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D00278

United Nations Industrial Development Organization

Distr.
LIMITED

ID/WG.50/5
2 December 1969

ORIGINAL: ENGLISH

Expert Working Group Meeting on the
Manufacture of Chemicals by Fermentation

Vienna, 1 - 5 December 1969

FERMENTATION PROCESSES EMPLOYED IN THE PHARMACEUTICAL INDUSTRIES AND THEIR ECONOMIC ASPECTS [1]

by

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id.69-6201

United Nations Industrial Development Organization

Distr.
LIMITED

ID/WG.30/[illegible]
28 October 1969

ORIGINAL: [illegible]

[illegible] Working Group Meeting on the
[illegible]ucture of Chemicals by Fermentation
[illegible]na, 1 - 5 December 1969

SUMMARY

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by

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The discovery of penicillin, the elaboration of its mass production and its introduction into therapy marked the emergence of a new branch of research and a new method of production in pharmaceutical industry, which as a result entered a phase of extremely rapid development. Today, 25 to 30 per cent of pharmaceutical products are produced by this technology, among others the most important antibiotics, vitamin B_{12}, ketosteroids and enzymes are manufactured by sterile fermentation processes. The advance of technology,

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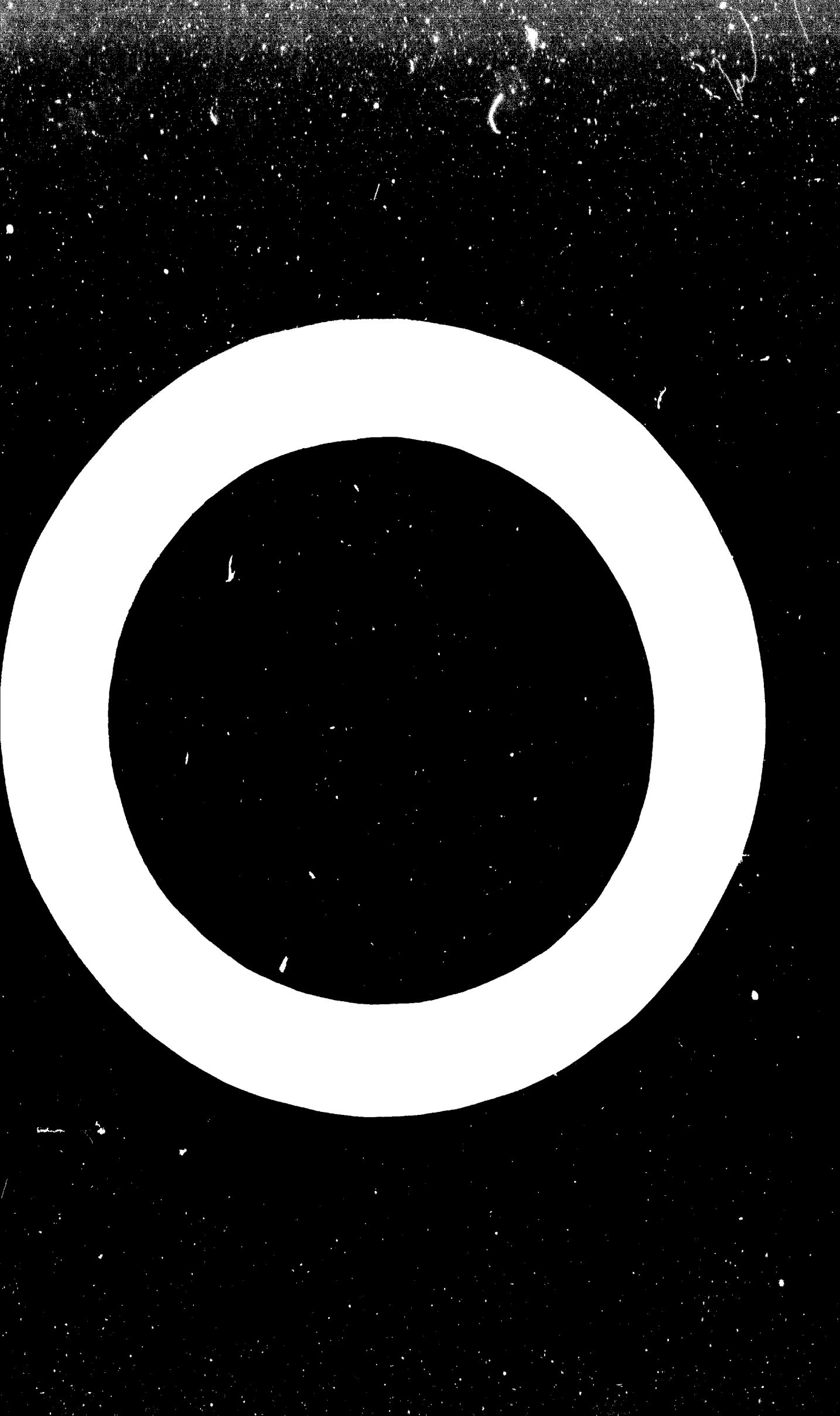

the isolation of biologically active high-yielding strains reduced the prime costs in the first place, which is demonstrated by the price levels that developed after the patents have expired. This fact ensures that the production of most antibiotics by chemical synthesis will not become economical even in the future. The different fermentation processes used in pharmaceutical industry are summarized in a table which gives the price, the probable cost of production and the patent situation.

Principles for the establishment of an industrial fermentation plant

In a developing country it seems reasonable to start fermentation technology with the production of important antibiotics, first of all penicillin, tetracyclines, and vitamin B_{12}. The raw material required for production are agricultural products or by-products available in every country. Apart from its therapeutical importance it enables the national development of medical breeding as well. Considering the current market prices, the smallest but still economically producing unit is estimated to be of 200 metric tons capacity of fermentation volume. The conditions for running a fermentation plant of such a volume are the following. /Fermentation technology and the most important microbiological processes are presented and discussed separately./

1./ General aspects of planning a fermentation plant.

2./ Ensurance of appropriate technology that the supply of strain/s/ used for production be provided for at least five years. /To achieve this the setting up of an international strain bank seems desirable./

3./ The training of experts which should include not only engineers and biochemists but skilled workers as well.

4./ To ensure the undisturbed operation of a fermentation plant care should be taken that besides raw materials high voltage electricity, steam and adequate cooling be available especially in the tropics.

5./ The control of production effected by a small experimental plant apart from the routine laboratory control tests. This plant is to adapt the manufacturing procedures to the local supply of raw materials.

The most important fermentation processes used in pharmaceutical industry

In this chapter a brief summary will be presented of the major manufacturing processes, which at the same time gives an account of the requirements for a processing division in a generally adaptable fermentation plant. In connection with the more important processes the main trends of development will be surveyed.

