to appear it becomes necessary to decide whether the improvement achieved should be regarded as worthwhile or not. Judgement on this point depends on individual circumstances. As a rule it is intended to use the new strain for plant work.

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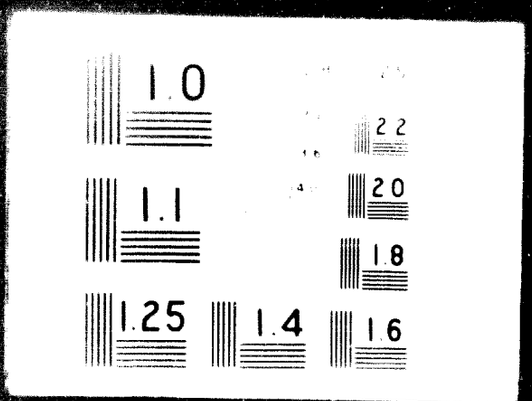

An increase of 10% may be difficult to demonstrate on the large scale, especially if only a few batches are available in the test programme. It may well be better to wait till an improvement of 20-30% or more is achieved, which will be more easily demonstrable.

This subject raises the question of the degree of testing that is required when a new strain has been isolated. The arrival of the new culture is usually a matter of considerable importance and brings equally great disappointment if it fails to justify itself. The testing programme should therefore involve the following steps:-

1. A repeat evaluation, fully controlled, using the method used in the final stages of screening. The writer prefers two or three small experiments (say six replicate flasks each) to a single large scale test which may by some unhappy chance give a misleading result. This test should confirm the original claim to have achieved an improvement.

2. Stirred culture trials should be carried out to check performance under conditions more closely resembling the plant. The size of the fermenters and the number of replicates is a matter of choice. This test will probably be carried out by another worker and it is mainly a matter of convincing him of the improvement. Care is needed at this point, lest too much time is spent or wasted, before the crucial plant trial is started. This is especially the case if the process is difficult to scale down and possibly less reliable than the plant itself. In some cases it may pay to proceed straight from shaken flasks to the full scale.

3. The culture should be plated on several media and a careful study made of variability and any other important points. The transfer of the culture from the development laboratory to the plant will soon bring questions about any differences from the original one and it is valuable if they can be answered clearly. Growth rate and growth form in surface and submerged culture are important.

It is also important to make sure that the culture is free from infection by tests under a variety of conditions of medium and temperature.

4. The culture should be put through the early stages of the plant seed-culture process. This will probably involve growing it in large shaken flasks and then transfering it at a high dilution to an inoculum culture. Often the levels of inoculation are lower in the plant than in the laboratory owing to the differing size of the apparatus. As a result of this, differing maturation times may occur. This can cause a good deal of trouble if it is not anticipated. Additionally the criteria used to judge maturity may have been upset and require re-adjustment. The mutator may regard the new strain as rather a blue-eyed boy, in the plant a more cynical attitude can prevail especially if the routine is upset.

5. When plant trials start it is advisible that someone who is familiar with the mutant takes a close interest in the initial trials. One recalls the alarm caused when the first sample of a new culture was taken from a large fermenter and turned out to be bright red instead of the expected white colour - expected by the process man that is - the mutator knew it would be red but had not bothered to say so.

Turning to other aspects, the importance of master cultures has already been mentioned. It has been recommended that at an early stage of a mutation programme a slope should be set aside as a master culture, and preferably put under oil so that it will keep fresh for at least a year. If the strain becomes important a new master should be set up, preferably by first plating, isolating and growing slopes of typical colonies, and then preserving them by a reliable method. These isolates should then be checked for productivity and agreement with the original culture.

Regular checks of master cultures, say at yearly intervals, are desirable though cultures
tho /usually last for several years or more. The methods used for culture preservation have already been indicated, see also "Methods in Microbiology".

