4/80 | CMCRCZ 9 (4), 247-352 (1980)

ΧΗΜΙΚΑ ΧΡΟΝΙΚΑ

ΝΕΑ ΣΕΙΡΑ

CHIMIKA CHRONIKA

NEW SERIES

AN INTERNATIONAL EDITION OF THE GREEK CHEMISTS ASSOCIATION

SPECIAL ISSUE CONTAINING INVITED PAPERS PRESENTED AT THE FIRST BALKAN CHEMISTRY DAYS, ATHENS 1980

CHIMICA CHRONIKA, NEW SERIES Volume 9, No 4, pp. 247-352, December 1980

CHIMIKA CHRONIKA/NEW SERIES

Published by the Greek Chemists' Association
27, Kaningos Street, Athens (147), Greece

Correspondence, submission of papers, subscriptions, renewals and changes of address should be sent to Chimika Chronica, New Series, 27 Kaningos street, Athens 147, Greece. Subscriptions are taken by volume at 500 drachmas for members and 1.000 drachmas for Corporations in Greece and 28U.S. dollars to all other countries except Cyprus, where subscriptions are made on request.
Printed in Greece by EPTALOFOS
Υπεύθυνος σύμφωνα μὲ τὸν νόμο: Μ. Καραγιάννης, Παρμενίδου 15, Ἀθήνα.

BALKAN CHEMISTRY DAYS
Athens, April 17-19, 1980

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CHIMICA CHRONIKA, *NEW SERIES*

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CHEMICAL AND CHEMICAL-TECHNOLOGICAL EDUCATION IN BULGARIA

KIRIL DIMOV
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The chemical and the chemical-technological educational institutions in Bulgaria train specialists with secondary and university education for the enterprises of the chemical industry, for other enterprises which apply chemical methods of processing their products, for the needs of the designers organizations and the scientific research institutes, for scientific – teaching work in the higher institutes of learning and for teachers in the secondary schools.

Training is carried out according to educational plans which are being continuously perfected with regard to the growing demands on the specialists – chemists, technologists, technicians, skilled workers. Growing attention is paid in the plans to the industrial education with a direct participation of the students in the material production and servicing. The plans contain a greater number of elements of combining the educational with the scientific – research work in training the students.

The secondary chemical-technological personnel in Bulgaria is trained in two kinds of educational establishments – technical schools and secondary professional technical schools.

The secondary professional technical schools (SPTS) are the lowest form of training specialists for the chemical industry. They are created on the territorial principle and train skilled workers (apparatus specialists etc.) for the needs of the corresponding territorial unit.

There are such SPTS in many towns. There they train personnel for the enterprises of the chemical industry and for such enterprises of the light industry as apply chemical processes. There are such schools in the towns of Vratza, Pleven, Stara Zagora, Yambol, Razgrad, Svishtov, Devnya, Plovdiv, Sliven, Lovetch, Sofia, Kazanlak – big centers of the technical and the light industry in our country. The course of training is three years. Specialists who have graduated from a SPTS are awarded the title of «apparatchik» (apparatus specialist) of the corresponding specific speciality from grade II to grade IV, (according to the scholastic abilities).

In the field of the chemical industry and the enterprises of the light industry which apply chemical processes, there function SPTS in the following special fields:

1. Production of inorganic substances.
2. Production of organic substances.

3. SPTS for chemical laboratory assistants.
4. Processing of oil and gas.
5. Chemical coatings.
6. Oil - chemical synthesis.
7. Cellulose paper and cardboard.
8. Chemical fibres.
9. Production of rubber and plastic products.
10. Production of glass products.
11. Production of porcelain products.
12. Ceramics — glass decoration.
13. Shoe production.
14. Leather - fancy goods production.
15. Fur industry.
16. Finishing and dyeing.
17. Chemical (dry) cleaning.

An average of 2500-2800 specialists are trained annually in the SPTS of Bulgaria. These are skilled workers and apparatus specialists for the needs of the enterprises of the chemical and the light industry. Teaching is carried out according to general educational subjects (chemistry, inorganic and organic) and special technological and technical subjects linked with the specific purpose of the field of study. Thus, for example the SPTS in finishing and dyeing train their students in the following special subjects:

— General textille technology, Technology of finishing, Technology of textile dyeing and printing.

The SPTS in the production of glass products:

— Technology of fine ceramics, Technology of glass production.

The technical schools, which train qualified secondary technicians — technologists for the needs of the chemical and the light industry (technologists - chemists) are 12 in Bulgaria. They function in the cities of: Varna, Sofia, Russe, Dimitrovgrad, Svishtov, Vidin, Bourgas, Novi Pazar, Lovetch, Vratza, Gabrovo and Sliven. The course of study is 4 years. The qualification of the graduates is Secondary - Technician - Technologist. About 1000 - 1300 secondary specialists graduate annually.

In the field of the chemical industry and the enterprises of the light industry which apply chemical processes, there function technical schools (technicums) along the following specialities:

1. Technology of inorganic substances.
2. Technology of organic substances.
3. Technology of the oil-chemical process.
4. Technology of oil and gas processing.
5. Technology of bio-chemical processes.
6. Technology of cellulose ane chemical fibres.
7. Technics and technology of glass production.
8. Technics and technology of porcelain and faience production.
9. Ceramics - glass decoration.

10. Technology of finishing, dyeing and dyeing technics.
11. Technology of rubber and plastic products.
12. Machines and apparatuses in the chemical industry.

It is envisaged that in the future the speciality «Technology of Cellulose» and «Chemical Fibres» to be sub-divided into two specialities.

1. Technology of paper and cardboard.
2. Technology of chemical fibres.

Training in the technical schools is carried out along the same special subjects as in the SPTS, the difference being that the students here get a much varied and versatile training, more profound knowledge in the field of general chemistry. The special subjects are based on definite theoretical bases.

The higher schools of learning — universities (HSL) which train specialists in chemistry and chemical technology (i.e. chemical enginering) are the following:

1. The Higher Chemico-technological Institute - Sofia.
2. The Higher Chemico-technological Institute «Prof. D-r Assen Zlatarov» — Bourgas.
3. The Higher Institute of Food and Gustatory Industries - Plovdiv.
4. The University of Sofia «Clement Ohridski».
5. The University of Plovdiv «Paissi Hilendarski».
6. The Higher Pedagogical Institute - Shumen.

The HCTI — Sofia and Bourgas train specialists in the following chemical - technological and metallurgical specialities:

1. Technology of inorganic substances (HCTI — Sofia and Bourgas) — it trains chemical engineers and technologists for the production of mineral acids, salts and fertilizers, mineral pigments, calcinated soda and soda products, production of industrial gases etc. The training is carried out according to the following subjects:

Physico-chemical fundamentals of the technology of inorganic substances.

Engineering shaping of the processes in the technology of inorganic substances.

Technology of inorganic substances.

Utilization and rendering harmless the waste materials of inorganic origin.

Installations and designing.

2. Technology of silicates and binding substances (HIGT - Sofia; HICT-Bourgas) — it trains engineer - chemists - technologists for the production of glass and fine ceramics, fire-proof materials, cement, construction ceramics etc. The training is carried on along the following special disciplines:

Mineralogy and petrography.

Physico-shemistry of silicates.

Heat processes, aggregates and equipment in the silicate industry.

Chemical technology of glass.

Chemical technology of binding substances.

Chemical technology of ceramic products.

Elective subjects: Technology of special ceramics, Technology of special binding substances, Technology of enamels.

3. Electro-chemical productions and resistance to corrosion (HICT - Sofia) trains engineers - chemists for the production that makes use of electro-chemical methods in the struggle against the corrosion of metals in the equipment and the transport installations, as well as in the chemical and the energy reactors. The training is carried out along the following special subjects:

Structure of metals and alloys.
Electro-chemical systems.
Kinetics of electro-chemical processes.
Corrosion and resistance of metals.
Electrosynthesis.
Galvanotechnics.
Chemical sources of current.
Methods of elctro-chemical research.
Electrical instruments and electro-chemistry.
Equipment and designing.

4. Technology of organic synthesis of fuels (HICT-Sofia and HICT-Bourgas) trains engineer -chemists for the production of semi-products, pharmaceutical preparations, means of protecting vegetation, dyes, synthetic parfume and cosmetic products, production of the fundamental organic and petrol-chemical sunthesis — for the enterprises which process solid, gas and liquid fuels. The training is carried out in two profiles along the fillowing subjects:

α. Profile «Technology of fuels and lubricating materials»:
Chemistry and technology of solid fuels.
Chemistry and technology of petrol.
Engineering-technical research in processing and exploitation of fuels.
Chemical processing of petrol products.
Coke-chemical production.
Chemical technology of fuels and lubricating materials.
β. Profile «Technology of light organic synthesis»:
Mechanism of organic reactions.
Functional analysis.
Technology of organic synthesis.
Pharmaceutical preparations.
Technology of dyes.

Elective subjects: Chemistry and technology of preparations for the rural economy, Chemical technology preparations of industry, Fundamentals of biochemical synthesis, Chemistry and technology of the parfume substances.

5. Technology of rubber and plastics (HICT-Sofia, HICT-Bourgas) trains engineer- chemists- technologists. Technologists for the production of rubber and rubber products, of plastics and plastic products, synthetic glues and film-forming substabces etc. The training is carried out in two profiles along the following special disciplines:

α. Technology of rubber:

Synthesis of semi-products and ingredients.

Theoretical fundamentals of production and processing of elastomers:

Technology of rubber.

Technology of rubber products.

Technology of synthetic rubber.

Machines and apparatuses in the rubber industry.

β. Profile «Technology of Plastics».

Technology of semi-products.

Chemistry and physico-chemistry of polymers.

Technology of plastics.

Processing of plastics.

Analysis and testing of polymers.

Technology of film-forming substances.

6. Technology of fibres and leathers (HICI-Sofia) trains engineers-chemists- technologists for the production of chemical fibres, for finishing textile materials, for the leather-fur production, the shoe industry, for the manufacturing of leather and fur products etc. The training is carried out in two profiles in the following disciplines:

a. Technology of chemical fibres and textile finishing:

Chemistry and physico-chemistry of fibre-forming polymers Material knowledge of fibres.

Machines and apparatuses in textile finishing.

Apparatuses and equipment in the manufacturing of chemical fibres.

Elective subjects: Technology of chemical fibres, Technology of preparation and dyeing. Technology of printing and special finishing (for the specialization - Technology of textile finishing). — Technology of textille finishing, Technology of man made fibres and fibres of special purposes and Technology of synthetic fibres (for specialization - Technology of chemical fibres).

b. Technology of shoe production and leathers.

Chemistry and physics of high molecular compounds and the tanning substances.

Technology of leather production.

Technology of shoe production.

Elective subjects: Machines and apparatuses in the shoe production, Knowledge of materials, Modernisation and designing the leather products (for the specialisation in the shoe production), Machines and apparatuses in the leather and the fur industry, Technology of the fur production, Bio-chemical analysis and special leather-fur technologies.

7. Chemical technology of wood (HCTI - Sofia). Training is carried out along the following disciplines:

Chemistry of wood.

Chemistry and technology of cellulose.

Chemistry and technology of hydrolysis production.

Machines and apparatuses in the cellulose-paper and hydrolysis production.

Chemistry and technology of paper.

Chemistry and technology of carboards.

8. Technology of petrol and gas (HICT-Bourgas) trains engineers - technologists to process petrol, gas and petrol-chemical synthesis. The training is carried out along the following special disciplines:

Chemistry of petrol and gas

Technology of petrol and gas.

Technology of the fundamental organic petro-chemical synthesis

Calculating and sizing the processes and apparatuses in the petrol processing industry.

Kinetics and catalysis of the petrol-processing processes.

Chemistry and technology of the solid and the synthetic fuels.

9. Technology of water (HICT-Bourgas) trains engineers- chemists for the disinfection, discolourification and clarification of the natural waters, their rendering them soft etc. The training is carried out according to the following special disciplines:

Sorption processes and apparatuses.

Technology of purifying waste and highly stratified waters.

Corrosion.

Water preparation.

Fundamentals of designing and water preparatory and purification stations.

10. Metallurgy of ferrous metals (HICT-Sofia) trains specialists engineer-metallurgists for the blast furnace production, steel manufacturing, production of ferro-alloy and foundry products, chemico-thermal processing of metals etc. Training is carried out along the following subjects:

Theory of metallurgical production.

Metallurgical heat technics.

Technology of smelting.

Plastic deformation of metals.

Metallography.

Crystallography and mineralogy.

Fundamentals of designing metallurgical enterprises.

Protection of the natural environment.

Metallurgy of non-ferrous metals.

Physics of metals.

Metallurgy of steel.

Designing and equipping furnace aggregates and shops.

Metallurgy of cast iron.

Thermal processing of steel.

Electivve subjects: Powder metallurgy, Metallurgy of special steels and alloys, Thermal processing of special steels and alloys, Plastic deformation of special steels and alloys, Technology of heating the special steels and alloys, Technology of high quality pig iron and special methods of smelting.

11. Metallurgy of non-ferrous metals (HICT-Sofia) trains engineers - metallurgists for the manufacturing of non-ferrous metals and alloys - copper,

zinc, lead, rare and noble metals etc. The training is carried out along the following subjects:

Pyrometallurgy of non-ferrous metals.
Hydrometallurgy of non-ferrous metals.
Metallurgy of heavy non-ferrous metals.
Metallurgy of light metals.
Metallurgy of noble and rare metals.
Elective subjects: Powder metallurgy, Metallurgy of secondary metals (secondary metallurgy).

In addition to these specialities the HICT - Sofia also trains specialists in the line of Automation of production - engineers in designing, assembling and exploitation of systems of automatic regulation and management of manufacturing in all fields of the chemical and the metallurgical industry.

The course of study in the chemical- technological specialities is 5 years, including the work and defence of the graduation paper. Special attention is paid on acquiring habits of independent work and carrying on scientific research during the training of the chemical engineers. The lecture material is consolidated in the laboratories. The work in then is not only of a study character but includes scientific - research elements as part of the scientific problems and subject matter of the corresponding department. The best students, in the course of study, are included in scientific circles attached to the departments.

The students get practical habits during the time of their practical work at the big chemical and metallurgical enterprises and the enterprises of the light chemical industry, as well as at the scientific - research institutes. The students receive a broad general scientific, general educational, general technical, chemical and special technological training. In contrast to the other technical higher schools of learning, together with the technical subjects higher mathematics, knowledge of machines, technical mechanics electrotechnics etc., the students receive a profound theoretical training in inorganic, organic and physico-chemistry on which are built up the special technological disciplines.

The graduates of the Higher Chemico- Technological Institutes of Sofia and Bourgas are given the qualification of Engineer - Chemist. They can work in the enterprises of the chemical, chemical- pharmaceutical, petrol-chemical, rubber, textile, leather-fur, silicate nitrogen- fertilizer, paper-cellulose industries as well as in enterprises for the manufacturing of chemical fibres, plastics, acids, bases and other ones which apply chemical methods in processing their products. They can also work at designing organisations and scientific - research institutes.

Graduates of the metallurgical specialities recieve a broad training which permits them to work not only in metallurgical enterprises but also in the field of machine construction, energetics transport, on construction projects, instrument making, electric and electronic industries.

The Higher Institute of Food and Gustatory Industry- Plovdiv trains specialists for the needs of the food-gustatory industry along the following specialities:

1. Technology of animal food products, canning and catering establishments. Specialists are trained in this speciality in the technology of canning and industrial processing of foodstuffs and the technology of the products of catering establishments. The training is carried out in five profiles along the following subjects.

a. Profile: «Technology of milk and dairy products».

Technology of milk.

Technological equipment.

Micro-biology of milk and dairy products.

Technology of dairy products.

B. Profile: «Technology of meat and fish».

Raw knowledge of the meat and fish industry.

Technology of meat.

Technology of fish and fish products.

Technological equipment and designing.

Technology of meat products.

Microbiology of meat and fish.

c. Profile: Technology of fruit - vegetable canning.

Commodity knowledge of raw materials.

Technological equipment and designing.

Technology of canning fruits and vegetables - sterile and dry production.

Technology of canning goods and vegetables - juices and concentrated products.

d. Profile: Refrigerating technique.

Refrigerating machines and installations.

Refrigerating technological processes and equipment.

Refrigerating technology of food products.

Trade and transport refrigerators.

e. Technology of the products of catering establishments.

Economics and organization and administrative - economic management of the enterprises of catering.

Technological designing of enterprises of catering.

Technology of products for catering establishments.

Technological equipment.

Special microbiology and hygiene of feeding.

Comodity knowledge of food products.

Accounting reports.

2. Technology of microbiological and fermentation products trains specialists in the technology of wines and strong alcoholic drinks, of beer and non-alcoholic drinks and in some micro-biological products - bio-preparations, vitamins and god's goods. The training is carried out in three profiles along the following disciplies:

a. Profile «Technology of microbiological products».
Technology of god's goods and other protein concentrates.
Industrial microbiology.
Technology of antibiotics.
Technology of vitamins and enzymes.
Technological equipment and designing.
b. Profile: «Technology of wine and strong alcoholic drinks».
Ampelography.
Technology of wine production.
Microbiology of wine production.
Technological equipment and designing.
Technology of strong alcoholic drinks.
Enology.
c. Profile «Technology of beer and non-alcoholic drinks».
Technology of malt.
Technology of beer.
Technology of non-alcoholic drinks.
Technological equipment and designing.
Special microbiology and industrial disinfection.
Fundamentals of refrigerating technique.