1./ Production methods of penicillin G and penicillin V.

2./ Production methods of tetracycline group /oxytetracycline and tetracycline/, the therapeutic importance of new tetracycline derivatives.

3./ Production methods of basic antibiotics /streptomycin, neomycin/. Pharmaceutical importance of substances isolated on the basis of similar technological principles.

4./ Production methods of macrolide antibiotics /erythromycin, oleandomycin/.

5./ Production methods of antifungal antibiotics /nystatin, candicidin, griseofulvin/.

6./ Technological development in the production of vitamin B_{12}.

7./ Technology of the production of enzymes.

8./ Utilization of by-products.

Most important fermentation processes and their economic aspects

Informatory economic calculations for a fermentation plant of the most important products /penicillin, streptomycin, tetracyclines, vitamin B_{12}, etc./ on the scale of a 200 metric tons capacity. A brief summary of the attainable results with other fermentation.

INTRODUCTION

The discovery of penicillin, the development of its large-scale production by sterile aerob fermentation processes marked the origin of a new branch of research and a new method of production in the pharmaceutical industry. A great variety of pharmaceutical products are now being produced on a large-scale by sterile fermentation processes and by microbiological transformations amounting to 25 - 30 per cent of the total product of pharmaceutical industry. Continuing research programmes and progress in technology have resulted in the production of many compounds within the scope of pharmaceutical industry which are not marketed firstly as therapeutics. The various fermentation processes applied in pharmaceutical industry are summarized in Table 1. (it is to be found in the addendum), indicating the fields of application, the date of discovery rendering information as to the patent situation and in the case of most important compounds the internationally accepted up to date prices.

The production of various antibiotics result in the most important production of the largest scale, which together with the results obtained in recent years by chemical transformation of the best known antibiotics may

prove to be of manufacturing importance in the not too distant future.

Methods for the production of certain vitamins particularly the production of vitamin B_{12}, meant an entirely similar process which - considering that it can be produced by anaerob microorganisms as well - may also be achieved by simple fermentation technique.

The microbial transformation of certain compounds constitutes a significant field within the fermentation industry, among which the process of microbial oxidation of ketosteroids is of the greatest importance.

In recent years the large-scale production of numerous extracellular enzymes has been established. The smaller part of the total enzyme production is marketed as digestive aid, while the larger portion of the production is utilized by the light industry and is of great economic importance.

Research development was also extended to the field of ergot alkaloids synthetized by Claviceps purpurea. The latest researches are extended to newer and newer areas.

Finally, it should be noted that the sterile fermentation technique in the mass production of microorganisms has beneficial effect on the control of processes employed in the field of agriculture and industry.

Nevertheless, this simultaneous establishment of the above mentioned complex projects may appear to be disadvantageous in the case of an industrially just developing country. For potential future developments among the various fermentation processes the introduction of vitally important antibiotics and of vitamin B_{12} are considered to be of greatest economic advantage due to their therapeutic and animal feeding usage. Thus, in the discussion each process is dealt with in detail in a chapter of its own.

Apart from the therapeutic and economic importance the introduction of these production methods is motivated by the fact that the essential raw material required, the components of media used for the rational development of the production are agricultural products or by-products available in every country. Subsequent to the process development stage practice, the extension of production profile as a second stage operation appears reasonable, considering the extent adequacy of special raw material potences and other special requirements depending on the agricultural and industrial development of the country in question.

Conditions of the establishment of a fermentation plant

Within the scope of the Expert Committee a detailed account will separatoly be delivered on the subjects of fermentation techniques and microbiological operations employed in the fermentation industry. Therefore, I will not touch upon these procedures. Nevertheless, I think that for the sake of a better demonstration of the economic aspects it is necessary to discuss the design conditions for the establishment of a fermentation plant in the pharmaceutical industry.

Principle and design of a fermentation plant.

As it was mentioned in the introduction, when establishing a pharmaceutical fermentation plant it is advisable to commence production with one of the vitally important antibiotics. The market prices of these antibiotics: penicillin, tetracyclines and essential basic antibiotics vary between 20 - 40 dollars/kg. In the past few years a considerable decline in the market prices has occured as a result of basic patent expirations. In my opinion further declines may be anticipated. Today, the economic production of antibiotics on a commercial scale is

carried out in large-volume fermentors of 100 - 300 tons capacity. The general use and rational development of these large-volume fermentors will doubtless affect market prices. However, in a developing country it would not be reasonable to design such a large-volume fermentation plant. Due to considerations outlined below, the establishment of a fermentation plant of 200 tons capacity combined with a three-channel processing plant, operating with extractor, ion-exchanger and precipitating technique, appears to be most advantageous in viewpoint of broadening the profile of production. The expenditure of this type of plant requires an investment of 2.500.000 dollars, excluding licence costs and expenses spent on training employees with the following partitions:

Fermentation plant

2 fermentors of 50 and 5 of 20 metric tons working volume together with intermediate fermentors, airfilters, proportioning devices and medium preparators	400.000 dollars
3 compressors with capacity of 4.500 cu.m air/hr	150.000 "
Precoat filter 20 cu.m of acid-resistant stainless steel	50.000 "
total	600.000 dollars

Fermentation plant total	600.000 dollars
Extractor plant	300.000 "
Ion-exchange plant	250.000
Precipitation processing plant	200.000 "

(if the latter plant is designed for tetracycline processing only, its cost amounts to about 100.000 dollars)

Thus, the total expenditure of a fermentation and processing plant amounts to 1.350.000 dollars

in addition to 30 per cent installation, 10 per cent of appliance and 55 per cent for allotment, buildings and energy supply.

The reasons supporting an investment of the above volume are as follows: Production and fermentation volume both allow marketable production and at the same time allowing the simultaneous production of various products in varying volumes. This design of a three-channel processing plant will find a wide acceptance as far as rentability and market development are concerned, though at the same time it has a considerably increased investment expense compared to a singlefold processing plant.

Within the scope of a three-channel processing plant all the necessary procedures can be performed.

For many developing countries such a large-volume fermentation plant is beyond their initial requirements to start with, but market development justifies the necessity of establishing a unit of this type, possibly by the collective investments of a group of states. Another powerful argument in this matter is, that immediately after the internal production has started, an approaching requirement of therapeutics may occur. Furthermore, taking advantage of the economic results, promoted marketing tendency may emerge in the field of animal feeding as well.

It should be emphasized that the capacity of processing plant designed by this consideration is greater than that of the fermentation plant, even in the case of a single product manufacturing, thus it serves an inexpensive opportunity to broaden the existing fermentor capacity that has been taken into account in the design subsequent to proper training of skilled workers.