Strain improvement work usually involves fairly long-term programming, at least for several weeks in advance. For this purpose a large diary is convenient, which not only lays down daily programmes, material requirements etc. but also includes details of holidays, maintenance shut-downs (if they are allowed to happen) and the like. Such a diary is not only useful for regulating progress, it also provides a convenient record of past work.

The compilation of records also requires care, as with a great deal of data to be handled, the copying of results to and fro may waste time and energy. The use of specially designed sheets in loose-leaf binders may be advantageous.

A long term mutation programme involves a great deal of routine work and can become monotonous and difficult especially if results are not forthcoming. This is an aspect that requires managerial attention. It may be desirable for a group to operate more than one line of mutation and selection so that there is a better chance of variety and success. Not all workers are happy in mutation work and this needs to be considered when workers are engaged.

A subject worthy of discussion at this point is the desirability or otherwise of purchasing improved strains. This is more a commercial rather than a research subject, on the other hand in general review of this kind some reference should be made to it.

Stress has already been laid on the importance, in the first stage of fermentation work, of obtaining an organism which gives a yield which puts the process on a practical and economical basis. If the only strain available is a wild one giving a low yield it may be expected that a year or two will elapse before it is sufficiently improved to give the venture a good start. Whether this can be achieved in such a short time might be difficult to decide reliably. The purchase of a strain would thus save say two years of work at a considerable expense and ensure an early start up. The saving of time could itself be a key factor. The purchase of a strain is usually accompanied by at least a minimum of information as to working methods, and this too can be invaluable. When buying a strain it is of course necessary to obtain some sort of guarantee as to its productivity under large scale conditions.

Against this it can be argued that the purchase of a strain deprives the firm of valuable experience that would be gained by developing its own process, while at the same time it might find itself commercially restricted in its markets and in other ways. On the question of experience, this is a matter that can be exaggerated as a rapid start under good conditions may be worth more to the development group. As to any restrictions, this is a commercial rather than a scientific matter. On the whole, there are many circumstances in which the purchase of a culture can be very advantageous.

9. LONG TERM PROBLEMS IN SELECTION WORK.

While mutation work usually proceeds in a straight forward manner at the start, after a sound routine method of working has been established, and can continue for a considerable time without too much trouble, a number of difficulties eventually begin to appear. These arise partly from practical difficulties but also from the reaction of the organisms to repeated mutation.

The main practical difficulties arise from slight changes in medium constituents, changes in practice and the like which arise over the years and which may produce slight differences in the appearance of reactions of the organisms. Along with this may be modifications to the media or in methods of working which are introduced to save time or give better performance or greater convenience. The mutation process itself may result in colour changes and changes in the growth pattern of the organism. As a result of all this it may be difficult to compare current results with what was happening in the past, and this is awkward when it is necessary to refer back. Most of these difficulties can be overcome by careful comparative experiments at intervals and careful examination of the cultures and accurate recording. In spite of this it may be hard to overcome all the difficulties involved especially if there are also changes in the staff. It is important for those operating the programme to understand their cultures intimately and carry out a good deal of work to make sure that changes in appearance or performance do not pass unnoticed. These problems most often arise when a culture is being used by several departments, one of which may feel that the culture has changed in some way, or when local sub-culturing is practised without adequate control.

The most serious problem arises when a culture ceases to respond to mutation. This is again illustrated by the work of Backus and Stauffer (1955) on penicillin.

The initial 1951 B25 strain P.chrysogenum was discovered in 1943 and by two mutation steps productivity was increased 3-4 times giving the strain Q176 in 1945. The programme was then continued with the following results (data taken from Backus Stauffer's paper):-

Year	Strain	Yield (u/ml) in shaken flasks
1945	Q176	640
1947	47-638	980
1949	49-133	2230
1951	51-20	2510
1953	53-414	2580

There is clearly a marked slowing off in the rate of advance, giving the impression that no further progress is possible. Quite similar results are seen in other published work.