3. Technology of vegetable food and gustatory products trains specialists in the technology of industrial processing of vegetable, food and gustatory products. The training is carried out in five profiles along the following disciplines:

a. Profile «Technology of grain and bread products:
Technology of grain storage.
Grain-elevator and storing economy.
Technology of grain processing.
Technology of bread and cereal products.
Technological equipment.
b. Profile «Technology of combined fodders.
Knowledge of raw materials.
Physiology of animal feeding.
Technoloty of combined fodders.
Technological control.
Technological equipment.
c. Profile «Technology of sugar and sugar products, starch and glucose».
Technology of sugar.
Technology of starch and glucose.
Technology of sugar products.
Technological equipment.
d. Profile «Technology of vegetable fats and ethereal oils».
Technology of oil yield.
Technology of oil processing.
Technology of natural and synthetic aromatic products.

Technology of parfume - cosmetic manufacturing.
Technological equipment.
e. Profile «Technology of tobacco and tobacco products»:
Production and drying of tobacco.
Commodity knowledge of tobacco.
Manipulation and fermentation of tobacco.
Technology of tobacco products.
Technological equipment.

The course of study in all specialities at the Higher Institute of Food-Gustatory Industry is five years including the work and defence of the graduation paper. The students receive a broad general scientific and general educational training both in the general theoretical and in the general technical knowledge by studying the subjects: inorganic, analytical and organic chemistry, physical-chemistry, physics, higher mathematics, applied electrotechnics, technical mechanics etc. There is carried on also a special engineering and economic training corresponding to the speciality and the profile. The graduates of the technological specialities of the HIFGI-Plovdiv receive the qualification of engineer - technologist. In addition to the enumerated technological specialities, the HIFGI-Plovdiv also trains students in the specialities of «Machines and apparatuses for the food and gustatory industry» and «Automation of Industry».

The «Clement Ohridski» University of Sofia trains students in «Chemistry» which is sub-divided into:

1. Chemistry for training teachers (Students in this speciality are also trained at the Plovdiv University «P. Hilendarski» and at the Higher Pedagogical Institute — in the town of Shumen. Graduates of this speciality receive the qualification of teachers of chemistry, with a second speciality — physics, at the secondary polytechnical and special secondary schools, the technical schools, the institutes for teachers and at the higher schools of learning.

2. Chemistry in three profiles — inorganic, organic and kibernetics of chemical processes. Graduates of these specialities are specialists in the scientific and teaching work at the higher schools of leaning (universities), for scientific, control - analytical scientific - research institutes.

The existing, in our country, educational institutions of chemistry and chemical technology completely satisfy the needs of specialists of secondary and university education in our chemical and metallurgical industry. A considerable number of young men and women from the developing countries are trained in them, too.

We do not consider everything in our chemical and chemical - technological education to be on the necessary level. We make our contributions for perfecting it and widely make use of the experience of other countries, too. I must, however, admit that we know least the analogous higher schools of learning in the neighbouring countries, the countries in the Balkan Peninsula and naturally we exchange the least experience with them.

I hope that if we accept the practice, at our future conferences, to discusss problems connected with the chemical education, it will be of common interest. These discussions will serve as a good form of exchanging experience and will be an occasion periodically to inform one another about our achievements in training specialists with secondary and university education in the various branches of the chemical industry.

Chimica Chronika, New Series, 9, 259-267 (1980)

THE CHEMISTRY BACKGROUND IN THE EXPLORATION OF A LOW GRADE GOLD - SILVER ORE DEPOSIT

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Summary

Applied methods for the determination and extraction of gold and silver from a particular low grade ore are briefly described. The importance of suitable analytical methods and the role of sampling are discussed with reference to specific technical demands.

Introduction

It very often happens, that the role of chemistry in the development of the mineral industry tends to be under-estimated. In many cases the chemist is simply expected to bring about, as quickly as possible, the necessary analytical results. Modern mineral exploration and processing methods or disciplines like Geochemistry and Hydrometallurgy, which often deal with minute quantities of certain metals or minerals put hard demands not only on the analyst but more generally on the problem solving chemist as well.

First Part

Gold ores fall into three principal categories[1] (Table I).

TABLE I: *OCCURRENCE OF GOLD.*

1. FREE.
2. PARTLY FREE & PARTLY ASSOCIATED WITH SULPHIDES.
3. ASSOCIATED WITH BASE METALS (Pb, Zn, Cu).

(A) Gold ores, in which oxidation of the sulphides has progressed to such an extend that practically all the gold is set free.
(B) Gold ores in which only a part is free, the remainder being associated with sulphides, such as pyrites.
(C) Base metal ores, which, besides lead, zinc or copper, contain gold.

The particular ore, which is the subject of our investigation lies morphologically between the first two groups. Most of its gold is found free

with a very small part associated with sulphides. An average chemical analysis of this ore is shown on Table II.

TABLE II: *AVERAGE CHEMICAL ANALYSIS OF THE ORE.*

Loss on ignition	6.4%
SiO_2	79.0%
$Al_2O_3 + Fe_2O_3 + TiO_2$	9.2%
SO_2	3.0%
Au	1.4ppm
Ag	5.0ppm
Alkalis & Alkaline Earths	2.3%

This kind of gold bearing ore is usually found close to sulphide ores. The exposed parts of the sulphide deposits have a reddish colouration, called «gossan» or «chapeau de for»[2] which resulted from the oxidation of the sulphide minerals.

This covers the unaltered sulphide mineralization. In many cases there is a gradual change from «gossan» to mineralization with layers of a more or less leached material of different composition and thickness.

In our case, as in many other similar cases, the leached material contains precious metals. The occurrence of gold and silver in such ores is normally spotty. This has for the exploration scientist two important implications:

(1) It is almost impossible to distinguish between the gold bearing material and the gangue without a chemical analysis and

(2) Extremely careful sampling is necessary in order to establish the average content of gold. We shall examine this point later.

The principal methods used in gold ore beneficiation to-day do not differ much from the methods used fifty or more years ago. These are: the cyanidation, the amalgamation, the different ways of gravity concentration and the flotation. In practice one or more of these methods may be used in a combination which depends on the composition and the properties of the ore.

The product of these primary concentration methods is a gold-silver concentrate or precipitate of variable content, ranging from say 50 ppm gold, or even less, to 40% gold or more, depending on the method and the ore. These concentrates are then smelted to produce gold and silver of different purities.

There have many efforts to introduce other leaching agents, besides cyanides, mainly in order to avoid the slow action of cyanides and their toxicity. One such agent is thiourea[3], first proposed by a number of Russian scientists. Although thiourea's properties are promising, its introduction as a leaching agent for gold is not yet known to be satisfactory.

Perhaps the most remarkable innovation in recent years is the rebirth of two processes related to cyanidation:

(1) The in situ leaching with cyanide solutions[4] and

(2) The carbon in pulp process[5,6] for the recovery of gold and silver from the pregnant solutions.

Second Part

It is self evident, that accuracy and precision are of the greatest importance when one has to do with precious metals. The difference of few tenths of a part per million gold in the analysis of a mill feed might be decisive for a multi-million dollar project.

Thus the important question arises: Is trace element analysis advanced enough so as to provide the necessary reliability in precious metal metallurgy to-day?

The scientists do not seem to be very happy with the results of trace element analysis. For example in July 1979 at the 8th Scandinavian Trace Element Analysis Meeting in Veile, Denmark, J.C. Tjell[7] stated in the course of a discussion that «the great majority of results of trace element analysis is not very good». In their excellent book «Trace Element Analysis of Geological Materials»[8] R.D. Reeves and R.R. Brooks devote several discussions to the different sources of error and their theoretical handling. They state at the end of the book that «the choice of acceptable levels of analytical errors is arbitrary» and that «the relative importance of accuracy and precision varies in different situations».

Indeed a precious metals analyst does not have much choice. In most cases his results must be both accurate and precise. In some other cases accuracy may not be absolutely necessary but precision, or reproducibility of the results, must be guaranteed.

Probably the most commmon source of error in a low grade gold ore analysis lies in sampling. When handling traces of materials a very careful procedure of selection of a representative sample, grinding, homogenizing and sample parting has to be followed. But still the most serious sampling error can occur when a small subsample is finally selected for analysis[8].

This is understandable, because there can be no guarantee that the few gold and silver grains present are evenly distributed in the sample. This becomes perhaps more critical for the free precious metals in an ore, than for any other trace element, as they are soft and malleable, and any effort to grind the whole sample to a very fine powder in order to have gold and silver evenly distributed, fails, because these are smeared on the tools used for grinding[9]. The best remedy against this final sampling error is to use as big a sample as possible and take the average of several analyses.

That is the main reason why the 400 years old fire assay method, which uses up to 30 g of sample, is still considered to be the most accurate. When coupled with modern instrumentation the method enables gold determinations even in the ppb level. A short description of this method follows:

It is essentially the same procedure that smelters use to extract precious metals from bulk minerals and consists mainly of two steps: The *crucible and*

TABLE III: *FIRE ASSAY.*

FUSION:
Sample + Na_2CO_3 + Borax
+ C (altern. + KNO_3) + $CaF_2 \rightarrow$
Pb containing precious metals
+ Slag

CUPELLATION:
Pb cont. precious metals
O_2
$\rightarrow$PbO + precious metals
ΔT

the cupellation. Both take place in muffle furnaces at temperatures around 900°C. The object of the fusion is to obtain a two phase melt consisting of slag and metallic lead. The metallic lead phase contains the noble metals gold and silver and most of the platinum metals, which are separated from lead during the cupellation.

Fusion: Aproximately 15 to 30 g of sample are mixed with an excess of litharge (PbO) in a fireclay crucible. Other ingredients mixed with the sample include sodium carbonate, pure silica, borax and flour. The purpose of the lead oxide is such, that when reduced by the carbon of the flour to lead, it alloys with the precious metals. The droplets of lead settle through the molten charge extracting the precious metals with high efficiency. The molten charge is poured into a mould where the lead button collects at the bottom under the now barren slag. After cooling and solidification the lead button is broken from the slag and cleaned. At this stage gold and silver have not been concentrated, but have only been extracted from a sample of complex composition into almost pure lead.

Cupellation: This is the concentration step in the fire assay method. The lead button is placed in a preheated bone ash cupel in the hot furnace with a good air supply. The lead is reoxidized to litharge, which is absorbed by the cupel, leaving a pure bead of precious metals. In cases that the content of the sample in gold and silver is expected to be low, a few milligrams of silver is added in the original sample in order to obtain a larger bead.

The addition of silver serves also to facilitate the later dissolution of silver in nitric acid and to reduce gold cupellation losses[10]. The analysis of the precious metal bead is quite simple:

In the case of samples rich in gold and silver, the latter is dissolved in nitric acid and the remaining gold is determined by weighing, using an electronic microbalance. When the sample contains very small amounts of gold (it is known that the human eye can hardly discern a 5mg piece of gold), either Atomic Absorption or emission spectrography is applied.

Other methods of gold analysis

It has been mentioned, that fire assay is the most accurate method for the determination of gold. However, there are cases in which other analytical practices are preferable.

1. When only a qualitative to semi-quantitative test for gold is required, a very simple method can be applied: The sample is ground to pass 300 mesh. 5g sample is leached for 24 hours with 25ml of a 2% NaCN + 1% NaOH solution. The gold content of the filtrate is determined by Atomic Absorption using an instrument with background correction and checking the results by fire assay at regular intervals.

W.J. Price in his recent book[11] mentions that «cyanide itself appears to give rise to differences in the response of gold by atomic absorption». Our experience supports this statement. The cyanide extraction of geological samples is a simple qualitative method, whose main advantage is its capability of screening a large number of samples. The exact content of gold has to be determined by fire assay.

2. The whole procedure of the fire assay takes 6 to 7 hours. This time is too long in certain cases. For instance in flotation plants the grade (ppm gold) of the product concentrate can often vary considerably from to time. It is, however, important for the plant operators to know as quickly as possible the approximate quality of the concentrate so that they may be able to make the necessary adjustments.

In such cases a quicker method of gold analysis is necessary. Emission spectrography might be the best method, but practical difficulties urged us to develop a new method, namely the extraction of the flotation concentrate with alkaline cyanide solution in which hydrogen peroxide is added during refluxing to accelerate the dissolution of gold. The so dissolved gold is determined by Atomic Absorption. The table IV shows that the agreement between fire assay and cyanide - peroxide - reflux - A.A. is quite good. Our method is in brief as follows: 0.5 g of finely ground flotation concentrate is heated under reflux and continuous stirring with 48ml of a solution containing 2% NaCN and 1% NaOH. One ml 30% H_2O_2 is added at the begining of the extraction and another ml one hour later. After a total of two hours refluxing, the liquid is cooled, filtered and the gold content measured by Atomic Absorption using alkaline - cyanide gold standards.

3. Of course there are several other analytical methods for gold. For example the dissolution of gold with aqua regia and then conversion into the gold bromide complex. This is extracted in methyl isobutyl ketone and the organic phase used for the determination of gold by Atomic Absorption. Some other methods start from gold cyanide solutions and use also extraction in organic solvents[13,14]. These methods might be useful in some cases, but have not found a wide application in our Laboratories.

TABLE IV: *GOLD ANALYSIS COMPARISON OF METHODS. Numbers represent ppm gold (all single, plant determinations).*

F.A.-A.A.	C.N.-A.A.	F.A.-Ag dis.
58	62	
78	78	70
62	64	
83	83	
91	90	
101	102	95
78	78	70
159	145	140
408	388	380
318	316	318
230	226	216
240	240	240

Silver trace analysis

Silver usually accompanies gold in nature and often constitutes a considerable economic factor when discussing the feasibility of a project. Therefore, it is obvious that accurate silver determinations at very low concentrations are of primary importance as well. Fire assay gives for silver fairly good results, although silver losses during the analysis, especially cupellation losses, are usually higher than gold losses. When the sample contains only a few parts per million —as it is normally the case for feeds or tailings— this problem becomes critical. One must consider that silver is used as a collector for gold in cupellation, as already mentioned. Theoretically gold can also serve as a collector for silver, but then the dissolution of the gold - silver bead would become very difficult as silver does not dissolve in nitric acid when the proportion of silver to gold is less than 3 to 1.

The use of aqua regia would complicate things further because of the possible precipitation of AgCl. On the other hand it is not easy to extract silver in an organic solvent and so improve its limit of detection by Atomic Absorption, as in the case of gold.

Nevertheless it is possible to extract silver directly from a solid sample with nitric acid digestion for Atomic Absorption determination[15]. Our experience shows that this method does not work always well and often gives incomplete extractions and too low results. Better results are obtained when bromine is used as an oxidation means before adding nitric acid. One must of course keep in mind, that the accuracy of silver determinations for low grade silver ores by Atomic Absorption is small because of the difficulty to bring the silver concentration up to the detection limit of Atomic Absorption.

Third part

The mineralogical examination of our ore revealed that gold occurred in

native form as cauliflower - like grains of a high variation in grain size (from about 5μ to approminately 120μ). Gold was not tied to pyrite.

These findings did not exclude the possibility of cyanidation of our ore. Still cyanidation did not give promising results.

At this stage it should be pointed out that a metallurgist usually checks his tests and the work of an enrichment plant by compairing the analyses of feed, tailings and product. The sum: product + tailings = feed must be approximately right.

When working with gold in low concentrations this is not always the case. While the analysis of gold concentrates may be quite accurate, the analyses of feed and tailings are not always accurate enough for the metallurgist, mainly because of the inherent difficulties in sampling and the limits of the method of analysis.

Our ore was a stock pile of approximately 100,000 tons. Apart from this we knew that we had to consider a larger quantity of the same auriferous ore which was not yet mined.

Three different samples were taken from the stock pile. Their average gold content was found to be approximately 2.4 ppm. After exploitation the average of all daily analyses of the feed had shown approximately 1.3 ppm. On the other hand while the tailings of the tests were shown to contain in the average 1.0 ppm, the average analysis of all tailings after the flotation of the ore was 0.4 ppm. These serious discrepancies are typical of the errors in sampling analysis, which are to be expected when working with gold in very low concentrations.

In general the gold ore beneficiation combines cyanidation with other methods. Direct cyanidation, bulk flotation and subsequent cyanidation of the tailings or of the concentrate are some typical examples of auriferous ore treatment. The tests we carried out were in short as follows:

I. Cyanidation tests

Agitation tests were conducted in rolling bottles at a pulp density of 40%. Different cyanide strengths were used (0.1%, 0.05% and 0.025% NaCN) and the progress of the gold extraction was followed by Atomic Absorption. Lime and cyanide consumption were found by oxalic acid and silver nitrate titration respectively.

It was very soon apparent that the ore was not liable to cyanidation. Very fine grinding (to - 300 mesh) was necessary in order to obtain an acceptable recovery and it was estimated, that the cost for this undertaking would run too high (Table V).