The manpower requirement of a fermentation plant of the suggested capacity of 200 tons should be de-

termined to local requirements. In the calculation presented in the last section of this discussion the following claim for manpower has to be expected for:

Managing director	1
Plant engineers	3
Foremen	18
Trained workers	60
Unskilled workers	30
Altogether	112 persons

Provision of technical operations. In securing the processing technology of a fermentation plant, according to existing conditions, it is possible to purchase know-how for the manufacturing of the vitally important antibiotics. However, it is very difficult to determine the exact sum of expenses. In general, for about 50.000 dollars a standard method could already be purchased.

The sound operation of the process depends on the standard yield of strain and to maintain this high yield a constant selective work, requiring great experience is needed.

Obviously, this continuous strain supply im-

poses a constant burden which may result in the dependence of a stronger firm having more technical and economical advances, thus it does not stimulate further process development. Thus one has to resort to the idea, that if several governments plan the realization of a pharmaceutical fermentation plant, the establishment of an international institution appears expedient, that provides for the continuousity of the strain supply and is capable of developing research work simultaneously.

Such an international strain-supplying and research development section should be situated next to one of the full-scale plants to be built but it should be run independently. This, however, is rather difficult to manage due to requirements of highly qualified experts.

Another powerful argument against the set-up of this project is its extremely high costs. But if this section will be capable to operate on a proper level, the fermentation plants that are rationally working on the basis of developments of this section may be able to shoulder a considerable part of its maintainance costs within a short while.

Supposing that the plan for the setting-up of

this central institution would get realised, it will have to be equipped with a small-scale producing pilot plant of 200 - 1000 litre order of magnitude volume similar in its design to full-scale fermentors. This volume ought to be sufficient enough to serve for process-technology research as well.

Training of experts. In a developing country the basic condition for the establishment of a new line of industry is the proper training of experts, i. e. the special training of engineers and microbiologists and the training of skilled workers who will be responcible for the most important operations. This can be managed by the combination of the purchased know-how with reservations in the contract concerning the training of experts for the putting into operation of a plant, also it may provide the final training of experts on the spot, adopting it to local conditions.

The other possibility suggested in the previous paragraph, is to organize the strain-supply and process-development section in such a way as to have capacity left for the training of experts required by the putting into operation of a plant and at the same time its task could be extended to carry out continuous expert train-

ing as well, simultaneously with process development requirements.

Power, water and raw material supply. When designing a fermentation plant the safety of the energy and proper water supply should be thoroughly investigated. The energy requirement of the proposed 200 metric tons fermentation plant is 6.300.000 KWh/year, 3.200 tons steam/year and 1,400.000 cubic m water/year. These factors represent the largest charge of production costs as it will be indicated later on.

Raw material requirements of a production may be covered in almost every country since the main parts of raw material required as medium-constituents are agricultural by-products. In order to maintain a constant high-level production, the extent adequacy and uniformity of all possible sources may be investigated though most of them are oil-industrial by-products applicable without any former preparation (soybean meal, peanut meal) also starch and flour may serve in largest proportions as constituents of medium. It has to be considered, that the constancy of the quality of these may decisively influence the whole production levels. This fact is proved by that, that

some food-producing plants arrive to these raw products with technique developed, that take into account the requirement of fermentation industry, thus when determining the local medium, this has to be reckoned with.

Supervision of manufacturing processes. For the satisfactory supervision of processes, the establishment of a microbiological, analytical and pharmaceutical (for toxicity tests, pyrogenity determinations) laboratory is necessary. Apart from these routine tests the possibility for checking operations in pilot plants should also be secured. In all cases of know-how bought, the processes employed must be adopted to the local raw material supply and in the case of continuous production these supplies must be checked in pilot plant scales. This pilot plant should be placed into the fermentation hall by its proper designing. This of course means further expenditure but in the pilot plant, design and operational mistakes, made on small scale are much less costly and less time consuming than the same errors in a full-scale plant.

A BRIEF SUMMARY ABOUT THE MAJOR FERMENTATION PROCESSES

Production of penicillin G and penicillin V

6-Aminopenicillanic acid; semisynthetic penicillin derivatives, cephalosporin C; semisynthetic cephalosporin derivatives.

Among the antibiotics penicillin, active against Gram-positive bacteria, was discovered first. At present, penicillin G and penicillin V are produced on large scale. The advantage of penicillin V against penicillin G is its acid stability, consequently it may be administrated orally, which in the case of penicillin G was possible only in the form of insoluble salts. After the wide range administration of penicillin into therapy a great resistancy developed in the microorganisms against penicillin which was due to penicillinase an enzyme inactivating penicillin, produced by the resistant strains. Cephalosporin C resembles penicillin in many respects, being also effective against penicillin-resistant bacteria, but its antibiotic yield in production is much lower than those reported for penicillin.

The production of 6-aminopenicillanic acid, responsible for the effects of penicillin has allowed the production of numerous semisynthetic derivatives which because of their structure, cleave with very low velocity upon the effect of penicillanic enzyme and consequently affect resistant microorganisms, and so the spectra of some semisynthetic penicillin derivatives grow more favourable, the semisynthetic production of material, affecting Gram-negative microorganisms also became possible.

The process for the isolation of 7-aminocephalosporanic acid from cephalosporin C in many respects resemble those used for 6-aminopenicillanic acid and resulted in the synthetization of semisynthetic cephalosporin derivatives.

A new outstanding result of research development is that the nucleus of 6-aminopenicillanic acid may be changed by chemical processes to cephalosporin structure.

Fermentation of penicillin G and penicillin V. Penicillium chrysogenum was found to be a superior productive strain for penicillin G and V. Today, the level of economic production in the case of penicillin

G is ranging between 12 - 16.000 U/ml and in the case of penicillin V between 8 - 12.000 U/ml.

In the early stages of penicillin production the main components of the medium were corn steep liquor (2 - 4%) and lactose (3 - 6%). Production of penicillin G was enhanced by the addition of phenylacetic acid, while penicillin V production by the addition of phenoxyacetic acid. The most recent developmental work has been concerned with increasing antibiotic yield which can be influenced by the skillful operation of fermentation techniques corn steep liquor is largely replaced by peanut meal, and in order to maintain the prolonged production period required, the addition of lactose takes place in the second phase of fermentation depending on pH values. Fermentations are generally run for 130 - 150 hours. By continuous addition technique the production of penicillin can be maintained on medium containing glucose and sucrose as well.

Preceeding to isolation, the fermentation liquid is filtered through a filterdrum on the very pH that the fermentation stopped.

In the development of the various operations involved in controlling fermentation techniques the following viewpoints should be considered, which result

in the fermentation of high yields of the desired products. In the case of high production values the microbial activity attained can no more be regarded as the sole characteristic of fermentation technology. The technology should be analysed in every case by the final output correlated with fermentation period and unit volume, respectively. By utilizing culture media of excessively high concentrations and very long fermentation periods yet higher microbial activities may be attained, as mentioned before. The high filtering losses, furthermore the emulsions occuring in the course of processing, these fermentations do not appear economical.