This situation can easily arise in a mutation-selection programme after a few years, depending of course on the rate of working and the culture being used. It can give rise to considerable concern as to the advisibility of continuing with the selection programme. One difficulty is to judge whether the level of production has reached a theoretical maximum which prevents any further increase whatever. This would seem unlikely in the case of antibiotic production when conversion of raw materials is low. With a direct conversion such as that with citric acid or glutamic acid the theoretical limit could well be approached. It is known, however, that with glutamic acid an important limiting factor is diffusion of product through the cell walls.

An analogous problem arises when only a small advance in productivity can be obtained in spite of a good deal of work. It seems that some species of micro-organisms are particularly difficult to mutate.

As it turned out in the case of penicillin considerable further advances were made during the next few years, mainly in industrial laboratories, titres

of 10,000 u/ml being reported in the sixties. These high levels of production were mainly achieved in stirred fermenters using highly developed medium and stirring conditions, though high titres (6000-8000 u/ml) were obtainable in shaken flasks. It is clear that penicillin production by the mutants had only reached an apparent limit. The application of a wide range of mutagens and mutation conditions overcame the limitation and a further advance was made. It is probable however that in this field the rate of advance is still quite slow.

When progress becomes very slow a variety of mutagens should be tried and numerous strains should also be tested in order to find one that mutates more readily. It is possible to think of a number of theoretical ideas, for instance that a hitherto unused mutagen should be tried, perhaps neutrons if they have not been employed previously.

It would seem that the trouble arises because the long series of mutations (which may number 30-40 or more) saturates the nucleus so that it is impossible to induce any further changes. It might be assumed that perhaps a single important block has arisen with little hope of its removal. The possibility of restarting is sometimes suggested, but the idea of waiting until current production levels are reached before there is any hope of an advance is rather daunting.

Alikhanian (1962) has suggested that a change in the test medium used for screening can expose new lines of progress. This is certainly an idea worth trying.

Another possibility is to introduce a hybridisation step. This may take time, but if progress is very slow there is little to be lost. Some care must be taken since if the mutation and screening steps are allowed to stop for a long time, the time lost can never be regained. Prolonged mutation often seems to weaken the strain, resulting in slower growth, reduced sporulation and difficulty over inoculation generally. Hybridisation may be able to help here, since diploids often show increased sporulation.

It is unwise to give in too soon since progress is often resumed after a time. With a mutation programme that has been in progress for a long time, it may be necessary to carry out five or ten or even more mutation steps before another rise begins. After this there is another period of progress. Sometimes, before yields again begin to increase, a diminution in productivity may occur temporarily. It is however worth while to check procedures to see that advances are not being missed. If the assay is rather erratic it is possible to select for rescreening only the falsely high results, all of which are followed by failure on retest. In all this it helps if more than one mutation programme is in operation, since if one is working it implies that the general methodology is satisfactory.

It can be disturbing at times, when reading the literature, especially patents, to see quoted very high production levels. These may well be higher than the reader thinks possible, or at any rate higher than has been achieved in his own experiments, which he believes to be of good quality. It seems reasonable to believe that the published results are true; on the other hand the assays may have been by a non-specific method and include impurities which interfere with the analysis. This could occur with some of tetracycline broths when measured by a ferric chloride assay. Alternatively, with amino acids isolated and weighed, impurities from the medium may be co-precipitated or the amino acid may contain a percentage of other amino acids. Yields actually obtained are usually rather lower than the highest data recorded.

GENERAL DISCUSSION

In the first part of this review the general methods used for strain improvement are outlined. In the second part a number of methods and problems are discussed in more detail. There is a danger that the impression may be given that strain improvement work is excessively difficult. This is not in fact the case although a high standard of work is needed for success to be achieved. The situation is that at first many practical difficulties may be encountered in establishing sound working methods, after which a period of progress is made. Finally the more fundamental problems begin to assert themselves and a reassessment of the position and a review of methods may be necessary. The beginner's problem is usually the failure of improved strains to appear. This can lead to enquiries and discussions as to why this situation has arisen, possibly to stopping the programme. A programme which has been stopped will not produce results and it is usually best to press on, at the same time checking wherever possible that all is in order. An early stage when progress is difficult is not uncommon.