Concurrently to the agitation –leaching other cyanidation tests were conducted. A simulated heap– leaching test was designed during which 25 kg of ore were continuously spread by a weak (0.025%) cyanide solution. Lime was used as a pH regulator (pH≧9), but after several weeks the idea of heap leaching was abandoned as the recovery was very poor. (less than 10%).

TABLE V: *AGITATION LEACHING RECOVERIES.*

Mesh size	Recovery in 24hrs	NaCN g/ton	Lime g/ton
80% – 35	~14%		
80% – 65	~25%		
80% – 240	~60%	800	650
80% – 450	~88%		

TABLE VI: *ANALYSIS OF FLOTATION CONCENTRATE.*

SiO_2	22.5%	Ag	800ppm
S (as S)	22.5%	Au	200ppm
Fe (as Fe)	40.0%	Se	300ppm
Al_2O_3	1.5%	Cu	0.5%
CaO	0.6%	Ni	100ppm
MgO	0.3%	Pb	80ppm
		As	50ppm

II. Other tests: Flotation

Gravity concentration showed also no promising results and separation of the magnetic minerals did not give a significant improvement. On the contrary the flotation tests were promising from the very begining. Different mesh sizes of the ore were tested in a laboratory flotation cell and it was established that a small quantity of a pyritic material was floatable. The analysis of one of the first flotation concentrates showed values of approximately 170 ppm gold and 460 ppm silver. This result was confirmed by other laboratories and a long series of flotation tests was started which aimed at finding the optimum conditions for the flotation of the ore. In particular the effect of mesh size reagents pH and pulp density were tested.

Satisfactory results were obtained at pH - values between 6 and 8.5 with a mixture of xanthates and dithiophosphates acting as collectors at a pulp density of approximately 30%.

Sodium carbonate was used as a pH - regulator where necessary, and the presence of lime was avoided it is a depressant for gold.

Aknowledgments

I would like to thank the management of Hellenic Mining Company and the Pancyprian Association of Chemists for encouraging and supporting me to underkate this work.

Literature

1. American Cyanamid, Mining Chemicals Handbok, Mineral Dressing Notes 26, page 1-3.
2. C.F. Blain and R.L. Andrew, Sulphide weathering and the evaluation of gossans in mineral exploration, Mineral Sci. Eng, vol 9, No. 3, 119-150 (1977).
3. T. Groenewald, The dissolution of gold in acidic solutions of Thiourea, Hydrometallurgy 1:277-290 (1976).
4. F.F. Applan, W.A. McKinney and A.D. Pernichele, Solution Mining Symposium, Proceedings, AIME Annual Meeting, Dallas, Texas, 1974.
5. World Mining, Nov. 1974, p. 44 to 49.
6. World Mining, Aug. 1977, p. 56 to 59.
7. F.M. Nicolaisen and P.W. Jense, European Spectroscopy News 27, 25-26 (1979).
8. R.D. Reeves and R.R. Brooks, Trace Element Analysis of Geological Materials, John Wiley and sons, Chemical Analysis volume 51, New York, 1978.
9. C.J. Williams, H.J. Seidemanh, Jr. and R.E. Hawley, Precious Metal Assay. Is fire assay here to stay? American Laboratory, August, 1975.
10. J. Haffty, L.B. Riley and W.D. Gose, A manual on fire assaying and determination of the noble metals in geological materials, U.S. Geological Survey bulletin 1445, U.S. Government pringing office, Washington 1977.
11. W.J. Price, Spectrochemical Analysis by Atomic Absorption, Heyden, London, 1979.
12. C. Huffman, Jr., J.D. Mensik and L. B. Riley, Determination of gold in geologic materials by solvent extraction and atomic-absorption spectrometry, U.S. Geol. Survey Circ. 544 (1967).
13. F.W.E. Strelow, E.C. Feast, P.M. Mathews, C.J.C. Bothma and C.R. van Zyl, Anal. Chem., 38, 115 (1966).
14. T. Groenewald, Anal. Chem. 40, 863 (1968).
15. C. Huffman, Jr., J.D. Mensik and L.B. Riley, Determination of silver in mineralized rocks by atomic-absorption Spectrophotometry U.S. Geological Survey Prof. Paper 550-B p. B189-191 (1966).
16. J.V.N. Dorr, Cyanidation and Concentration of gold and silver ores, Mc Graw-Hill, New York, 1936.

Chimica Chronika, New Series, 9, 269-283 (1980)

COMPARATIVE MERITS OF THE IMPEDANCE AND RESONANCE METHODS IN STUDYING PROPERTIES OF ELECTRIFIED INTERFACES

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Summary

The impedance method and the resonance method of identifying parameters of the complex impedance of an electrochemical cell have been analyzed in their capacity to render accurate results. This has been done by deriving relationships between the errors in determining electrode capacitance, C, the resistance to the charge transfer, Θ and the resistance of the electrolyte R, as cell parameters and the errors in measured quantities in the impedance and the resonance method. Using computer programs, and assumming one and the same precision of instrumentation for both methods (0.1%), the relationships have been applied systematically to all points of a three-dimensional matrix C-Θ-R covering the range of those parameters that are likely to be met in electrochemical systems. Space has been separated into that part in which the parameters can be determined with an acceptable accuracy (10%) and this where the error is likely to be larger. It was shown that the resonance method has an advantage over the impedance method in determining electrode capacitance and resistance of the electrolyte in that a wider portion of the matrix falls into the acceptable error space.

Key- words:
- Electrochemical cell, impedance of
- Electrochemistry, resonance method in
- Electrochemical measurements, accuracy of.
- Electrode capacitance, determination of
- Faradaic resistance, determination of.

Introduction

a) The Impedance Method

Chemists are interested in electrical properties of interfaces solid-solution, since these reflect the structure of those interfaces and the mechanism and kinetics of processes taking place there[1,2].

As far as the passage of current through an interface metal-electrolyte is concerned, the situation can in general be represented by an equivalent circuit, as this shown in fig. 1., where C is a reactive component of this complex impedance, reflecting capacitive properties (accumulation of decrease of charged

particles at the interface), Θ is the resistance to charge transfer across the interface, resulging in a chemical change (reduction or oxidation), W - is a complex so-called Warburg impedance, which is best approached by a transmission-line electrical model and which reflects diffilculties in transport of the reacting material towards and away from the interface, and R is the resistance of the electrolyte.

The active and the reactive components of the impedance, Z′ and Z″ resp., can be measured by set of instruments, shown in fig. 2, the core of which represents a phase selective voltmeter (a lock-in-amplifier).

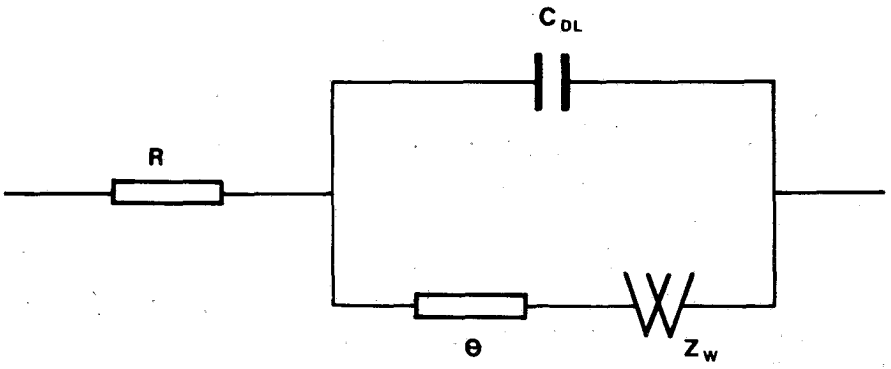

FIG. 1: *Randles equivalent circuit of an electrified interface with electrochemical process. C - capacitance; Θ -resistance to charge transfer process; W -Warburg - impedance; R -ohmic resistance ot the electrolyte.*

The components, Z′ and Z″ are related to the four properties of the interface, C, Θ, W and R by well defined relationships[3]

$$Z' = R + \frac{\Theta + \sigma\omega^{-1/2}}{(\sigma\omega^{1/2}\ C+1)^2 + \omega^2C^2\ (\Theta+\sigma\omega^{-1/2})^2} \qquad (1)$$

$$Z'' = \frac{\omega C\ (\Theta+\sigma\omega^{-1/2})^2 + \sigma\omega^{-1/2}\ (\sigma\omega^{1/2}\ C+1)}{(\sigma\omega^{1/2}\ C+1)^2 + \omega^2C^2\ (\Theta+\sigma\omega^{-1/2})^2} \qquad (2)$$

where ω is the frequency of the alternating current flowing through the interface. Conversly, by studying the former as functions of frequency the latter should be quantitatively identifiable. If, for example, one has a system in which difficulties in transport are negligible so that the Warburg impedance can be neglected over the whole range of conditions of an experiment, the equations are considerably simplified

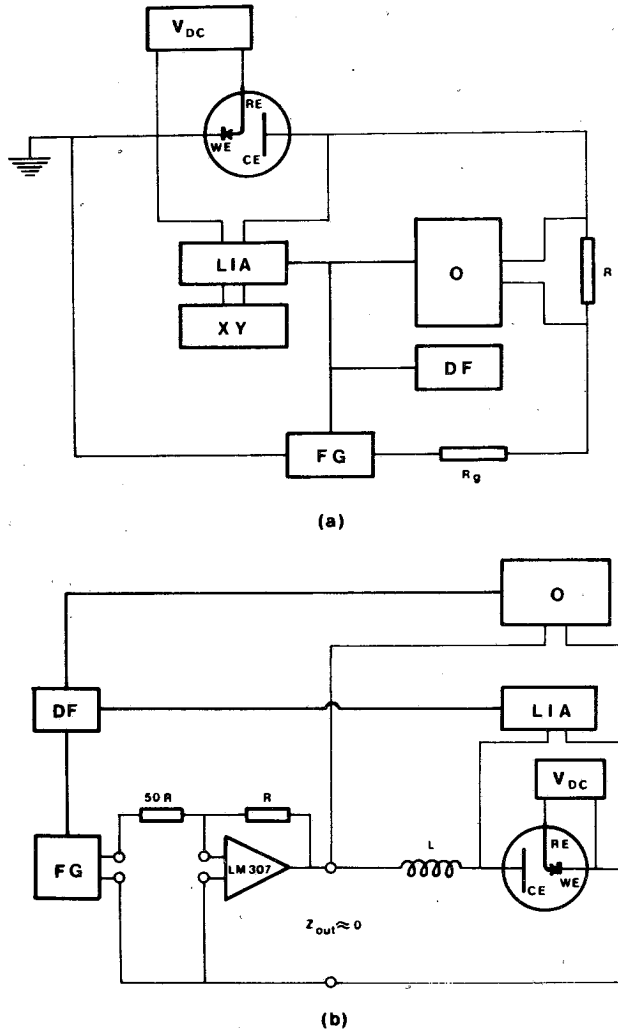

FIG. 2: *Impedance (a) and resonance (b) measuring circuits. FG - function generator, R_g - current controlling resistor, R - current measuring resistor, O - oscilloscope, DF - digital frequency meter, LIA - phase sensitive lock-in-amplifier, XY - recorder, V_{DC}-DC voltmeter, C - electrochemical cell.*

$$Z' = R + \frac{\Theta}{1+\omega^2 C^2 \Theta^2} \tag{3}$$

$$Z'' = \frac{C\Theta^2}{1+\omega^2 C^2 \Theta^2} \tag{4}$$

and the problem of identification of C, Θ and R can be resolved in the following manner:

Combining (3) and (4) to eliminate the frequency ω, one obtains the so-called complex plane (Z′ — Z″ plane) solution which is an equation of a semi-circle.

$$[(Z'-R) - \frac{\Theta}{2}]^2 + Z''^2 = (\frac{\Theta}{2})^2 \quad (5)$$

Indeed, such a situation is experimentally obtained, as shown in fig. 3, for example at a platinum electrode immersed into an aqueous solution containing ferrous and ferric-ions, where the chemical processes are those of reduction of ferris ions and oxidation of ferrous ions by the electric durrent[4].

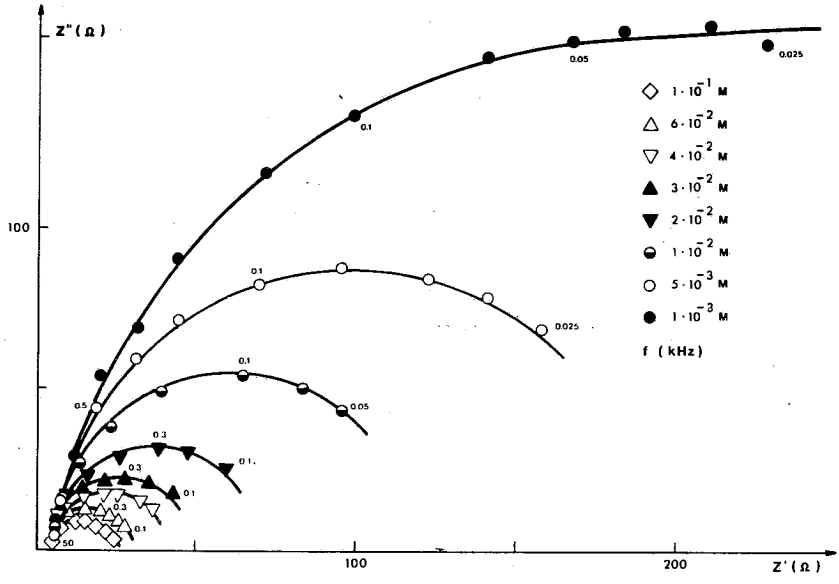

FIG. 3: *Complex plane representation of active (Z′) and reactive (Z″) impedances of the ferrous-ferric system on bare platinum at different concentrations (numbers at the points denote frequencies in kHz).*

By virtue of the properties of the equation of semi-circle the parameters Θ and R are found from the radius, which is equal to Θ/2 and the position of the center at the abscissa, which is equal to Θ/2 + R.

The capacitance, C, can be obtasined by rearranging equation (4) in such a way as to obtain equation (6). It is seen that C is the slope of a linear depen-

dence of ω/Z'' on ω^2. The above cited experiments have indeed rendered such a linearity as seen in fig. 4, for most concentrations of the redox couple.

$$\frac{\omega}{Z''} = C\omega^2 + \frac{1}{C\Theta^2} \tag{6}$$

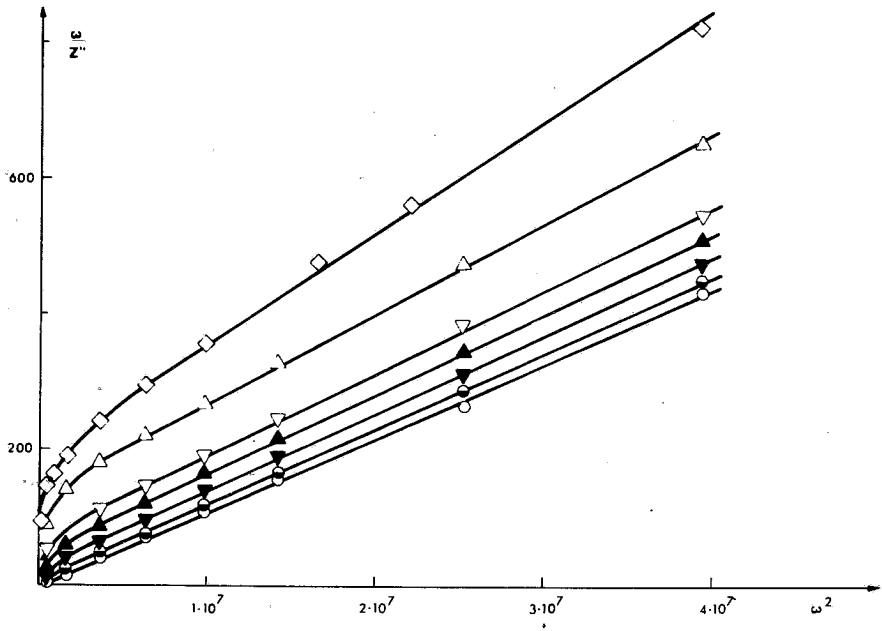

FIG. 4: *Plot of measured reactive impedance of the bare platinum electrode in the* $\omega/Z'' - \omega^2$ *plane for different concentrations of the ferrous and ferric ions.*

b) The Resonance Method

Not too long ago[5,6,7], we have realized that the capacitive nature of the metal-solution interface should produce resonance phenomena if the electrochemical cell is connected to an induction coil in series or in parallel as shown in fig. 5. The resonance is seen as a rather sharp amplification of the voltage across the cell for a constant voltage at the terminals of the resonance circuit, the amplification factor $A = U_c/U$, attaining a maximum value, Ar, at, a given value of frequency ω_r.