Isolation of penicillin G and V. Chemically, penicillin is a weak acid which may be extracted from the fermentation beer at pH 2 - 2,5 and in a mildly alkaline medium reextracted into the aqueous phase at pH 7,5 - 8,3. Based on these characteristics the following isolation process was developed:

Because of the heat-sensitivity of penicillin the filtered culture broth was cooled to 5°C continually acidified to pH 2,0 - 2,5 and extracted with butylacetate. In the course of extraction a five-fold reduction in volume may be attained. In large-scale production the extraction may be affected with separators and Podbielniak type ex-

tractor, respectively. Penicillin was transferred from butylacetate into the aqueous phase with the help of buffer of pH 7,5, then again extracted with butylacetate at pH 2,5. The organic phase was dried by means of anhydrous sodiumsulphate, then penicillin G was precipitated by the addition of saturated solution of potassiumacetate. Approximate output is between 60 - 80%.

The raw potassium salt may be purified by decolorizing it with Norit in aqueous solution and precipitation with an organic solvent.

The above procedure may be applied without any modification to the isolation of penicillin V too.

Production of 6-aminopenicillanic acid. 6-Aminopenicillanic acid is the moiety of the penicillin molecule, and responsible for its microbiological penicillin activity. It may be manufactured from penicillin G (or eventually from penicillin V) by means of enzymatic cleavage or chemical breakdown, respectively. The enzyme necessary for the cleavage of penicillin G may be produced from Escherichia coli cultivated in large volumes. The cleavage is effected by using the cells directly or by the free enzyme following autolysis, at high concentrations of penicillin G (2 - 5%) resulting a yield of 70 - 80%.

6-Aminopenicillanic acid can be produced by chemical

cleavage at low yields, which however are supposed to be improved at present.

Semisynthetic penicillins. Theoretically, 6-aminopenicillanic acid may be acylated by any acylchloride. The acylating agent determines the microbiological activity, acid-stability and the behaviour towards penicillinase enzyme of the resulting penicillin. The best known semisynthetic penicillins are: methicillin (2-6-dimethoxybenzyl-penicillin), oxacillin (5-methyl-3-phenyl-4-isoxazolil penicillin) and dicloxacillin (which is the dichloro derivative of the last one).

Methicillin is acid-sensitive but resistant to penicillinase. Oxacillin and dicloxacillin are acid-stable and resistant to penicillinase. Thus, all three compounds are effective against penicillin-resistant strains.

By acylating 6-aminopenicillanic acids with radicals containing basic groups, the microbiological spectrum of penicillin broadens towards the direction of Gram-negative bacteria. The most important member of this group is ampicillin.

Cephalosporin C and cephalosporanic acid derivatives. The strains of Cephalosporium produce cephalosporin C. The structure of cephalosporin C is very si-

milar to that of the penicillins. The moiety of the molecule responsible for the activity is 7-aminocephalosporanic acid. Cephalosporin C is not inactivated by penicillinase thus it exhibits microbiological activity against penicillin resistant strains too.

Microbiological processes for the production of cephalosporin C in many respects resemble those used for penicillin production, but the antibiotic yields are much lower than those reported for the penicillins. Manufacturing operations to produce 7-aminocephalosporanic acid from cephalosporin C by its acylation resulted in semisynthetic cephalosporanic acid derivatives (cephalotin, cephaloxin).

The exceptionally high prices of cephalosporanic acid derivatives together with their low fermentation levels prevents their general use in therapy, the more so, as these derivatives do not offer more advantageous effects when compared to semisynthetic penicillins.

It is a new possibility to transform the penicillin nucleus of low production cost to the cephalosporanic nucleus by chemical means. The therapeutic and economic importance of this new result may prove to be of manufacturing interest in the not too distant future.

Production of tetracyclines

Almost simultaneously, three similar antibiotics with similar structure and wide-range spectra have been discovered: chlortetracycline, tetracycline and oxytetracycline. All three antibiotics are manufactured by fermentation procedures using species of Streptomyces, and their therapeutic possibilities are almost identical. Nowadays tetracycline and oxytetracycline account for the largest sale volume.

Apart from the therapeutic application a large amount is used for animal feeding purposes.

Fermentation of tetracyclines. Medium requirements of the various Streptomycetes suitable for the production of tetracyclines show extreme variability. In fact strains producing the same antibiotic, being individual variants of the same species may present quite different requirements in media. The characteristics of medium used for the production of tetracyclines may be summarized as follows: The most currently employed nitrogen sources of the media are soybean meal or peanut meal. In the case of cultures exhibiting higher yields inorganic nitrogen sources, in the form of ammoniumsulphate are applied. Most generally starch or sometimes its amylase

digest starch form is utilized as energy source, or other carbohydrates e.g. in the case of chlortetracycline; sucrose. Apart from the current nitrogen and energy sources the medium is always supplemented by industrial by-products which have favourable effect on fermentation due to their vitamin and growth factors. For this purpose corn steep liquor, yeast or yeast extract and distiller solubles are employed. Besides, these components sodiumchlorides and for buffering purposes $CaCO_3$ is also added to the medium. The addition of antifoam agents i.e. fats or oils frequently stimulates production.

Today, a yield of 12 - 16.000 U/ml is considered to be sufficient for the economic production of oxytetracycline and tetracycline, respectively. The antibiotic produced is bound to the mycelium, therefore the first step of processing is filtration, by means of acidifying the broth. This procedure is usually performed by oxalic or sulphuric acid or by means of simultaneous use of oxalic and sulphuric acids, respectively, adjusting the pH to 2.0. At these instances $CaCO_3$ changes into calciumoxalate or calciumsulphate, respectively, resulting in an insoluble precipitate which may act as filter aid. Apart from fermentation conditions the structure of the precipitated insoluble salts strongly depends on the temperature and speed of acidification procedures, therefore the

mode of acidification affects the rate of filtration.

It should be noted that for the production of tetracyclines tube fermentors without stirrer are employed in some plants. The yield of production of tube fermentors is always lower than those obtained by fermentors equipped with mixers. However, due to lower investment costs and power requirements it is nevertheless employed. If a fermentation plant is equipped both with mixed and tube fermentors, better results are attainable when the process starts in fermentors equipped with mixer and the second phase following growth is continued in tube fermentors. In these cases antibiotic yields may become similar to those obtained by mixed fermentors.