The sections on mutations, mutagens and methods of screening are intended to give typical examples of work methods. There is an extensive literature on the subject that can be consulted. Usually it is necessary to work out the most suitable procedures for a given case. There are very many papers describing mutations leading to the production of improved strains and it is impossible to test all the methods that are suggested. Often a choice is a matter of common sense. The accounts of hybridisation may seem rather pessimistic. There is no doubt that hybridisation methods have been of the greatest value in genetic work, but for strain improvement results have been more limited in value. None the less in some cases useful if small

improvements in production have been achieved, or a move towards successful mutations started. Increases in vigour of growth may also be obtained. Provided the extra time and effort is available hybridisation work may be worth while. Yeast hybrids are used commercially.

In this review a number of basic and practical aspects of the subject have been stressed. These are, the choice of objectives, the importance of the strain used and the need for high standards of technology in mutation and selection work. It is not desired to overstress the latter points as this might make the task seem insuperable, but the need, which is achievable, remains.

On the subject of objectives the value of a clear, straight forward objective has been stressed. Such an objective can usually be reached if a serious attempt is made, though it may be long and expensive. Where the target is vague the difficulty is as great but its worthwhileness, being diffuse, the effort and finance may be harder to justify. Where the objective is to start a process already being worked elsewhere there is the possibility of buying a suitable strain, but to rely on this limits the possibility of breaking into unexplored fields.

As a field in which objectives were foreseen and attacked, one may cite the case of the Japanese work on glutamic acid and lysine. Here a large amount of work together with biochemical acumen gave valuable results. Generally speaking, the foreseeing of new microbiological processes, the isolation of the strains required for them and the improvement of the strains to give worthwhile results is a difficult matter.

Throughout the review of mutation and hybridisation work the importance of of the strains used has been stressed. While good techniques are valuable their effects are limited by the starting strains involved and by choices of strain made at different stages of the programme. The obtaining of the

initial strain is of course a vital step in the initiation of a project. In improvement work the availability of alternative strains can also be important especially when progress becomes difficult. In the obtaining of strains the national culture collections may help as attempts are made to keep a wide range of strains to meet possible future needs. The obvious step is to buy a strain which has already been used successfully. While in many cases this may be quite feasible, with a new process, to be worked under special local conditions, this may be impossible.

The methods of selection which have been described are based on ideas which are basically sound and which have proved successful in practice. It has to be recognised however that a great deal still depends on the ability to "spot the winner" and additionally to understand the behaviour of the complex types of population which are produced by mutation. That is to say these populations contain abnormal distributions of strains with only a low proportion of improved mutants. Selection work with them is not only difficult but requires a special knowledge of the subject which is at present largely lacking.

Looking at the future, the writer cannot but observe that the selection of microorganisms has much in common with plant-breeding, where there is the same insistence on the importance of parental strains, combined with difficulties of selection from special types of populations. A great deal of work is being done in plant breeding on both these problems, but it is as yet probably too remote from microbiology to be helpful in strain improvement work.

In the Synopsis it was suggested that U.N.I.D.O., because of its central position, might be in a favourable position to help with strain improvement work, for instance in obtaining strains and obtaining advice on breeding and selection problems. In connexion with this the interest of United Nations organisations in crop improvement might be helpful.

The development and exploitation of new strains of wheat and rice have been of great importance in the activities of F.A.O. In this instance the new strains were developed independently under the aegis of the great philanthropic scientific foundations, so that this particular work is probably not relevant to the present case.

One can see tremendous difficulties in a proposal of this kind. Many groups might be unwilling to participate, for a variety of reasons. In addition to this there is the practical problem of how to run and co-ordinate such a scheme, especially if the objectives were unclear, as they probably would be in the first place. However, in considering the problem of starting new industries in the developing countries, the possibilities of new forms of collaboration are thought worth mentioning.

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