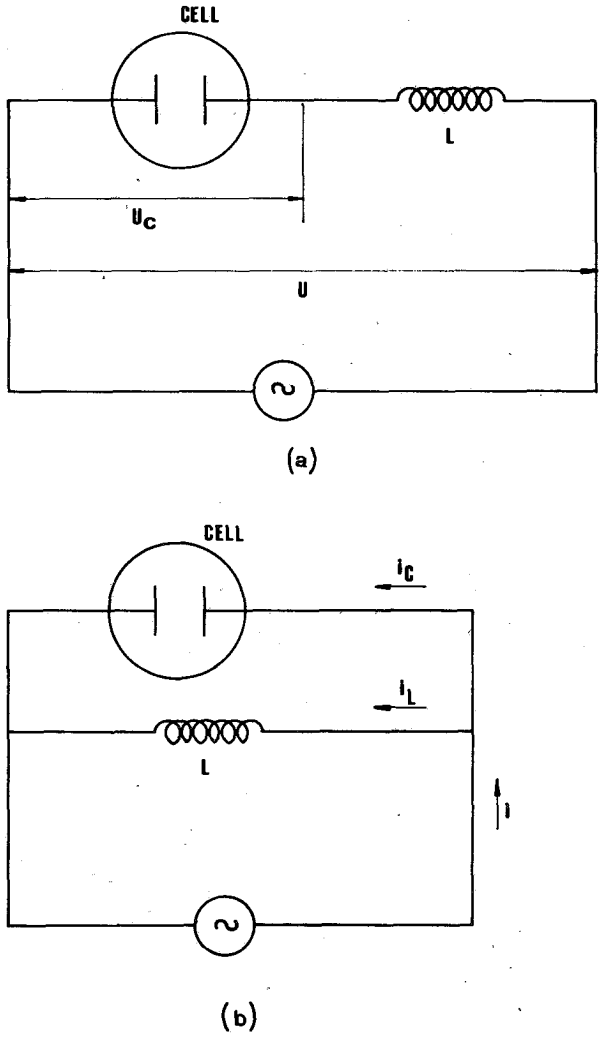

FIG. 5: *Electrochemical resonance circuit.*

Measurement of the resonance spectrum and detection of A_r and ω_r requires a less sophisticated instrumentation than the impedance measurements in that it does not require phase sensitive devices. Ordinary RMS voltmeter should suffice. As an example, the resonance spectrum for the same- experimental system as described above, is shown in fig. 6.

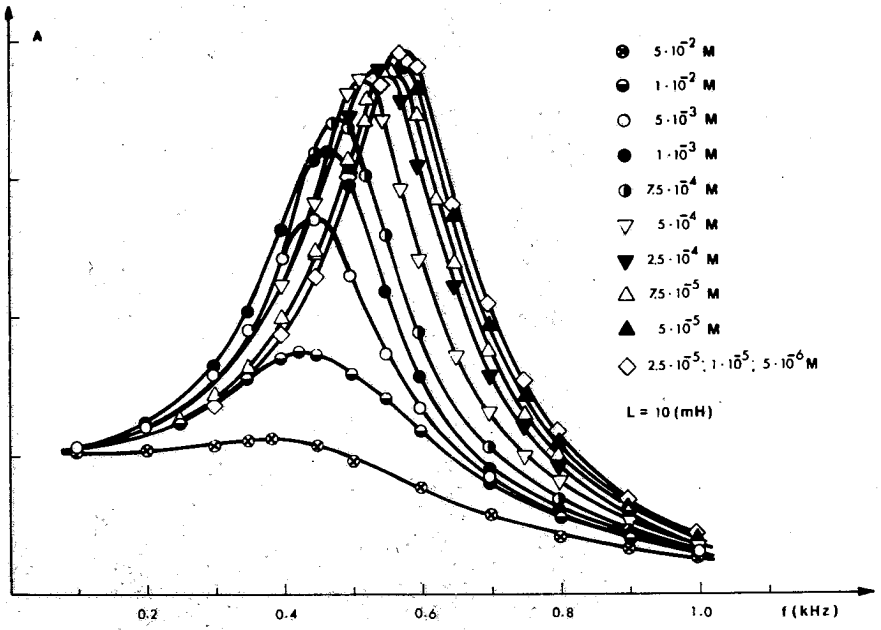

FIG. 6: *Experimentally obtained resonance spectrum obtained with bare platinum in different concentrations of ferrous and ferric ions. (L = 10 mH).*

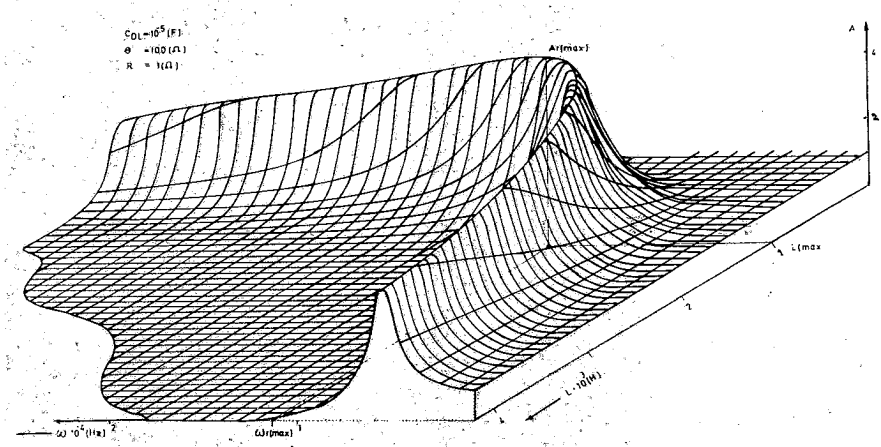

FIG. 7: *Dependence of the amplification factor, A, on inductance, L, and frequency of the applied alternating current, ω, when an electrochemical cell (with $C = 10^{-5}$ F, Θ = 100 ohms, R = 1 ohm) and an induction coil are connected in series.*

The resonance spectrum follows a well defined relationship between A and ω which contains the elements of the equivalent circuit simulating the electrochemical cell, as parameters. It depends also on the applied inductance of the coil. Fig. 7 shows a computer calculation of resonance spectra for different inductance values ranging between 0.1 and 4 mH. It is seen that there is a definite L value, $L_{(max)}$, at which the resonance amplification factor is at maximum, i.e. there is a maximum maximorum of A, $A_{r(max)}$, at a given frequency $\omega_{r(max)}$. Relatively simple equations relate these quantities to the elements of the equivalent circuit.

$$L_{(max)} = \frac{CR\Theta^2}{\Theta + 2R} \tag{7}$$

$$\omega_{r(max)} = \frac{(R + \Theta)/R^{1/2}}{C^2\Theta^2} \tag{8}$$

$$A_{r(max)} = \frac{(\Theta + 2R)^{2 1/2}}{4\ (\Theta+R)R} \tag{9}$$

Conversly, those elements can be quantitatively identified by measuring the above resonance parameters if equations (7), (8) and (9) are appropriately solved

$$C = \frac{A_{r(max)} + [A^2_{r(max)^{-1}}]^{1/2}}{2\omega^2_{r(max)}\ L_{max}\ A_{r(max)}} \tag{10}$$

$$\Theta = 2\omega_{r(max)}\ L_{(max)}\ A_{r(max)} \tag{11}$$

$$R = \frac{\omega_{r(max)}\ L_{(max)}\ A_{r(max)}}{A^2_{r(max)} - 1 + A_{r(max)}\ [A^2_{r(max)^{-1}}]^{1/2}} \tag{12}$$

II. Analysis of errors in identifying electrode properties

The question arises, if both the impedance measurements and the resonance measurements can be made (and the latter can be done by the same instrumentation as the former with the addition of an induction coil only), which ones of the two are likely to give more accurate values of the equivalent circuit ele-

ments? The answer to this question is the main subject of this communication.

The analysis of possible errors is made in the following way:

Let the precision of the measuring instrument be (%). This is equal to the relative indeterminacy of the measured impedance values. The probable error should be smaller than that by the factor 0.6745. Hence, if one has a set of measurements plotted in the graph, as shown in fig. 8, there is likely to be a scatter of points within the shaded area. This results in an indeterminacy of the slope, so that it can be anywhere in between the value of $C + \Delta C$ and $C - \Delta C$. This enables deriving the expected relative error, $\Delta C/C$ as depending on the precision of the instrument S, and also on the values of all of the elements of the equivalent circuit C, Θ and R, since measured Z'' values in the graph are essentially determined by these values.

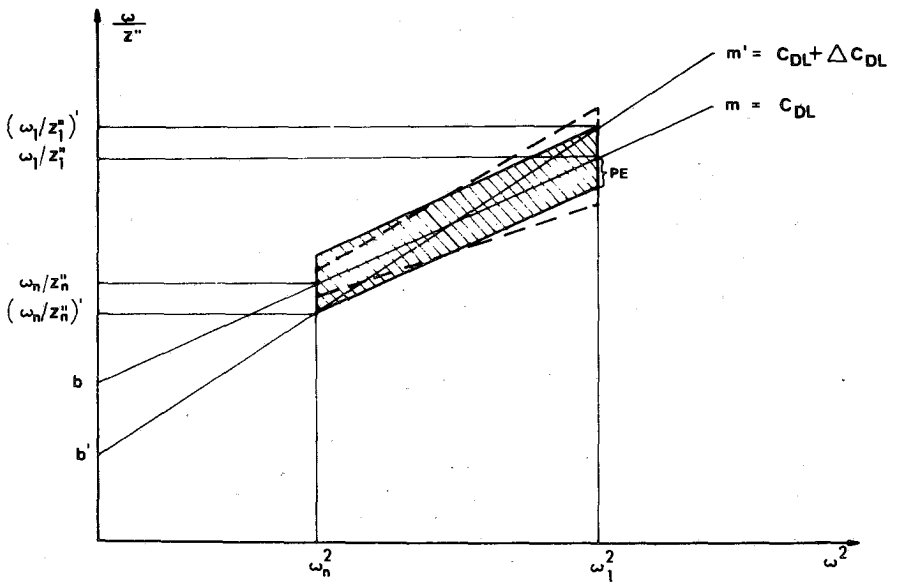

FIG. 8: *A schematic representation of the indeterminacy in the evaluation of the slope and intercept from the ω/Z'' vs. ω^2 function.*

A similar situation is with the complex plane representation of the two impedance components. Although in principle the measured points should cover a semi-circle, in reality and because of lower and upper limits of frequency that can be used only parts of semi-circle are covered, as seen in fig. 9 for three different sets of C, Θ and R values. Hence, indeterminacies arise in the evaluation

of the radius of the semi-circle and of the position of its center, shown in fig. 10 based on similar reasoning as that used for estimating the error in determination of C. Thus, equations for $\Delta\Theta/\Theta$ and $\Delta R/R$ could be derived, again as function of S and of the values of equivalent circuit elements.

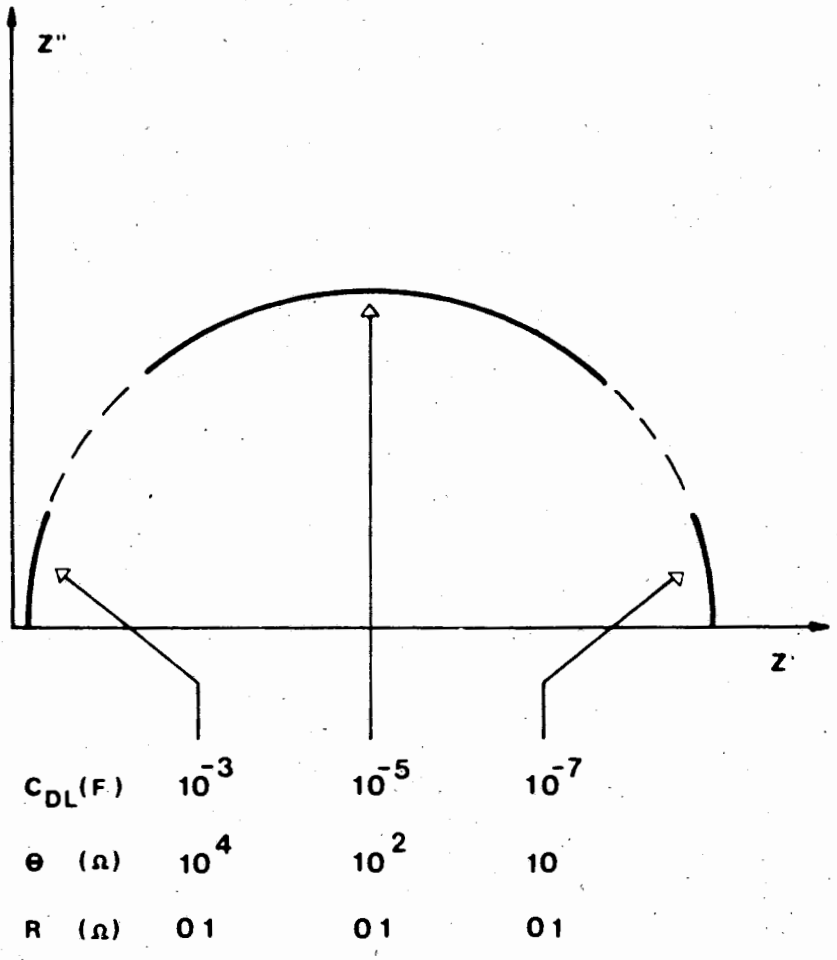

FIG. 9: *Segments of the semicircle of impedances covered by experimental points for three different cases of electrodes with activation controlled electrode processes when impedance measurements are made in the frequency range of 1 - 10^5 Hz.*

A computer programme has been set which provided the calculation of $\Delta C/C$, $\Delta\Theta/\Theta$ and $\Delta R/R$ for all the points in a three-dimensional matrix C-Θ-R covering the range of values of those three elements which are likely to be found in electrochemical systems. A limit of 10% was chosen as an acceptable

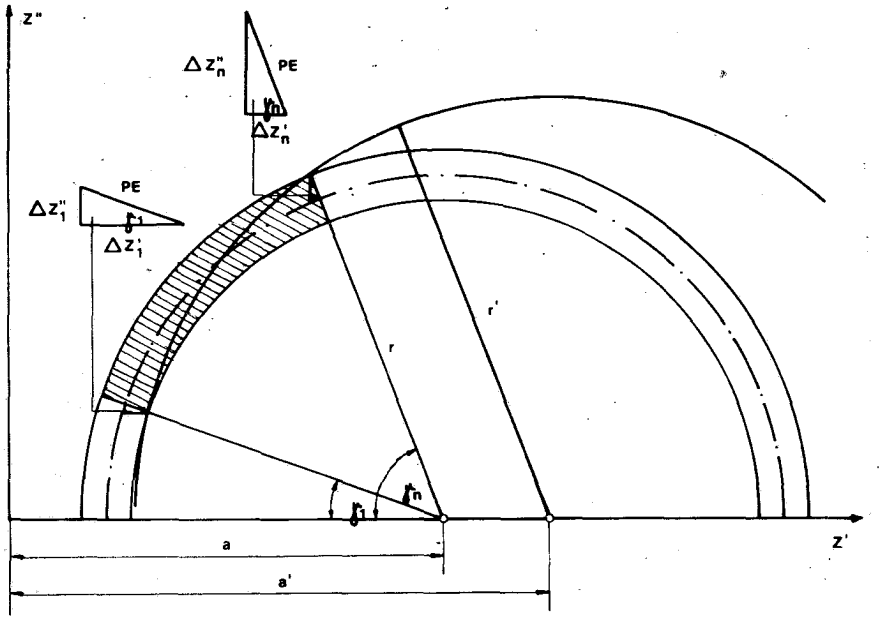

FIG. *10: A schematic representation of the indeterminacies in the evaluation of the position of the center (a) and of the radius (r) of the semicircle of impedances of an activation controlled proces in the complex plane.*

error in determining C, Θ or R from impedance measurements and the space C-Θ-R was divided into a box within those parameters can be determined with an accuracy equal to or better than that, and a space outside where the error is likely to be larger. The result is shown in fig. 11. It is seen that limitations in determining R and especially C are very serious. It comes out that the impedance method can satisfactorily be used for capacitance determinations only in a very limited range of conditions.