Isolation of tetracyclines. Tetracyclines are amphoteric compounds and dissolve very poorly in water at pH 4,0 - 5,0. In the case of low yield fermentation broth isolation procedures start with extraction by butanol at mildly alkaline pH values. The thus obtained extract is concentrated and treated with anhydrous methanolic-HCl resulting a crude hydrochloride. A more advantageous process has been developed for the isolation of tetracyclines by means of precipitation with quaternary ammonium salts. Similarly to the former procedure, after filtration of the complex salt and its treatment with anhydrous methanolic hydrochloric acid,

tetracycline-hydrochloride is obtained. Following dissolution of the hydrochloride and its neutralisation, the amphoteric form is precipitated and after filtration a final product of good quality is gained. (Nowadays, these procedures may be employed to obtain by-products for animal feeding.)

Today, the following economical procedure is applied for the isolation of oxytetracycline from beers of an output of 10.000 µg/ml. Fermentation broth are filtered at acidic pHs, then clarified by various adsorbents. These steps were followed by adjusting the pH to 4,0 - 5,0. After these procedures oxytetracycline and tetracycline of pharmaceutical grade can directly be precipitated.

For animal feeding purposes the mycelium obtained through the neutral filtration of fermentation broth is dried and marketed in this form. In rare cases fermentation broths are partially evaporated then the total lot of fermentation broth is evaporated again to dryness by using Niro atomizer.

Research prospects. Many investigators have attempted to adopt microbiological and chemical modifications in the production of tetracyclines. Some methyl derivatives of tetracyclines have gained wide clinical acceptance. Among them the most important is doxicycline which is derived from oxytetracycline. It is stable

against acids, therefore its discharge is prolonged thus, it has therapeutical advantage.

Production of water-soluble basic antibiotics

A great many substances with varying chemical structures belong to the group of water-soluble basic antibiotics. Since their isolation methods are rather similar, they are going to be discussed within this group. The most important antibiotics of this group are produced by Streptomyces. Their chemical structure is built up by amino sugars, aminocyclitols or oligopeptides. Streptomycin is active against Gram-positive and Gram-negative bacteria and is a powerful drug against tuberculosis. Antibiotics belonging to the neomycin group (neomycin, kanamycin, paromomycin, gentamicin) strongly affect both Gram-positive and Gram-negative bacteria. Viomycin and capreomycin are applied exclusively against Mycobacterium tuberculosis.

The polymyxine type antibiotics (polymyxin B, colistin) are active against Gram-negative bacteria.

Basic antibiotics do not absorb from the gastro-intestinal tract so they have to be administrated parenterally.

Fermentation of basic antibiotics. Media for the production of streptomycin produced by basic antibiotics

currently in commercial use are generally based on those media established for the production of tetracyclines. High yields may be attained in the case of some individual antibiotics by means of continuous carbohydrate addition. Production outputs ranging between 7.000 - 10.000 µg/ml in the case of streptomycin and neomycin, and 3.000 - 4.000 µg/ml in the case of viomycin are considered to be economical.

Production of antibiotics belonging to the polymyxine group requires media of high phosphate concentration in addition to inorganic nitrogen source. Apart from these components the medium contains soybean meal or peanut meal, low concentration of corn steep liquor and occasionally hydrolyzed animal protein. Glucose is used as energy source. Antibiotic yield ranging between 8 - 12.000 U/ml are claimed to be economical.

Microorganisms of basic antibiotics are bound to the cell, therefore prior to processing, fermentation broth is acidified to pH 2,0 and then filtered as in the case of tetracyclines.

Isolation of basic antibiotics. The major principles for the isolation of basic antibiotics are the following: The formerly acidified fermentation broth is first filtered then neutralized. The precipitate thus obtained is filtered again and the clear solution is allowed to

flow on a carboxyl-type ion-exchange resin of sodium or ammonium form. After washing the saturated column, it is eluted either by aqueous or by methanolic-HCl or by sulphuric acid. This procedure results in an at least tenfold increase in concentration.

For the isolation of various basic antibiotics the carboxyl-type ion-exchange resins marketed by different firms are adequate. However, there may exist some slight differences among the products available in the market which may affect the subsequent steps of purification.

The isolation of antibiotics by ion-exchange process requires the exhaustion of column capacity with a maximum accuracy. Before binding on the resin the bi- and tri-valent cations - if necessary - should be suitably removed from the broth, since they interfere with the ion-exchange. The ion-exchange is properly executed by columns aligned one after the other. The first column in the row is separated for elution when it is binding no more antibiotics. By utilizing the full capacity of the columns in this manner, very concentrated eluates are won.

To remove impurities before elution several processes employ a washing with dilute acid or ammonia

after a thorough washing with water.

The final purification steps become all the more effective if the antibiotic is in the smallest possible volume of eluates.

Fermentation beers of very high microbiological activity may furnish, by means of this ion-exchange technique, eluates of such purity that they may be directly freeze-dried after decolorising it with Norit and deacidifying it by ion exchange.

In the case of lower fermentation levels the eluates may process in the following ways:

a) Repeated ion-exchange furnishes sufficiently pure endproduct;
b) Precipitation of the eluate with water miscible organic solvent and recrystallization may give satisfactory results;
c) Some isolated cases may require chromatography in the final purification steps. This may be accomplished on ion-exchange, alumina or activated carbon columns;
d) The active substance may be precipitated from the eluate by means of organic salt insoluble in water (naphtalinsulphoric acid, pamoicacid) may be applied. The resulting salt may be purified by recrystallization and subsequently a sulphate salt is formed.

For therapeutic purposes the substance produced may be finally freeze-dried.

Research tasks. For the restriction of therapeutic application of the basic antibiotics there are two reasons: The therapeutic index of the two groups; neomycin and polymyxine, though they possess the widest range spectra, is very low. Both antibiotics are specifically toxic to the kidney, furthermore at prolonged administration, secondary neurotic effect is detectable.

In order to discover analogues possessing lesser toxic effect further experimental studies are needed.

Production of antifungal antibiotics

Among the antibiotics possessing antifungal properties, polyene antibiotics and griseofulvin are of the greatest importance in clinical therapy. Polyene antibiotics: nystatin, amphotericin B and candicidin are produced by various strains of Streptomyces. These substances proved to be effective on local applications, while parenterally they exhibit moderate effect only. These antibiotics are not absorbed orally, therefore they are extensively applied after administration of antibiotics of wide range spectra to suppress the amount of fungi in the intestinal flora.

Griseofulvin is produced by fungi belonging to the Penicillium group and is especially highly active in the case of dermatomycosis because it accumulates in the epidermis.

Fermentation of antifungal antibiotics. Fermentation processes for the production of polyene type antibiotics of Streptomyces are very similar to those developed for other Streptomyces antibiotics. A marked increase in antibiotic yield is followed after continual glucose addition. Economic production of nystatin amounts to 10.000 U/ml.

The medium requirement of griseofulvin production by Penicillium is quite different. Fermentation is maintained on a medium of high carbohydrate but at the same time low nitrogen concentration to accomplish nitrogen starvation of the microorganism. Economic griseofulvin production amounts to 8 - 12.000 µg/ml.