A similar analysis has been undertaken for the maximum resonance method. There, as seen schematically in fig. 12, the indeterminacies in determining $L_{(max)}$, ΔL_{max}, and $\omega_{r(max)}$, $\Delta\omega_{r(max)}$, depend not only on the probable error in measuring $A_{r(max)}$ but also on the sharpness of the resonance peak in both ω and L directions.

$$\Delta A_{r(max)} = 0.6745 \cdot \frac{S}{100} \cdot A_{r(max)} \qquad (13)$$

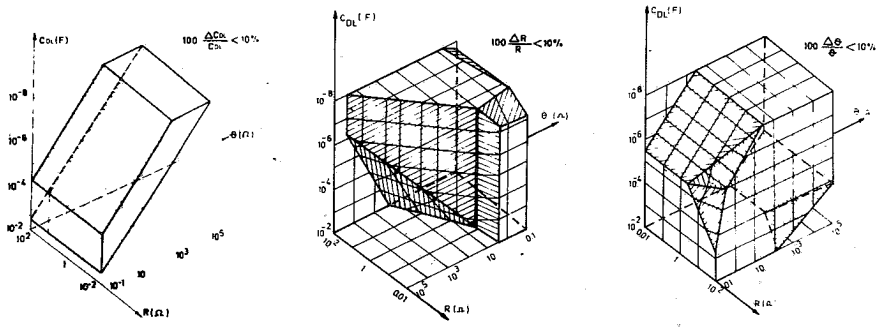

FIG. 11: *Boxes of C-Θ-R points in which those parameters can be determined with an accuracy better than 10% by the impedance method, when the precision of the measuring instrument is 0.1%.*

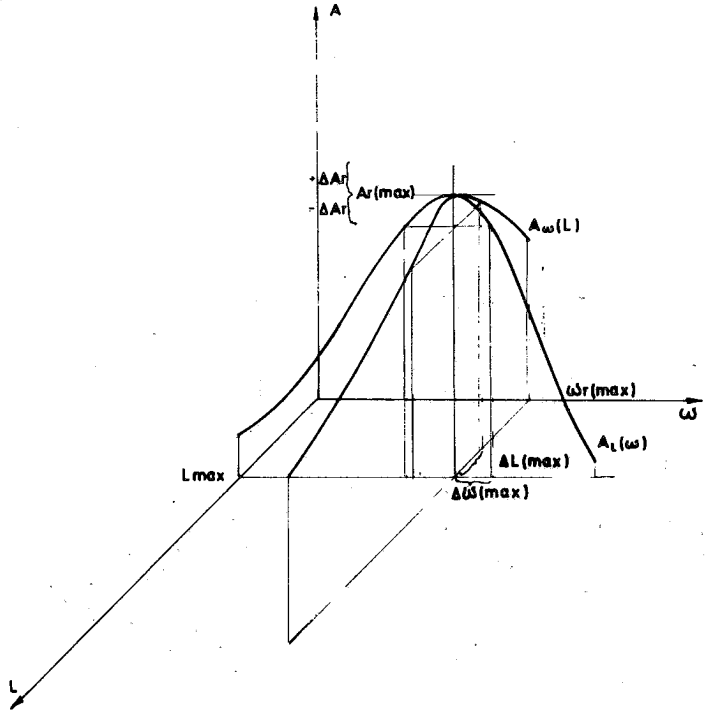

FIG. 12: *A schematic representation of indeterminacies in determining $\omega_{r(max)}$ and $L_{(max)}$, which are caused indeterminacy in measuring the maximum resonance amplification factor, $A_{r(max)}$.*

Thus equation (13) shows the probable error in determining $A_{r(max)}$, while $L_{(max)}$ and $\omega_{r(max)}$ can be obtained by solving first the equation for the resonance spectrum for $L_{(max)}$ and then for $\omega_{r(max)}$, and then finding for each set of C, Θ and R values, both true (with $A_{r(max)}$) and erroneous values (with $(A_{r(max)}) - \Delta A_{r(max)})$) of $L_{(max)}$ for constant $\omega_{r(max)}$ and vice-versa. The indeterminacies $\Delta L_{(max)}$ and $\Delta\omega_{r(max)}$ can be composed as shown in equation (14) and (15).

$$\Delta L_{(max)} = f\,[\omega_{r(max)},\ (A_{r(max)} - \Delta A_{r(max)})] - f\,[\omega_{r(max)}\ A_{r(max)})] \qquad (14)$$

$$\Delta\omega_{r(max)} = f\,[L_{max},\ (A_{r(max)} - \Delta A_{r(max)}] - f\,[L_{max},\ A_{r(max)})] \qquad (15)$$

Maximum probable errors in deriving C, Θ and R from resonance measurements are obtained when the probable erroneous values of $A_{r(max)}$, $L_{(max)}$ and $\omega_{r(max)}$ obtained as $A_{r(max)} \pm \Delta A_{r(max)}$, $L_{(max)}$, $\pm\ \Delta\Lambda_{(max)}$ and $\omega_{r.(max)} \pm \Delta\omega_{r(max)}$, are placed into equations (10), (11) and (12) in the worst combination, as to obtain maximum deviation of $(C+\Delta C)$, $(\Theta+\Delta\Theta)$ and $(R+\Delta R)$ from C, Θ and R obtained from the same equations using correct values of $A_{r(max)}$, $L_{(max)}$ and $\omega_{r(max)}$, and contrasting the former with the latter.

All this has been built into a computer programme, so as to calculate probable errors in C, Θ and R for a given precision of the measuring instrument S and for all combinations of C, Θ and R in the same three-dimensional matrix as used in the previous calculation.

Another limitation had to be built the programme: the resonance peak has to be within those limits of ω and L which can be used in practice. The same limits of ω have been taken as in impedance measurement evaluation, while L was limited to a range between 10^{-6} H and 10 H. Three signs have been denoted for the computer response for each C-Θ-R point: one for the resonance maximum being within the measuring range and the probable error being within acceptable limits, e.g. 10%, another one for the maximum being outside the measured range and the third for maximum being in the measuring range but the error being outside the acceptable limits.

The numerical result showed that the C-Θ-R space built of the points rendering the first sign is the same for determining all the three equivalent cell parameters, and is shown in fig. 13. The major reason for some space staying outside the acceptable measurement box, proved to be in that the maximum resonance fell outside the measuring range, rather than in the error being larger than 10%. In any case the space in which acceptable measurements can be made by the maximum resonance method proved to be much larger than that for the impedance method when electrode capacitance is concerned. In R and in Θ determinations the two methods partly cover different parts of the space. This indicates that the methods should be considered as complementary. Hence, a good impedance measuring device should have an inductance decade box at hand, to use one or the other method, according to fabour indicated by the space in which the C-Θ-R combination to be measured, is found.

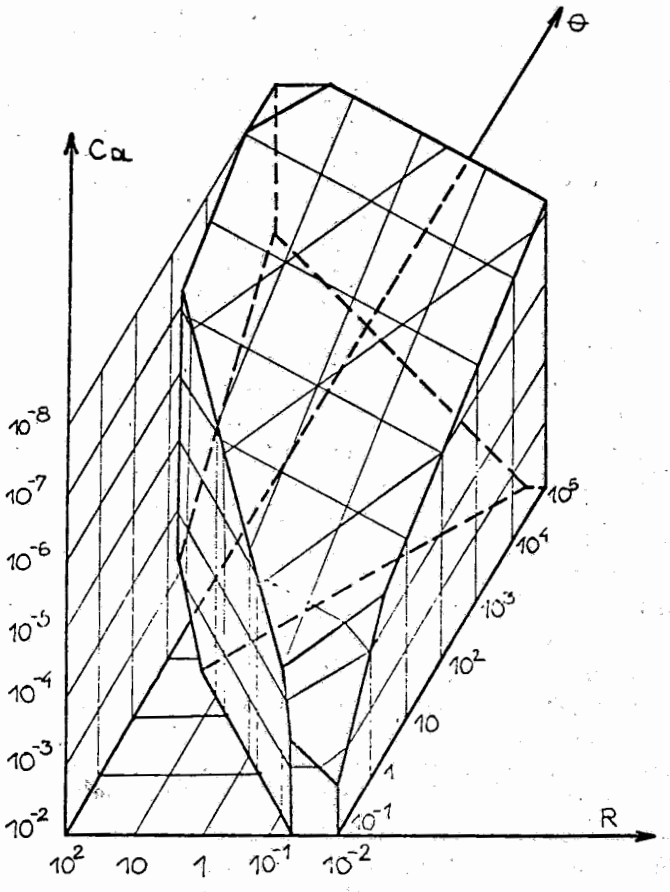

FIG. 13: *The box of C-Θ-R points in which those parameters can be determined with an accuracy better than 10% by the resonance method, when the measuring instrument has a precision of 0.1%.*

Acknowlegements

The author is indebted to Mr. V. Jović of the Institute of Technical Science in Beograd and to the colleagues mathematiciens, Mr. M. Djorović and M. Drazić for invaluable help in performing this work.

References

1. Bockris J.O' M. and Reddy A.K.N., Modern Electrochemistry, vol. 2., Plenum Press, New York 1970.
2. Delahay P.: New Instrumental Methods in Electrochemistry, p. 146 Interscience Publ. inc., New York, 1954.
3. Sluyters - Rehbach M. and Sluyters J.H. in Bard. A.J. ed. «Electroanalytical Chemistry», vol. 4, M. Dekker, New York 1970.
4. Despić, A.R., Jović V.D. and Djorović M.V., Israel J. of Chem., **18,** 65 (1979).
5. Despić A.R., Bull. Soc. Chim. (Belgrade) **39,** 539 (1974).
6. Despić A.R., Atanasoski R.T., and Djorović M.V., J. Electroanal. Chem., **70,** 1 (1976).
7. Despić A.R., Jović V.D. and Djorović M.V., Z, physik. Chem. (N.F.), **112,** 167 (1978).

Chimica Chronika, New Series, 9, 285-302 (1980)

STUDIES ON OLIVE OIL AT THE GENERAL CHEMICAL STATE LABORATORY

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Greece is the third olive oil producing country in the world and covers 15% of the olive oil world production. Olive oil represents ca. 12% of the total agricultural production in our country, and covers 70% of the needs of the greek population for lipid consumption with the known beneficial results as far as atherosclerosis etc. is concerned. Another indicative figure is that ca. 38% of the agricultural population of Greece is occupied in the cultivation of the olive-tree. Olive oil is thus a national product of Greece with exceptional economical and sociological value.

Together with olive oil, olive-residue oil is produced. Olive-residue oil is obtained by extraction with solvents from the remaining seeds of olives, after the extraction of olive oil which is achieved by application of pressure and mild heating conditions. Olive-residue oil is an edible oil which is consumed after refining as it happens with all other vegetable oils, except with virgin olive oil which is consumed without any industrial treatment.

At the General Chemical State Laboratory research on olive oil aims the scientific knowledge of its composition, its constituents structure, and consequently an adequate protection of the product. My talk will mainly point towards two directions:

A. Comparative Studies on the Glyceride Structure of OliveOil, Olive-Rsidue Oil, and Reesterified Oils.

B. Studies on the Non Glyceride Constituents of Olive Oil and Olive-Residue Oil.

A. Comparative Studies on the Glyceride Structure of Olive-Oil, Olive-Residue Oil and Reesterified Oils.

Reesterified oils are produced by esterification of glycerol and distilled fatty acids or by direct reesterification of oils with high percentages of free fatty acids. For the production of reesterified oils, olive oil or rather olive-residue oil are usually used. The products thus obtained have identical fatty acid composition with olive oil (fatty acid composition of olive oil and olive-residue oil is practically identical). Since reesterified oils are cheap, adulteration of the expensive olive oil or even the less expensive olive-residue oil with these oils is attractive. The production of reesterified oils is prohibited in Greece as in other countries like Italy, France, etc.

Olive oil or olive-residue oil and reesterified oils can be distinguished from their triglyceride structure. Saturated fatty acids i.e. palmitic and stearic acid are esterified almost exclusively at the combined 1,3 positions of olive oil and olive-residue oil triglycerides, as it was found to happen with other triglycerides of vegetable origin. However, in reesterified oils, fatty acids have a statistical distribution on the three positions of glycerol.

In our studies we approached triglyceride structure of the above mentioned oils by the following powerful analytical techniques:

a. Enzymatic Deacylation by Pancreatic Lipase.
b. Silver Ion Adsorption Chromatography.
c. Differential Scanning Calorimetry.

a. Enzymatic Deacylation by Pancreatic Lipase

Determination of palmitic acid at position-2 has been used for detecting adulteration by reesterified oils in olive oil and olive-residue oil. Procedures published comprise selective enzymatic hydrolysis by mammalian pancreatic lipase, separation of hydrolytic products by thin-layer chromatography, conversion of isolated 2-monoglycerides to methyl esters, and their fatty acid analysis by gas-liquid chromatography. IUPAC has published the method II.D. 27 for this purpose which was provisionally agreed by the Codex Alimentarius Committee on Fats and Oils (FAO/WHO) to be vorwarded for assessment to the Codex Committee on Methods of Analysis and Sampling and was adopted by the European Economic Communities (EEC). The percentage of palmitic acid at position-2 in the glycerides of 62 samples of genuine virgin olive oil from the main oil producing areas of Greece (years of production 1975 and 1976) was determined by the method II.D.27 of the IUPAC. Among the 62 samples 48 were edible olive oil samples i.e. having free acidity up to 3.3% (as oleic acid), and 14 having free acidity 3.7-9.2% which would be consumed after refining.

The determination was also performed to 30 samples of genuine olive-residue oil from the islands of Crete and Corfu, produced in 1975, 1976, and 1977. The samples of olive-residue oil were either crude or neutralized by alkali, soda or distillation. The results are summarized in Table I.

From the results of Table I it is seen that the limit of the EEC may be considered adequate for edible (acidity up to 3%) virgin olive oil and that the limit proposed by the International Olive Oil Council (100C) is too low. More interesting is the observation that for oils with higher than 3% acidities which according to II.D.27 have to be neutralized with alkali in the presence of a solvent prior to hydrolysis and for oils which have been neutralized in industry both limits appear too low. According to this observation, we have investigated the effect of the following neutralization steps, described by II.D.27, on the glyceride structure of oils and consequently on the results of the method:

(a) Neutralization with alkali in the presence of a solvent, for oils having acidities higher than 3%, and

(b) passage through a column with activated alumina to purify all the samples and also to neutralize oils having acidities up to 3%. Thus, several samples of

TABLE I: *Percentage of Palmitic Acid at Position-2 in the Glycerides of 62 Samples of Genuine Virgin Olive Oil and of 30 Samples of Genuine Olive-Residue Oil fom Greece*

Results from 48 samples of edible olive oil (acidity up to 3.3%)		
min.: 0.6	max.: 2.7	mean value: 1.5
number of samples exceeding 1.5 (limit proposed by the 100C)[a]: 20		
number of samples exceeding 2.0 (limit of the EEC[7]): 3		
Results from 14 samples of olive oil (acidity 3.7-9.2%)		
min.: 1.4	max.: 3.6	mean value: 2.6
number of samples exceeding 1.8 (limit proposed by the 100C for refined olive oil)[a]: 9		
number of samples exceeding 2.0 (limit of the EEC): 9		
Results from 30 samples of olive-residue oil (crude of neutralized)		
min.: 1.2	max.:4.0	mean value: 2.4
number of samples exceeding 2.0 (limit of the EEC): 18		
number of samples exceeding 2.2 (limit proposed by the 100C for refined olive-residue oil)[a]: 12		

a: The limit proposed by the International Olive Oil Council (100C) is expressed as saturated acids, i.e. palmitic+stearic acid, but the percentage of stearic acid is too small to be measured accurately.

olive oil and olive-residue oil with acidities ranging from 3.3-35.5% were neutralized with alkali according to (a), with soda, over alumina according to (b), and over Kieselgel. The results shown in Table II indicate that steps (a) and (b) clearly affect the glyceride structure of the oils, probably, because some hydrolysis of ester bonds followed by acyl migration is taking place. The effect is stronger with alkali, but alumina also results in higher percentages of palmitic acid in position-2 when compared to Kieselgel. By alumina it was reported that some hydrolysis of triglyceride ester bonds may occur. In order to verify the effect of alkali, three samples were left with the neutralizing solution for three hours; the results are indicative.

Further, since it is expected that the structure of partial glycerides may be altered with time, the effect of storage on the glyceride structure, especially of oils with high acidities and consequently with considerable content in partial glycerides, was investigated. Thus, samples of crude olive-residue oil, with acidities ranging from 10.5 - 35.5%, stored for 1-2 years, were examined by II.D.27, and after isolating the pure triglycerides of the oils by a Kieselgel chromatographic column. The results are shown in Table III.

Clearly, samples stored for 1-2 years (Table III) show considerable increase in the percentage of palmitic acid at polistion-2 of their glycerides when examined by II.D.27, but no increase when enzymatic deacylation is performed on the pure triglycerides at least of oils containing considerable percentages of partial glycerides, the structure of which is altered during storage. In Table III are included also two samples of olive oil with acidities 0.9% and 2.1% which have not shown any alteration.

TABLE II: *Effect of the Neutralization Steps on the Percentage of Palmitic Acid in Position-2 in the Glycerides of Olive Oil and Olive-Resudue Oil*

Sample	Acidity %	Alkali	Soda	Alumina	Alkali 3 hours	Kiesel-gel
Olive oil	3.3	2.0	—	1.4	—	—
» »	4.4	3.4	2.6	2.6	—	1.8
» »	4.8	1.4	—	1.1	—	1.0
» »	5.5	2.9	2.3	1.5	—	1.2
» »	5.6	1.6	—	1.0	—	—
» »	5.9	2.8	2.1	1.4	—	—
» »	7.0	1.5	—	0.9	—	—
» »	8.0	2.1	—	1.1	—	—
» »	9.2	3.1	2.3	1.4	—	1.1
Olive-residue oil	16.6	3.9	—	1.8	8.0	1.4
» »	18.8	3.5	2.7	2.0	5.9	1.4
» »	19.1	2.6	—	1.6	5.7	1.2
» »	22.5	2.7	2.1	—	—	—
» »	35.5	4.0	3.4	—	—	—

TABLE III: *Effect of Storage on the Persentage of Palmitic Acid at Position-2 in the Glycerides of Olive-Residue Oil and Virgin Olive Oil*

Acidity as % oleic acid		According to II.D.27		Neutralization and isolation of pure triglycerides over Kieselgel
Samples of olive-residue oil produced in 1975 and examined in:				
1976	1978	1976	1978	1978
10.2	15	2.2	3.1	1.5
18.9	25	1.8	4.4	1.3
Samples of olive-residue oil produced in 1976 and examined in:				
1977	1978	1977	1978	1978
22.5	24.6	2.7	4.5	1.4
18.8	24.2	3.5	5.8	1.3
16.6	18.6	3.9	5.4	1.8
35.5	38.5	4.0	5.0	1.7
Samples of virgin olive oil produced in 1976 and examined in:				
1977	1978	1977	1978	1978
2.1	2.2	1.5	1.6	1.4
0.9	0.9	1.1	1.2	1.1

Finally, in order to compare possible alterations brought about by different refining procedures, palmitic acid in position-2 of glycerides was determined according to II.D.27 and according to the proposed by us improved procedure i.e. neutralization and isolation of pure triglycerides over Kieselgel, in three crude olive-residue oils. The determination was repeated in the same oils after one part of each of them was neutralized industrially with soda and the other part by steam distillation, and in their final refined products. The results are shown in Table IV.

TABLE IV: *Effect of Different Refining Processes on the Percentage of Palmitic Acid at Position-2 in the Glycerides of Olive-Residue Oil and Olive Oil*

Sample		Crude	Neutralized with soda	Refined	Neutralized by distillation	Refined
Olive-residue oil	I	4.2[a]	3.1[a]	3.3[a]	5.4[a]	5.7[a]
		2.0[b]	1.1[b]	1.2[b]	4.0[b]	3.9[b]
Olive-residue oil	II	2.6[a]	2.9[a]	2.8[a]	4.2[a]	4.4[a]
		1.8[b]	2.0[b]	2.0[b]	3.7[b]	3.7[b]
Olive-residue oil	III	2.9[a]	2.9[a]	2.8[a]	5.8[a]	5.7[a]
		1.5[b]	1.8[b]	1.9[b]	4.2[b]	4.2[b]
			With alkali			
Olive Oil	I	2.0[a]	1.4[a]	1.4[a]	—	—
Olive Oil	II	1.8[a]	1.6[a]	1.6[a]	—	—
		0.9[b]	0.9[b]	1.1[b]	—	—

a: According to II.D.27.
b: According to the proposed improved procedure i.e. neutralization and/or isolation of pure triglycerides over Kieselgel.

Once more it is seen the necessity of eliminating steps (a) and (b) of the method II.D.27 and their replacement by Kieselgel column chromatography. Further, by comparing results obtained by the two different industrial neutralization procedures, it is seen that steam distillation clearly affects the triglyceride structure of the oils to a considerable extent. Thus, the method is found not to be applicable to oils neutralized by steam distillation. Otherwise refining procedures have not been found to affect the results obtained to a detectable extent.

The above described observations and some others of minor importance resulted in an improved method which we have submitted to the 100C and the Codex Alimentarius Committee on Fats and Oils, where the observers of the IUPAC and the EEC are taking part. All four bodies have decided to discuss amendment of the method II.D.27 in the light of our findings.

b. Silver Ion Adsorption Chromatography

Detection of reesterified oils by the determination of palmitic acid in position-2 of oil triglycerides is a quite tedious procedure, it needs about two working days and the sensitivity of the method lies in the range from 10-20%, when taking under consideration variations of olive and olive-residue oil and of reesterified oils in palmitic acid at position-2. The 100C has also characterized the sensitivity of the method as not satisfactory. For this reason, we tried a rapid qualitative and semiquantitative determination of reesterified oils in olive oil by silver ion thin-layer chromatography.

Silver ion adsorption chromatography of triglycerides is based on the weak interaction between Ag^+ and the π-electrons of double and triple bonds. The Ag^+/olefin complex is of sufficiently low energy that it can be made and broken during standard lipid chromatographic procedures. Argentation chromatography is accomplished by impregnating $AgNO_3$ into normal lipid adsorbents such as silicic acid. After suitable adjustment of the solvent system, a given class of lipids can be fractionated according to the number of double bonds per molecule.

We thought of fractionating the positional isomers of the monoenoic and dienoic triglycerides of olive oil, olive-residue oil and reesterified oils, i.e. palmito-oleo-palmitin (010) and oleo-palmito-palmitin (100) (monoenoic fraction) their proportion being found in olive oil 90:10, while in reesterified oils 30:70 respectively; palimito-oleo-olein (011) and oleo-palmito-olein (101), their proportion being in olive oil 97:3, while in reesterified oils 65:35. Separations of positional isomers of the types 001 and 010, 011 and 101, and 002 and 020 (where 0: saturated, 1: monounsaturated, and 2: diunsaturated acid) by Ag^+ TLC were reported previously; the 010, 011, and 020 isomers having the higher R_f value in each case.

We tried several solvent systems and achieved the best separation with toluene: ether 96:4. Figure 1 shows schematically the separation achieved.

Starting from the front of the solvent downwards (Figure 1) for all the three oils, triglycerides are fractionated in the following order: Saturated (000), monounsaturated (010), diunsaturated (011), and triunsaturated (111) triglycerides. With the reesterified oil, we clearly observe additional bands due to the non symmetrical triglycerides 001 having lower R_f as compared to the symmetrical 010 triglycerides, and the symmetrical 101 (below 011) both characteristic of these oils. As already mentioned percentages of 001 and 101 triglycerides in olive and olive-residue oil are negligible (except in steam distilled olive-residue oil). In addition, in the reesterified oil, we observe bands due to the triglycerides containing trans oleate (E) of the type 01E, 11E etc. The percentage of trans oleate present in reesterified oils varies and is not characteristic of these oils, since steam distilled and otherwise treated oils may contain variable amounts of it.

TLC analysis of mixtures of 10, 15, 20, 25 and 30% of reesterified oil in olive oil and olive-residue oil has shown that the sensitivity of the method lies

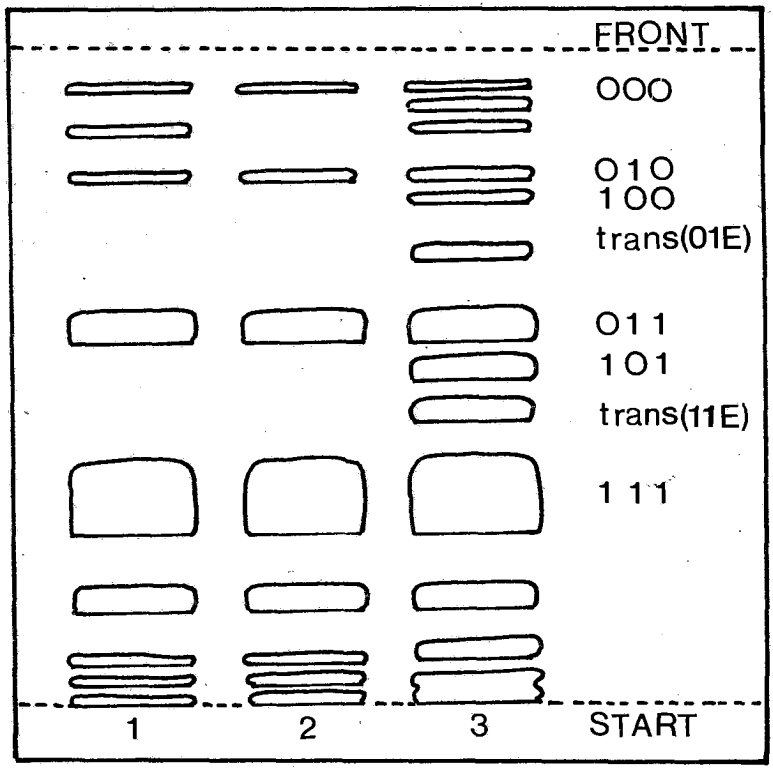

FIG. 1: *Separation of triglycerides of (1) olive-residue oil (2) olive oil, and (3) reesterified oil on Kieselgel G/silver nitrate (plate 20×20cm). Solvent system: toluene: ether, 96:4. Amount of oil 5mg.*

from 15-20% which is comparable to the sensitivity of the enzymatic deacylation method. The value of the silver ion chromatography method lies thus on its simplicity and speed: Kieselgel TLC plates Merck type 5721 are very rapidly plunged into a solution of 1:1 ethanol: 20% w/v silver nitrate in water for half a minute, drained, all excess liquid wiped from the surface by a soft paper tissue and the plate dried at about 70°C for 15 min. The plates may be used immediately. Thus, the time needed for a test does not exceed two hours and it can be performed in every laboratory without sophisticated equipment.

c. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry heating or cooling curves of triglecerides show multiple peaks due to their polymorphic behaviour. Procedures for using DSC curves for the analysis of triglyceride mixtures are developed but are limited. The precise crystallization temperature for each triglyceride type depends on the cooling rate, but the relative positions of the peaks in the curve are constant. Thus, it is possible to obtain a qualitative idea on the triglycerides

in a mixture by running a simple DSC cooling curve. This promising technique merits further development.

We have studied comparatively both the cooling and heating curves of olive oil, olive-residue oil and reesterified oils by different cooling and heating rates. The thermograms obtained with 15mg samples and at a rate 2.5°/min are shown in Figure 2. Thermograms are conventionally presented having the abscissa normally calibrated in degrees Kelvin and ordinate calibration in millicalories per second: The area under the transition peak represents the energy absorbed or produced and is independent of the scanning rate, the temperature and the nature of the transition, and the scanning direction.

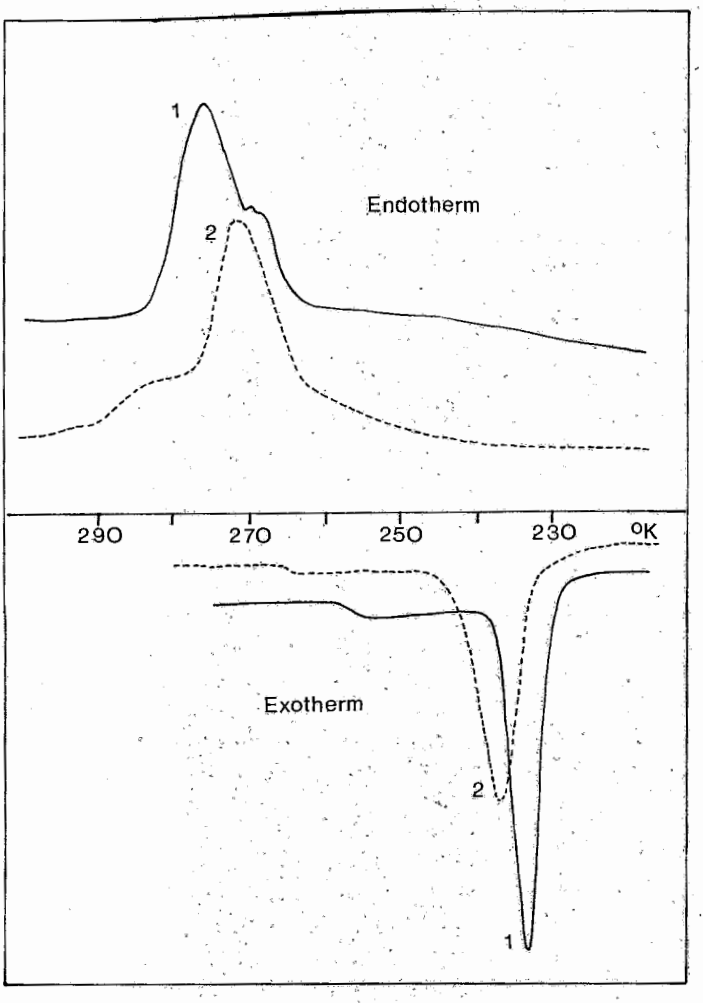

FIG. 2: *DSC transition curves of (1) olive oil and (2) reesterified oil. Cooling and heating rate 2.5°/min. Heating curves were recorded after annealing at 260°K for 30 min. Amount of oil 15 mg.*

Endotherms were recorded after annealing at 260°K for 30 min. Comparison of the thermograms of Figure 2 shows that there is a difference in the crystallization temperature of olive oil 237.5° to that of the reesterified oil 243°K. However, the difference is small. Fusion curves seem to be differentiated better; both curves appear with two maxima but in opposite order, for olive oil the main peak is at the higher temperature maximum (277°K) accompanied by another peak at 269°K, while for the reesterified oil the main peak is at the lower temperature maximum (272°K) with a shoulder at 283°K. The ratio of the areas under the transition peaks in the cooling curves (representing the energy evolved during crystallization) of reesterified oil/ olive oil is 0.9. Olive-residue oil shows similar to olive oil behaviour.

Further, we are interested to find out if the isolated monoenoic, dienoic and trienoic triglycerides of the oils studied will show differences in their crystallization or fusion curves because of the different geometrical or positional isomers they contain. As already mentioned, reesterified oils contain monoenoic and dienoic glycerides with high percentages of saturated acids at position-2, and glycerides with trans oleate.

B. Studies on the Non Glyceride Constituents of Olive Oil and OliveResidue Oil

The unsaponifiable fraction of vegetable oils varies from 0.2-2.0% and contains nornally sterols, terpene and aliphatic alcohols, tocopherols, carotenoides, carotenes, unsaturated hydrocarbons, mainly squalene, and saturated hydrocarbons. Certain fractions may contain also vitamins (A, D, K).

The unsaponifiable matter of oils is usually isolated after saponification of the oil by alcoholic solution of potassium hydroxide, extraction of the unsaponifiable matter with ether, which is washed several times with water in order soaps to be removed, dried over anhydrous sodium sulphate, and the solvent removed by distillation. When the unsaponifiable matter of oils is fractionated on Kieselgel G TLC plates by the solvent system petroleum ether: ether 1:1, the bands shown schematically in Figure 3 are clearly distinguished.

We will further refer to our analytical studies on the following fractions of the unsaponifiable matter of olive oil, olive-residue oil and some other vegetable oils:

a. Sterols.
b. 4a-Methylsterols.
c. Fluorescent Unknown Fraction.

a. Sterols

The unsaponifiable matter of olive oil contains 15-20% sterols. The amount of sterols varies in olive oil from 80-240mg/100g of oil. Sterols occuring in olive oil are shown in Figure 4.

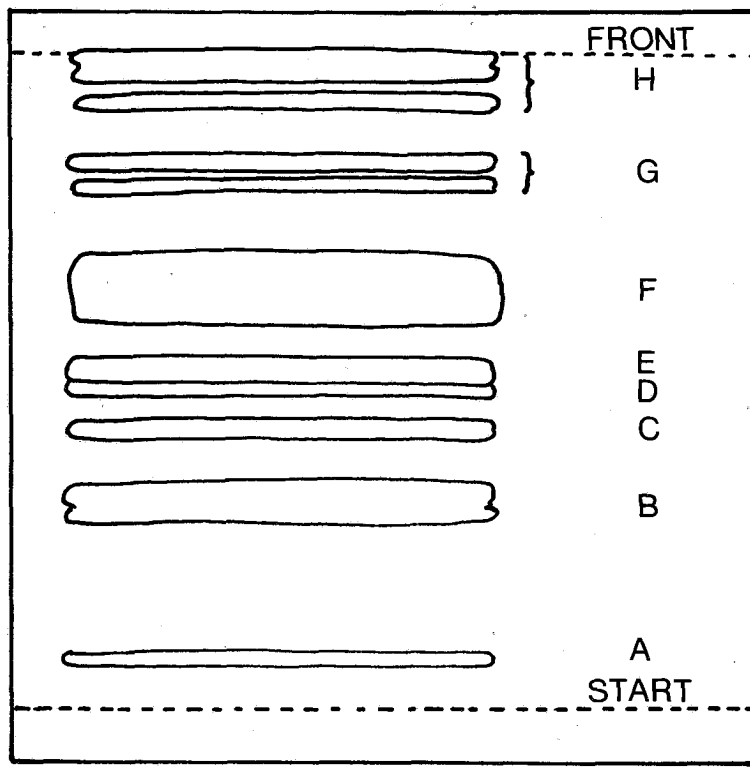

FIG. 3: *Fractionation of the unsaponifiable matter of olive oil on Kieselgel G TLC plates 20×20cm by petroleum ether: ether, 1:1. (A) pigments, (B) sterols, (C) 4α-methylsterols, (D) fluorescent band (mainly present in olive-residue oil), (E) terpene alcohols, (F) aliphatic alcohols, (G) tocopherols, and (H) hydrocarbons.*

Determination of the composition of sterols % is performed by GLC analysis of the material extracted from the TLC sterolic band. Our results from a statistical study on 38 samples of genuine virgin olive oil and on 21 samples of refined olive-residue oil from Greece are summarized in Table V.