Isolation of antifungal antibiotics. Antifungal antibiotics are substances insoluble in water and bound to mycelium. Their isolation is achieved by extracting the filtered mycelium.

In the case of polyene type antibiotics the fermentation broth is filtered at pH 4,0 - 5,0. The filtered mycelium after drying is extracted by methanol or acetone.

To achieve higher activity of the extract, the addition of 1% calciumchloride at defined wetness proved to be useful. The extract is evaporated in vacuum and the precipitated antibiotic is washed and dried by acetone and apolar solvents.

The product obtained by this procedure is generally not further purified, its activity depends mainly on the activity of the fermentation.

Polyene antibiotics are extremely unstable and photosensitive. The stability of each compound depends on the property of the producing strain, the fermentation technique and the process of isolation.

In the case of griseofulvin the fermentation broth is filtered without altering its pH, the mycelium may be dried, then the active substance is extracted by alcohol, acetone and chlorinated solvents. After the evaporation of the extract, griseofulvin in crystalline form is obtained. By the aid of carbontetrachloride the fats are removed and the antibiotic is recrystallized from the aqueous acetone.

In certain countries the mycelium of griseofulvin producing strains is dried, ground and marketed without further purification for veterinary practice and for animal feeding.

Research tasks. The need for substances with antifungal properties offering equivalent promise in human and animal therapy to those of antibacterial antibiotics has become of great importance, therefore further experimental studies in this field are required.

Production of macrolide antibiotics

All members of this group are active against penicillin resistant Gram-positive bacteria. Their medical use decreased since the discovery of synthetic penicillins. Most important members of this group in medical applications are erythromycin and oleandomycin.

Fermentation of macrolide antibiotics. Macrolide antibiotics are produced by Streptomyces, the media generally applied for this purpose have to be similar in their characters to those discussed above. The most economic production level is ranging between 2 - 4.000 µg/ml.

Isolation of macrolide antibiotics. The chemical property of the macrolides is as follows: The amino sugar moiety in the structure of macrolide antibiotics accounts for their basic character, the macrolide part linked to the amino sugar is lipophylic. Basic principles for their isolation: The fermentation broth

filtered at pH 4,0 - 5,0 contains the major part of the antibiotic which is extracted at pH 9.0 into organic phase using solvents such as ethylacetate, butylacetate or chlorinated hydrocarbons and extracted at pH 5,5 into water. To attain appropriate concentration of the aqueous phase it is practical to concentrate the organic phase. The free basic group of the antibiotic can be precipitated at alkaline pH then crystallized from the organic solvents.

Production of vitamin B_{12}

Among the vitamins produced by fermentation techniques vitamin B_{12} is of the greatest importance for clinical as well as animal feeding purposes. For therapeutic administration crystalline vitamin B_{12} or its coenzyme form can only be used. The quality of the products are microbiologically checked.

Vitamin B_{12} is synthetized by a wide range of bacteria and Streptomycetes. Formerly, recovery of vitamin B_{12} as by-products of antibiotic fermentation was accomplished, nowadays only those of high yield are used in large-scale industrial production.

Production of vitamin B_{12} by fermentation processes. The most generally accepted process for

vitamin B_{12} production is based on the use of strain of Propionibacterium shermanii. A fermentation technique different of those used for antibiotics is practized by cultivating Propionibacterium anaerobically. For the production of vitamin B_{12} a medium including corn steep liquor, ammoniumchloride and glucose has been developed. Intermittent addition of alkaline is used to neutralize the large amount of acid formed during the anaerobic process. Periodically, vigorous aeration in the second stage has increased the cell production. A marked increase in vitamin B_{12} activity is detectable when cobalt salts and 5,6-dimethylbenzimidazol were added to the medium.

It should be noted that sewage sludge contain a useful amount of vitamin B_{12} which can be influenced by adding waste materials and by subsequent anaerobic fermentation. To these subsequent anaerobic fermentations the intermittent addition of precursors i. e. cobalt salts and 5,6-dimethylbenzimidazol were found to be useful. The economic aspects of vitamin B_{12} production are decisively governed by the local conditions and by wage costs.

Isolation of vitamin B_{12}. The vitamin B_{12} formed in the course of fermentation is retained in the cells. The separation of cells is attained by centrifuging the

fermentation broth or by filtration of the gel-like precipitates preceedingly formed in the broth. The vitamin B_{12} activity is released from the cell mass by disrupting it by heat in the presence of potassium-cyanide. After filtration B_{12} is adsorbed by the aid of charcoal and ion-exchange resins, then it is eluted in alcohol or acetone either in mildly alkaline or in mildly acidic stage depending on the characteristics of the adsorbent. The elute thus obtained is evaporated and extracted either by benzylalcohol or by phenol-chloroform mixture. By the aid of apolar solvents vitamin B_{12} is extracted into the aqueous phase and finally it is chromatographed on an aluminiumoxide column. By this procedure vitamin B_{12} is separated from the substances exhibiting similar structure, then it is crystallized from the aqueous-acetone solution.

The vitamin B_{12} analogues having similar effects and structures are used for animal feeding purposes.

Microbial transformation of steroids

The steroid hormone industry utilizes microbial transformations to produce hormones and hormone analogues. Among these processes the following steps attained greatest importance:

Production of Reichstein's compound S (17 α,21--dihydroxy-pregn-4-ene-3,20-dione) and Reichstein S--17 α-acetate from 3β,17α-21-trihydroxypregn-5-ene-20-one, and from its 3β,17 α,21-triacetate, respectively, as well as the production of hydrocortisone (11β,17 α,21-trihydroxypregn-4-ene-3,20-dione) by means of hydroxylation of Reichstein's compound S or Reichstein S-17-acetate. In the case of the latter substrate this step is followed by deacetylation.

Prednisolon (11β,17α,21-trihydroxypregnene-1,4--diene-3,20-dione) is manufactured by the Δ^1-dehydrogenation of hydrocortisone.

Several processes were elaborated for the production of androsta-1,4-diene-3,17-dione from cholesterol or progesterone which enable the production of estrone. The importance of these processes, however, diminished lately since estrone and contraceptive norsteroid production was solved by total synthesis. Nevertheless, in total synthesis microbial steps are applied too, for instance stereo-specific microbial reduction in the production of natural-estrone.

Processes referring to microbial transformation of steroids generally are patented. Their introduction requires a steroid industry of high level since they are always connected with chemical processes.

Culturing of microorganisms for steroid transformation. The different steroid transformations are carried out by a great variety of bacteria and fungi selected by a very extensive screening. The microorganisms contain the transforming enzymes intracellularly. They are capable to perform very different reactions. The screening aims to select strains performing the desired transformations with a high yield. These strains are supposed to produce the smallest possible quantity of by-products and to contain only small amounts of enzymes which are able to decompose the steroid molecule after having accomplished the reaction in question.