From the results summarized in Table V it is seen that when using GLC columns with less polar stationary phases (SE-30, OV-1, JXR), the minimum value of the main sterol, sitosterol, is 93.1% of the total sterols in olive oil, while in olive-residue oil the minimum value lies lower (90.2%). Based on statistical data obtained with non polar stationary phases the EEC excludes olive oils in which the peak area, having the retention time of sitosterol, represents less than 93% of the total area of the sterol peaks. The same lower limit is proposed for olive oil by the IOOC to the Codex Committee on Fats and Oils. However, with non polar or less polar stationary phases, separation of sitosterol and Δ^5 avenasterol cannot be achieved, which leads to the erroneous assumption that more than 93% of the sterols in olive oil consists of sitosterol.

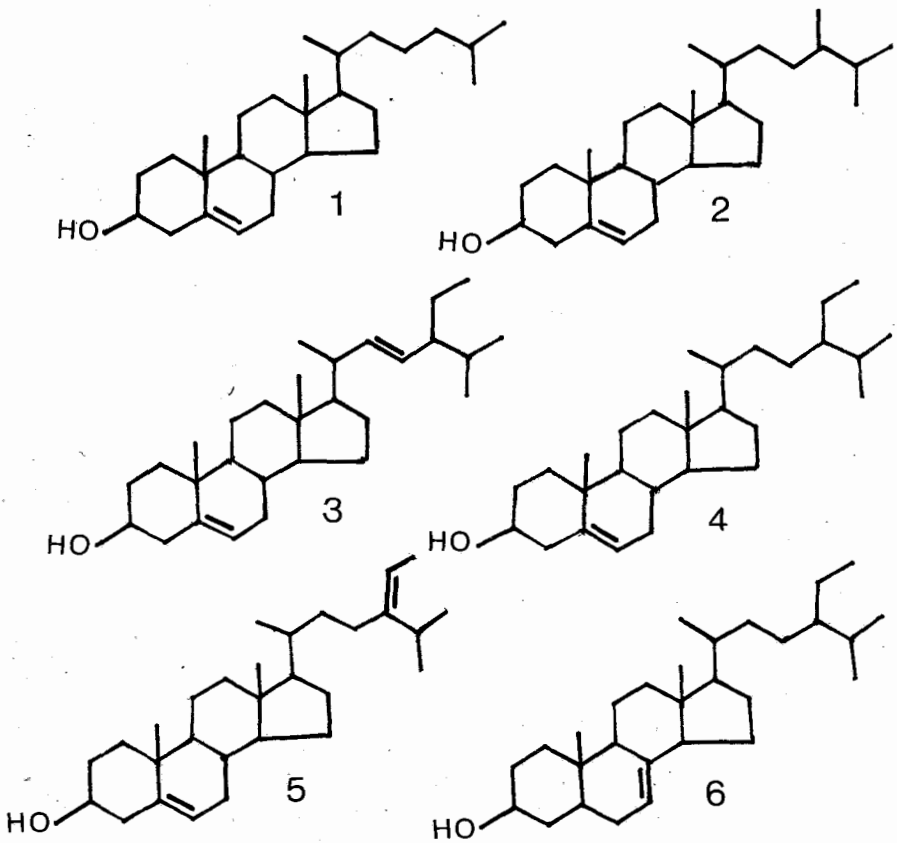

FIG. 4: *Sterols occuring in olive oil: (1) cholesterol, (2) campesterol, (3) stigmasterol, (4) sitosterol, (5) avenasterol, and (6) stigmastenol.*

By using more polar phases like methyl-phenyl silicon gum (OV-17) the peaks of sitosterol and Δ^5 avenasterol are resolved efficiently (R=1.1). The minimum value of sitosterol thus becoming 74% while Δ^5 avenasterol varies from 0.7–21.6%. Δ^5 avensterol seems important because it has been shown to minimize the oxidative polymerization of oils during heating.

More interesting is the finding that in olive-residue oil, mainly, the sterol fraction (separated on a TLC Kieselgel plate by petroleum ether: ether 1:1) contains in addition two at least other substances which are assigned to erythrodiol and to uvaol shown in Figure 5.

TABLE V: *Composition % of Sterol Fraction*

Campesterol	Stigmasterol	Sitosterol + Δ^5Avenasterol		Δ^5Avenasterol	Δ^7Stigmastenol + Δ^7Avenasterol
OV-1 or OV-17	OV-1 or OV-17	OV-1	OV-17[1]	OV-17	OV-1 or OV-17
Olive Oil (38 samples)					
min 1.8	0.9	93.1	74.0	7.0	0.5
max 3.7	3.2	96.3	88.6	21.6	1.2
mean 2.9	1.6	94.9	81.4	13.7	0.7
Olive-Residue Oil (21 samples)					
min 2.6	1.5	90.2	–	–	0.5
max 4.7	2.9	93.8	–	–	5.6
mean 3.4	2.0	91.9	–	–	3.0

1. Values refer only to sitosterol.

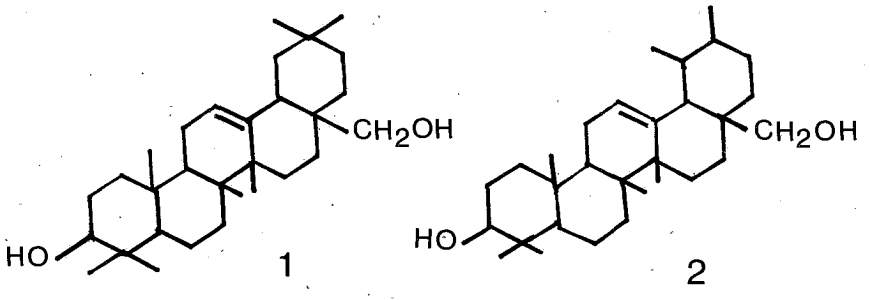

FIG. 5: *Terpenic diols occuring in olive-residue oil: (1) erythorodiol and (2) uvaol.*

In Table VI are summarized our results on the percentage of erythrodiol (and uvaol) in the sterol+diol fraction for 21 samples of genuine virgin olive oil and 21 samples of refined olive-residue oil from Greece.

The mean value of erythrodiol in the sterol+diol fraction of olive-residue oils is 14.1% (Table VI), while in olive oil 1.2%. The finding consists an excellent basis for determining olive-residue oil in olive oil. Adulteration of olive oil by olive-residue oil is very attractive as the price of olive-residue oil is about half of that of olive oil and methods of detection are indeed not satisfactory. Other vegetable oils like cotonseed oil, corn oil, soyabean oil etc. have not been

TABLE VI: *Erythrodiol* (RRT=1.9)[1] and Uvaol (RRT=2.1)[1] % in Sterol + Diol Fraction (Column: 3% OV-1)

	Erythrodiol		Uvaol	
	Olive Oil	Olive-Residue Oil[2]	Olive Oil	Olive-Residue Oil
min	0.6	11.8	–	0.9
max	1.9	16.0	–	4.5
mean	1.2	14.1	–	2.7

1. Relative to sitosterol.
2. Refined and after seasoning. Erythrodiol reaches 24% in crude olive-residue oil.

found to contain diols. We have extended analysis on hydrogenated products from olive-residue oil; the results obtained show that percentages of erythrodiol and uvaol in the sterol+diol fraction are not diminished during hydrogenation.

b. 4a-Methylsterols

4a-Methylsterols are a minor fraction in the unsaponifiable matter of vegetable oils; in olive oil it amounts ca. 2% (8-20mg/100g of oil). This means that collection of the amount needed for analysis is a tedious operation. This is probably the reason why this fraction has drawn less attention as compared to the sterol fraction which has been studied extensively. Another reason might be that the 4a-methylsterol fraction appears more complex and its constituents have not as yet been satisfactorily resolved by GLC analysis. However, some more or less detailed information exists in the literature mainly for other than olive oil vegetable oils. Probable 4a-methylsterols occuring in olive oil are shown in Figure 6.

In Figure 7 are presented the chromatograms of 4a-methylsteryl acetates obtained from olive oil and olive-residue oil. The assignment shown on the chromatograms is tentative and is based on literature data (RRT to cholesteryl acetate).

It is interesting to note that except lophenol, obtusifoliol, cycloeucalenol and/or gramisterol and citrostadienol, olive-rsidue oil contains a considerable amount of 4a. 14a. 24-trimethyl-9β, 19-cyclocholest-24-en- 3β-ol and/or ethyllophenol which might be used to differentiate olive-residue oil from olive oil. The composition of the 4a-methylsterols % of olive oil and olive-residue oil together with our results on cotonseed oil, and sunflower oil are compared in Table VII.

It can be concluded tentatively that the oils studied contain the same kind of 4a-methylsterols and differ only in their composition %. However, it is to be kept in mind that 4a-methylsterols are not well resolved by GLC analysis and conclusions drawn at this stage may be proved erroneous.

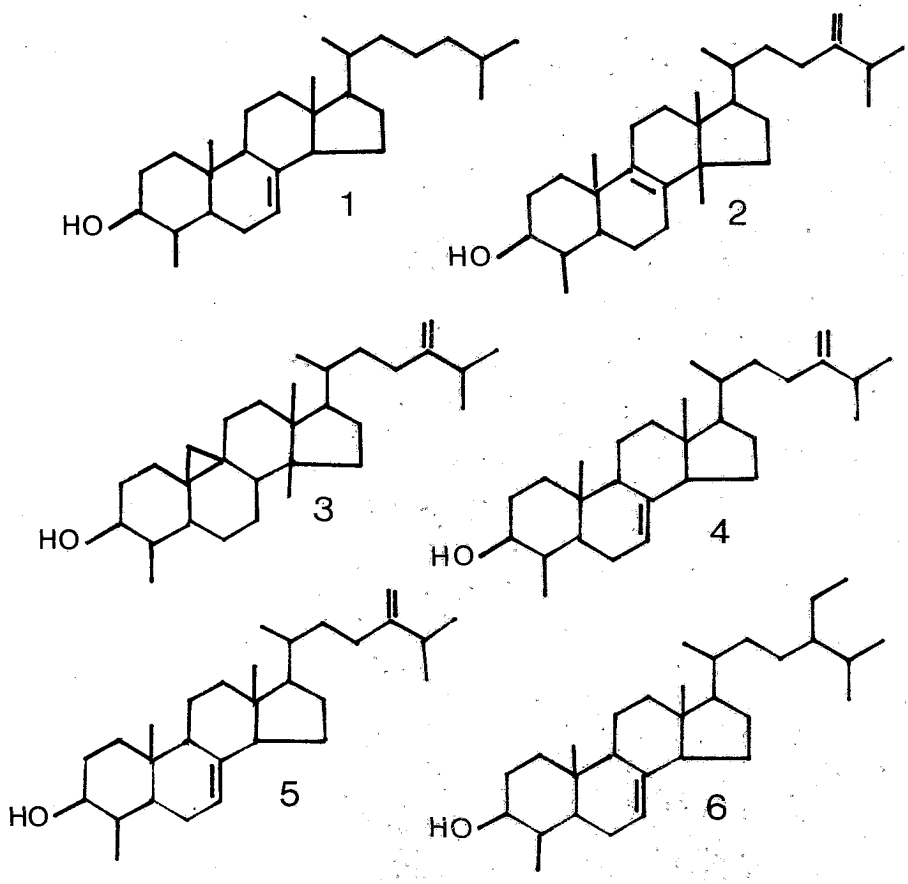

FIG. 6: *4a-Methylsterols occuring in olive oil (tentative): (1) lophenol, (2) obtusifoliol, (3) cycloeucalenol, (4) gramisterol, (5) citrostadienol, and (6) ethyllophenol.*

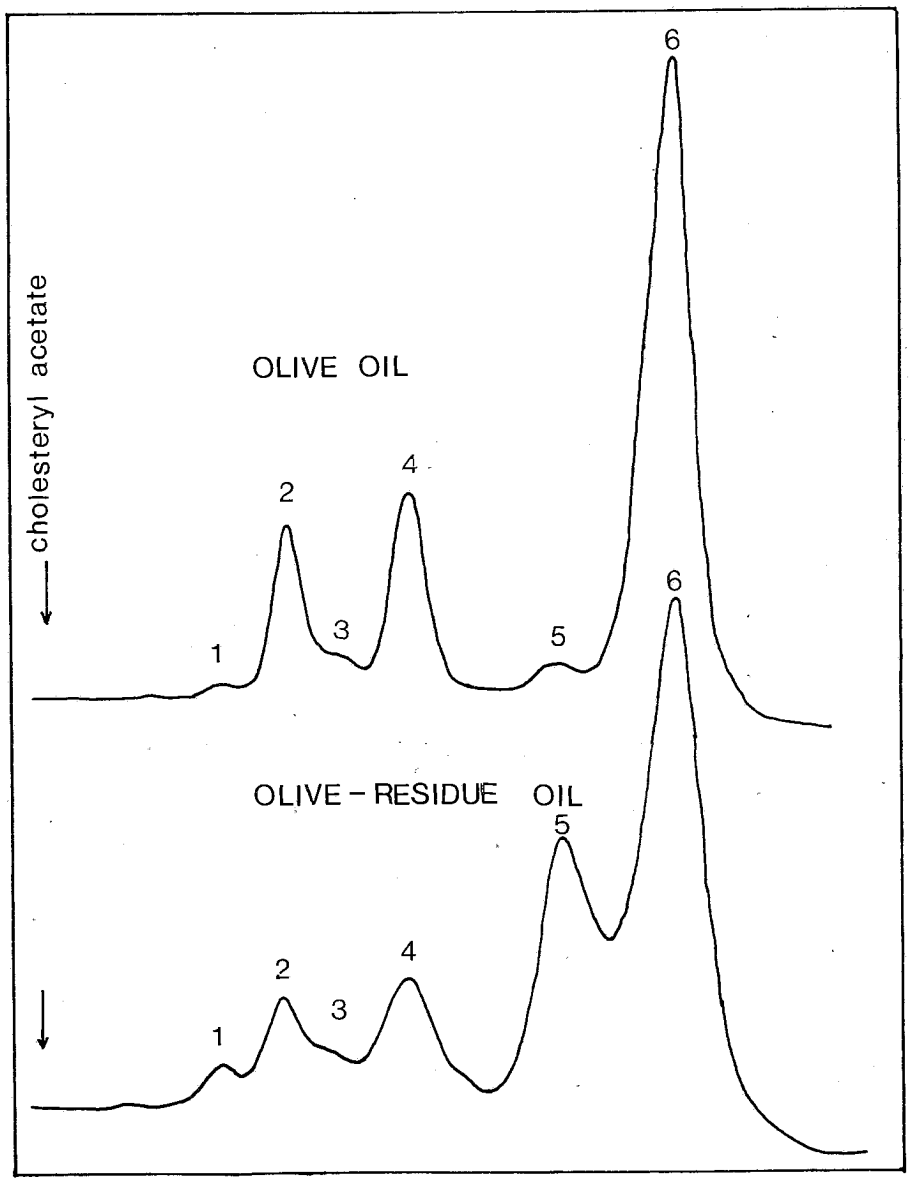

FIG. 7: *GL Chromatograms of 4a-methylsteryl acetates of olive and olive-residue oil (1,5% OV-17, glass 2m, 270°C). (1) lophenol, (2) obtusifoliol, (3) 31-norcycloartenol, (4) cycloeucalenol and/or gramisterol, (5) 4a-14a, 24trimethyl-9β, 19-cyclocholest-24-en-3β-ol and/or 24-ethyllophenol, and (6) citrostadienol. (Tentative assignment).*

TABLE VII: *Composition % of 4a-Methysterols of Some Vegetable Oils (analysed as acetates)*

	Olive oil	Olive-Residue Oil	Cotonseed oil	Sunflower oil
Lophenol [1]RRT: 1.32	0.7	3.6	5.2	2.1
Obtusifoliol [1]RRT: 1.41	12.7	8.6	19.3	36.5
Cycloeucalenol and/or Gramisterol [1]RRT: 1.76	17.4	13.4	29.6	18.4
4a, 14a, 24-Trimethyl--9b, 19-cyclocholest--24-en-3b-ol and/or Ethyllophenol [1]RRT: 2.1	2.3	23.6	3.5	5.2
Citrostadienol [1]RRT: 2.3	66.8	50.8	42.4	37.9

1. Relative to cholesteryl acetate.

Work is still in progress, we intend to continue the fractionation of 4a-methylsteryl acetates on silver nitrate TLC plates as each GLC peak might include more than one substances. Work will be completed by GC-MS analysis of the fractions separated on silver nitrate TLC plates for better assignment.

c. Fluorescent Unknown Fraction

Fluorescence is a general characteristic of oils. Among vegetable oils olive-residue oil shows a strong blue fluorescence characteristic of the species. In some laboratories still, fast detection of olive-residue oil in olive oil is helped by observing the sample under UV light.

The fluorescence spectrum of a sample of olive-residue oil is a broad spectrum consisting probably of several spectra of different species in the oil. When analysing the unsaponifiable matter of olive-residue oil on a Kieselgel TLC plate (petroleum ether: ether 1:1) and observing the plate under UV light before developing the chromatogram by 2,7 dichlorofluorescein, a strong fluorescent band appears between the band of 4a-methylsterols and terpene alcohols. Byextraction of the material held in this band and further purification by repeated TLC, a very small amount of the fluorescent material is obtained.

We have recorded the UV spectrum of this material and its fluorescence spectrum, which are shown in Figure 8.