The enzyme concentration within the cell is influenced, beside the inherent characteristics of the strain, by the culturing conditions. In certain cases the enzyme levels may be enhanced to a large extent by growing the microorganisms in the presence of steroids of small concentration. The transformation capacity is determined continually in the course of fermentation and the steroid substrate to be converted is added at its maximum level in one or several batches. In many cases the steroid conversion is performed by harvested cells without any carbon source,

in order to facilitate the isolation of steroid products.

Possibilities for steroid substrate addition.

Steroids poorly soluble in water are added to a fermentation broth suitable for transformation. The addition may be performed in two ways: either the steroid is dissolved in organic solvents (methanol, ethylene, acetone) and added in this solution to the fermentation broth. In this case the organic solvent content of the broth influences the enzymatic process. The usefulness of this method should be proved experimentally. The other possibility is, that the steroids are micronized in aqueous solution or in the presence of wetting agents and are added to the fermentation solution. From economic viewpoints it is advantageous to apply the possibly highest concentration of substrate in the course of fermentation. The optimum substrate concentration may be established experimentally, generally it may amount to 1 mg/ml but in rare cases a multiple of this may be converted.

In many microbial processes enzyme inhibitors are employed to diminish unfavourable side reactions.

All fermentation steps are checked analytically. At the rate of maximum accumulation of the useful product the fermentation is immediately interrupted. In

order to avoid undesirable side reactions the processing starts instantly.

Processing of steroid fermentation. At the end of fermentation the steroid depending on substrate concentration and solubility, is either in solution or is precipitated in the broth. In the first case the fermentation broth is filtered and extracted with water-immiscible organic solvent. Most suitable solvents are: ethylacetate or methylisobutylketone for steroids having many polar groups and chlorinated hydrocarbons for less polar steroids. After a possibly continuous extraction the extract is evaporated in vacuum to dryness. In some cases the extracted steroid is contaminated with large amounts of fatty impurities, originated from the microorganism. These impurities may be removed by extraction of the aqueous methanolic solution of the evaporation residue with petroleum ether. The steroid obtained after evaporation is crystallized in an organic solvent. If in the course of fermentation different steroid by-products are accumulated the purification may be performed by fractional crystallization or chromatography, respectively.

In cases when after conversion the main part of steroid is precipitated in the broth, it may be filtered together with the cells. It is reasonable to saturate broth with salt before filtration to diminish the solub-

ility of the steroid. The steroid-containing cell mass is dried, extracted with an organic solvent and purified as described above.

Production of microbial enzymes

Prior to the development of sterile fermentation techniques, all enzyme compounds employed in therapy were of animal origin. Recently, a great number of digestive enzymes are produced by fermentation processes for therapeutical purposes. In fermentation technology - as it was stated in the Introduction - the pharmaceutical industry can provide the most skilled experts, therefore, as an additional advantage pharmaceutical industry produces enzymes in large-scale volumes by various fermentation techniques for other industries as well (food, detergent, leather, textile and paper industries).

For enzyme production a great variety of bacteria and fungi furnishing high yields are used and enzymes of the greatest industrial interest are the extracellular products of these microorganisms.

Presently, enzymes produced for commercial scale are the following: with the aid of bacteria, α-amylase, neutral and alkaline protease (alkalise) and lipase. Enzymes produced by fungi: α-amylase, amyloglucosidase,

protease, lipase, cellulase, hemicellulase and pectinase.

Fermentation processes for the production of enzymes. Microorganisms used for the production of the above listed enzymes are produced in fermentors under sterile conditions employed for antibiotic fermentation processes. The composition of media is markedly different in the case of various microorganisms. Nutrients used for optimum production must be empirically established for each microbial system. In the first phase following inoculation, the microorganisms attain rapidly sufficient growth, in the second phase following growth the biosynthesis of the extracellularly produced enzyme develops.

After having achieved maximum enzyme levels, microbial cells are separated from the mash by filtration in the case of fungi or by centrifugation in the case of bacterial fermentation.

General principles of enzyme recovery and purification. Since the enzymes are protein-like compounds, procedures generally used for protein purification may be adopted for enzyme recovery. They are acid- and alkaline-labile substances, generally sensitive to heat.

Most enzymes of industrial importance are recovered either by precipitation or by adsorption technique. The obtained fermentation broth is concentrated by evapor-

ating it with vacuum. From the thus obtained solution the enzyme is fractionally precipitated, while cooling, by the aid of ammoniumsulphate or organic solvents (methanol, ethanol and acetone) at pHs of optimum stability. The obtained dried precipitate can directly be used for industrial purposes. Components for therapeutic use must be exposed to further purification.

The other processing possibility is as follows: To the filtered fermentation broth an adsorbent capable to bind protein is added. After filtration the adsorbed protein is eluted by an aqueous salt solution of defined concentration and pH value. Where enzymes of high purity are required the concentrated eluate may be subjected to further fractional precipitation.

Marketing of by-products

The different processes of the fermentation industry furnish several by-products utilizable as feedstuffs. Thus in setting up the plant, the sale of these by-products must be immediately provided for. The largest amount of these by-products is furnished by the filtered cell mass, which contains - in the case of antibiotic fermentations - appreciable amounts of the respective antibiotics, a variety of vitamins (from the

vitamins the quantity of vitamin B_{12} has to be taken mainly into consideration) and very valuable proteins. It is desirable to provide facilities for the drying of these by-products and, - considering its active material content - for its application as feedstuff.

The antibiotics which have proved to be useful as feedstuffs are: penicillin, tetracyclines, furthermore the by-products of vitamin B_{12}, - productions otherwise unfitted for human clinical purposes - may also be utilized similarly.

Economic aspects of the major fermentation processes

The production cost developed after the construction of the fermentation plant is influenced to a high degree besides technological parameters (fermentation volume, output of processes employed, concentration of useful products at the end of fermentation), by local raw material, employment conditions, as much as tax- and customs-regulations of the respective states. Based on these, informative economic calculations may be accomplished, which are to be demonstrated in the case of one of the most important antibiotic; tetracycline or oxytetracycline.

In case the proposed 200 tons fermentation plant manufactures exclusively tetracyclines, the following output may be expected in one year. At continuous fermentations 330 working days may be calculated, thus the production hours amount to 7.920 hr/year. A single fermentation period lasts for 150 hr, theoretically 52 batches may be expected in each fermentor. Considering the different technical difficulties a total of 45 batches may be calculated for, which means 45 x 200 cu. m. i. e. 9.000 cu.m. If average fermentation yield is 12.000 µg/ml and the output amounts to 70%, the plant may manufacture 75,6 tons of tetracycline. Its approximate world market price is 30 dollars, thus the production value may amount to 2.268.000 dollars.

Expected production costs. Calculated for one cubic meter fermentation broth:

raw material (culture media and processing costs)	45.00 dollars
salaries, 30 hrs 1 $/hr	30.00 "
power (700 kWh, 0,03 $/kWh)	21.00 "
steam, 3,6 tons	5.70 "
water 150 cu.m. (0,04 $/cu.m.)	6.24 "
total:	105,94 dollars

(Salaries, electric power, steam and water consumption was calculated from the data of the second part.)

In the case of an output of 70%, 8,40 kg tetracycline may be in one cubic meter fermentation broth. Thus the production cost of one kg product amounts to 12.60 dollars.

Overhead expenses assumed to be of 25%, furthermore, amortization and the various taxes have to be added to this value.

The data of the study prove that 50% of the overall price of fermentation product are energy, power and salary expenses. This is a comparatively constant value and independent of the quality of the product. The cost of raw materials depending on the production technique has comparatively little influence over the established price, it may vary between 40 - 60 % pro fermentation in the production of a great variety of substances.
Based upon the above data, Table 2. summarizes the price of penicillin G, streptomycin and vitamin B_{12} compared to tetracycline and to world market prices. (Table 2. is to be found in the addendum.)

(It is to be noted that because of the anaerob fermentation technique, vitamin B_{12} has extremely low power requirement: 100 kWh/cu.m. was calculated.)

The above scheme may be applied to the calculation of production cost of any antibiotic. No great mistake is committed if 100 - 130 dollars/cu.m. cost is calcul-

ated for a fermentation period of 140 hrs.

The decrease of fermentation period followed by the simultaneous diminishing of power requirement can considerably reduce the production cost of antibiotic produced in one cubic meter.

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Addendum

Table 1.

Important products of pharmaceutical industry

Groups of products	Name	Date of discovery	Comments
A. Antibiotics			
I. Antibiotics active against Gram-positive bacteria			
1. Penicillin and cephalosporine derivatives	Penicillin-G	1929	potassium salt, price 18 $/kg used for animal feeding
	Penicillin-V	1948	potassium salt, price 20 $/kg
	6-aminopenicillanic acid	1960	produced of penicillin-G, basic product of semi-synthetic penicillins
	Methicillin	1960	
	Naficillin	1963	
	Oxacillin	1961	
	Cloxacillin	1961	
	Dicloxacillin	1963	
	Ampicillin	1960	it has wide-range spectrum
	Carbenicillin	1967	" "
	Cephalosporin C	1956	
	Cephalosporidine	1961	
	Cephalotin	1962	
	Cephalexin	1968	

Groups of products	Name	Date of discovery	Comments
2. Macrolides	Erythromycin	1952	Price 180 $/kg.
	Oleandomycin	1954	
	Carbomycin	1952	
	Leucomycin	1953	
	Spiramycin	1954	
3. Other antibiotics	Novobiocin	1955	
	Vancomycin	1956	
	Lincomycin	1962	
	Chlorlincomycin	1967	
	Fusidic acid	1962	
	Pristinamycin	1960	
II. Antibiotics with wide-range spectrum			
1. Tetracyclines	Chlortetracycline	1948	used for animal feeding
	Oxytetracycline	1950	Price 30 $/kg
	Tetracycline	1953	"
	Demethylchlor-tetracycline	1956	
	Methacycline		
	Doxacycline		
2. Water-soluble basic antibiotics	Streptomycin and Dihydrostreptomycin	1944	Price 24 $/kg.
	Neomycin	1949	" 28 "
	Paromomycin	1958	" 38 "
	Kanamycin	1959	
	Gentamicin	1963	
III. Antibiotics active against Gram-negative bacteria	Polymyxin B	1947	
	Colistin	1950	
IV. Antibiotics active against TB bacteria	Viomycin	1951	Price 220 $/kg
	Capreomycin	1962	
	Cycloserine	1954	
	Rifamycins		

Groups of products	Name	Date of discovery	Comments
V. Antifungal antibiotics	Nystatin	1951	Price 53 $/Bou
	Amphotericin B	1956	
	Candicidin	1953	
	Trichomycin	1952	
	Hamycin	1960	
	Griseofulvin	1939,1958	Price 80 $/kg.
VI. Anticarcinogen antibiotics	Actinomycin D	1953	
	Mitomycin C	1956	
	Chromomycin A_3	1958	
	Olivomycin	1962	
	Streptonigrin	1960	
	Daunomycin	1965	
	Bleomycin	1965	
VII. Antibiotics applied locally	Tyrothricin	1939	
	Gramicidin	1939	
	Xanthocillin	1953	
VIII. Antibiotics applied for other purposes			
1. Animal feedings	Bacitracin	1945	
	Moenomycin	1964	
2. Plant protecting agents	Blasticidin S	1958	
	Cellocidin	1958	
	Polyoxin	1966	
3. Food-industrial preservatives	Nisin	1946	
	Subtilin	1946	
	Tylosin	1961	
4. Antibiotics used for other purposes	Hygromycin B	1958	

Groups of products	Name	Date of discovery	Comments
B. Vitamins	Vitamin B_{12}	1949	Price of crystalline vitamin B_{12} 4.1 $/g.
	Vitamin B_2		
	Gibberellic acid		
C. Compounds produced by steroid bio-oxidation			
	Hydrocortisone	1952	
	Prednisolone	1955	
D. Enzymes	Proteases		
	Amylases		
	Amyloglucosidase		
	Lipases		
	Cellulase		
	Hemicellulase		
	Pectinase		

Addendum

Table 2.

Cost of production in the case of most important antibiotics and vitamin B_{12}

Product	Fermentation level	Cost of one cu.m. broth in $ if fermentation term is 140 hr	Amount of isolated substance/ cu.m. (with a 70% yield)	Production cost
Oxytetracycline or tetracycline	12.000 µg/ml	106	8.4 kg	12.61 $/kg.
Penicillin-G potassium salt	15.000 U/ml	130	10.5 Bon	12.38 $/Bon
Penicillin-V potassium salt	12.000 U/ml	130	8.4 "	15.47 $/Bon
Streptomycin base	10.000 µg/ml	110	7.0 kg	15.71 $/kg
Neomycin base	8.000 µg/ml	110	5.6 "	19.64 $/kg
Erythromycin base	3.000 µg/ml	140	2.1 kg	66.70 $/kg
Nystatin	10.000 U/ml	140	7.0 Bon	20.00 $/Bon
Vitamin B_{12}*	30 µg/ml	60	20 g	3.00 $/g

* Production cost of vitamin B_{12} may considerably be reduced in easily realizable larger volumes and by simplifying fermentation technique.


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