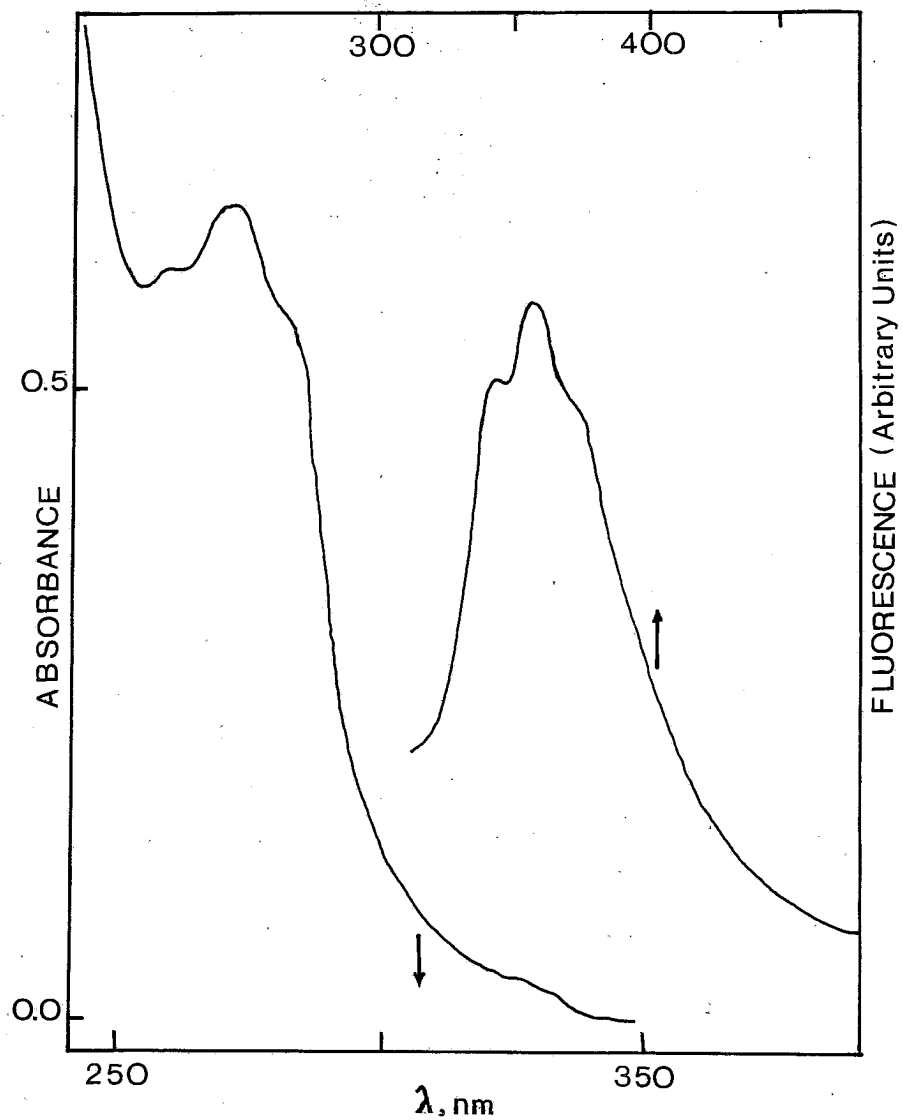

FIG. 8: *UV Absorption and fluorescence spectrum of unknown fluorescent material isolated from the unsaponifiable fraction of olive-residue oil.*

The fluorescence spectrum matches well with the UV spectrum obtained, being its mirror image as it normally happens (Figure 8). From the UV spectrum one judges that we probably are concerned with an unsaturated compound(s) containing conjugated double bonds. The IR spectrum recorded in a KBr pellet suggests that the compound(s) probably contains a carbonyl group. For the moment there are some doubts as far as the purity of the isolated material is concerned. It is obvious that we are just in the start of this work, which will be proceeded further.

Before closing my talk I like to mention my collaborators without the devotion of which these studies could not be accomplished. For the first part referred to the studies on the triglyceride analysis, Mrs MARY GEORGOULI, and for the second part of the studies on the non-glyceride constituents, Mr GEORGE DIONYSSOPOULOS M.Sc., and in part Miss HELEN KATSOULI.

Chimica Chronika, New Series, 9, 303-304 (1980)

CONTRIBUTION TO THE FIELD OF MACROMOLECULAR CHEMISTRY

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Chemistry of macromolecular compounds, regarded as a modern field of chemical science, and consolidated in the last half of this century, has proved to be a dynamic and fertile domain owing to which chemistry has penetrated into the mystery of nature and has afirmed itself in the contemporary technico-scientific revolution. Today, several scientific schools in the world tackles polymer chemistry as an independent territory, as a mature discipline which witnesses a diversifying process. Its contribution to the development of other scientific fields, the appearance of new disciplines such as: mechanochemistry, plasmochemistry, polymer electrochemistry, biopolymerization etc. is very well known.

This lecture deals with only a few aspects of the new ways of access to the process of knowledge by means of the directions shown above. One presents in the field of mechanochemistry of macromolecular compounds the mechanochemical syntheses, the initiation of polymerization (blockpolymerization and grafting, polycondensation and complexation) as well as the degradation which have an impact on the structure and properties of carbo- and heterocatenary polymers. Particular attention has been paid to certain compounds with electrophysical, photo- and semiconducting properties that were obtained by polymerization of aryl-acetylenes and ferrocene derivatives. It is interesting to note the correlation between structural isomery of polyethylene, polymerization mechanism, crystallinity and electrical and magnetic properties.

The plasmochemistry of macromolecular compounds — a field that has emerged somewhat recently — offers large prospects for syntheses of nonconventional monomers, for the change of natural and synthetic polymers by grafting processes. At the same time plasmochemistry has proved to be the generator of certain compounds of biological importance (biomonomers, biopolymers, organized microsystems). One should expect that by using macromolecular plasmochemistry resources one obtains valuable results in the study of the origin of living matter. In this connection, an original model, the so-called «cold model» of the appearance of life is presented.

A final domain concerns electropolymerization of some acetylenic monomers. These few examples on the limits of mechano- and electrochemistry

of macromolecular compounds with implications in the synthesis of new polymers with semiconducting properties and of some bioprecursors of the living matter tend to show the maturity of polymer chemistry and the start of its diversification.

Chimica Chronika, New Series, 9, 305-324 (1980)

ON COMPLEX-FORMING PROPERTIES OF SOME HIGH MOLECULAR POLYETHERS

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Poly(ethylene oxide), poly(dimethoxyethylene) and poly(p-dioxene) can be considered as polymeric analogues of ethereal solvents, dimethoxyethane and dioxane. The cation binding properties of the macromolecular polyethers are studied by spectrophotometric investigations of complex formation with lithium, sodium and potassium derivatives of fluorene. The formation of two types of complexes is observed corresponding to different solvation states of the alkali cations. Poly(ethylene oxide) is shown to be an universal cation binding agent, while poly (dimethoxyethylene) and poly(p-dioxene) interact in a different manner with the cations studied, and direct evidence is found for selective complex formation. The conclusion is made that the solvating properties of polymeric ethers are superior to those of their monomeric analogues which is explained by the «polymer effect».

Key words: Cation binding, complex formation, ion pairs equilibria, alkali cations, fluorenyl derivatives, poly(ethylene oxide), poly(dimethoxyethylene), poly(p-dioxene), accelerating effect.

Abreviations and Terminology

Crown ethers: cyclic polyethers exhibiting pronounced cation binding properties.

Dibenzo-18-crown-6: 2,3,11,12-dibenzo-1,4,7,10,13,16- hexaoxacyclooctadeca-2, 11-diene.

DME: 1,2-dimethoxyethane.

DOX: 1,4-dioxane.

ESR: electron spin resonance.

EU: ethereal unit (monomer unit of high molecular polyethers).

FlMt: fluorenyl metal.

FlLi: fluorenyllithium.

FlNa: fluorenylsodium.

FlK: fluorenylpotassium.

Glymes (G): polyglycol dimethyl ethers.

NMR: nuclear magnetic resonance.

PE: high molecular polyether.

PDME: poly(dimethoxyethylene).

PDOX: poly(p-dioxene).

PEO: poly(ethylene oxide).

UV: ultraviolet.

When speaking of solvation in ionic systems we have in mind the interaction between the solvent or additives to the solvent and the ions present. In this an equilibrium exists between different ionic species which can be represented by the well-known scheme (Fig. 1). The chemical activity of the different ionic species is different, that of the free ions and the solvent-separated ion pairs being the highest. In almost all cases cations are the solvated entities. In the case when the cations are solvated by additives, a complex is formed between ionophore and solvating agent and thus the cations are bound with the additive molecules. Since solvation (complexation) proceeds without any substantial change in the dielectric properties of the solvent, possibilities are offered for influencing nucleophilic reactions (both substitution and addition), their kinetics (e.g. rate)[1-3], selectivity[4], stereospecifity[5,6], and even their mechanism[7,8].

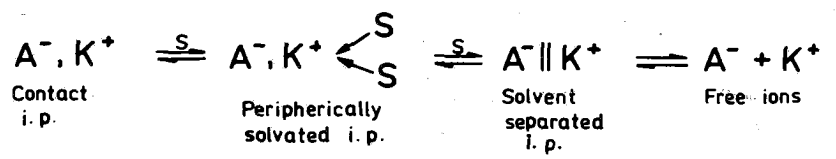


FIG. 1: *Ionic equilibria.*

Ethers are among the most widely used polar solvents and especially dioxane (DOX) known as a weak solvating agent, and tetrahydrofuran (THF) and dimethoxyethane (DME) possessing very good solvating properties. In the last decade some cyclic ethers, the so called «crown ethers» attract the attention of the chemists (Fig. 2)[9]. The solvating power of crown ethers is superior to that of open chain ethers.

In our Laboratory we have been studying for several years the solvating properties of certain polymers which can be considered as high molecular

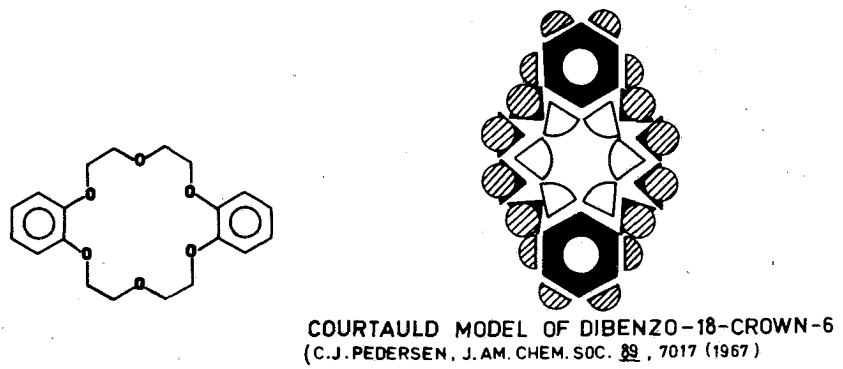


FIG. 2: *Crown ethers: dibenzo-18-crown-6.*

weight analogues of low molecular solvents. We have been particularly interested whether the polymers would be better solvating agents than the corresponding solvents, i.e. whether a «polymer effect» will be registered. In my talk I would like to present to your attention some results obtained recently in my Laboratory in the study of the solvating properties of three polymeric analogues of two commonly used ethereal solvents: poly(p-dioxene) (PDOX), which can be regarded as a high molecular analogue of DOX, and poly(dimethoxyethylene) (PDME) and poly(ethylene oxide) (PEO) - the high molecular weight analogues of DME - a very good solvating agent (Fig. 3). We expected the solvating properties of polymeric ethers to be superior to those of the monomeric analogues because of the possibility the polymer chain to form conformations favourable for improving their complexing ability. Thus it could be expected that the flexible chain of the simplest representative of this group, PEO, will be able to form under the action of the electrostatic field of the cation favourable conformations resembling crown units.

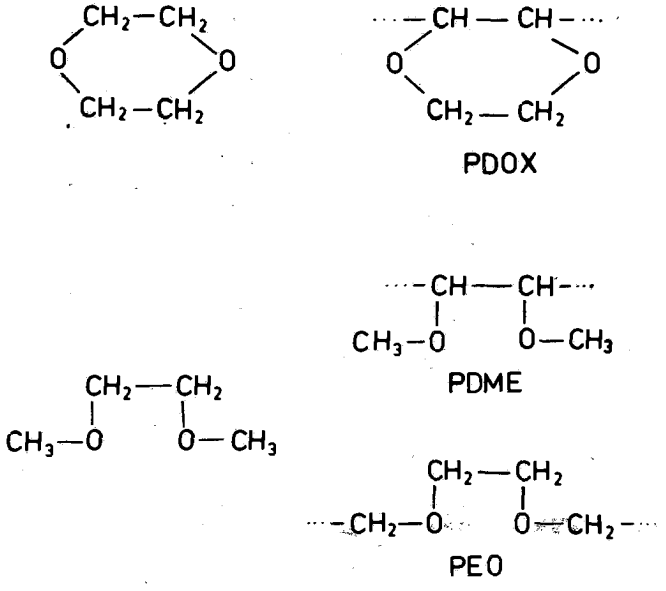


FIG. 3: *Ethereal solvents and their polymeric analogues.*

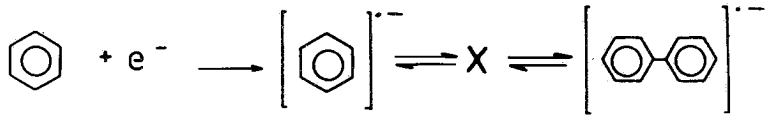
As a matter of fact we were aware of the good solvating properties of PEO from our previous investigations, which I will mention briefly. It is known that with reagents of highest purity and in full absence of moisture, oxygen and protic impurities, potassium solutions («blue» solutions) can be prepared in THF and DME, the amount of dissolved metal being of the order of 10^{-5} gatom/l. High vacuum technique is usually used. Potassium is deposited as a

mirror on the walls of the glass vessels to insure a larger surface. The solubilization of the metal can be represented by the equilibria:

$$2M_s \rightleftharpoons M^- + M^+$$
$$M^- \rightleftharpoons M + e^-$$
$$M \rightleftharpoons M^+ + e^- \text{ etc.}$$

All species taking part in the reaction are solvated and solvation is the driving force in the process. In the presence of PEO potassium solutions can be easily prepared by simple shaking of metal, cut into small pieces. The concentration of the PEO is 0.05 – 2%. No vacuum technique is necessary. The solutions have a dark blue colour and the concentration of the metal dissolved (determined titrimetrically) reaches up to 10^{-3} gatom/l[10].

But whereas potassium has a certain solubility in THF and DME even in absence of PEO, it does not show the even slightest tendency to dissolve in aromatic hydrocarbons like benzene and toluene. The interaction of potassium with benzene containing PEO results in the formation of intensely coloured red-brown solutions («red» solutions)[11]. The amount of the metal dissolved determined titrimetrically as total alkalinity reaches 10^{-2} gatom/l (2% PEO). The «red» solutions are paramagnetic and their ESR spectra are identical with the spectrum of benzene anion-radical, the signal intensity being considerably lower as corresponding to the total alkalinity of the solution. On exposure to sunlight or upon UV irradiation the red-brown colour rapidly changes to green. The green solution is also paramagnetic, the ESR spectrum in this case being identical with the spectrum of biphenyl anion-radical (biphenyl potassium). The visible spectrum also coincides with the spectrum of biphenyl anion-radical. These results indicate that the first product obtained in the interaction of potassium with benzene in the presence of PEO is benzene anion-radical. Under the reaction conditions (ambient, i.e. relatively high temperature and low polarity medium) the paramagnetic potassium salt of benzene anion-radical is in equilibrium with its diamagnetic dimer. Benzene anion-radical being unstable under these conditions readily transforms into the more stable biphenyl anion-radical (Fig. 4)[11].



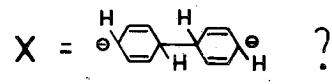


FIG. 4: *Proposed mechanism of benzene interaction with potassium.*

These results clearly show that in the presence of PEO reactions are possible which cannot proceed in its absence.

In order to get a quantitative picture of the solvating ability of polyethers we studied their interaction with the lithium, sodium and potassium derivatives of fluorene by the aid of electronic spectra - a method developed by Szwarc, Smid and Hogen-Esch (Fig. 5)[12]. They showed that the optical spectra of fluorenyl carbanion are very sensitive to the changes in the interionic distance in the ion pir. For example, the contact (tight) ion pair of fluorenylsodium (FlNa) is characterized by a sharp absorption maximum at 356 nm (room temperature), whereas the solvent-separated ion pair is characterized by an absorption at 373 nm (low temperature, −50°C)[13,14]. To avoid the concurent solvation of the lithium cation by THF[14] and to make possible the comparison of the results with the three cations, the investigations were carried out in DOX instead of THF. The concentration of polyethers (PE) was expressed with respect to their constituonal repeating units. Thus Q represents the concentration ratio of polyether and fluorenyl metal (FlMt) (—EU-: ethereal unit, monomer unit of PE):

$$Q = [-EU-] \,/\, [FlMt]$$

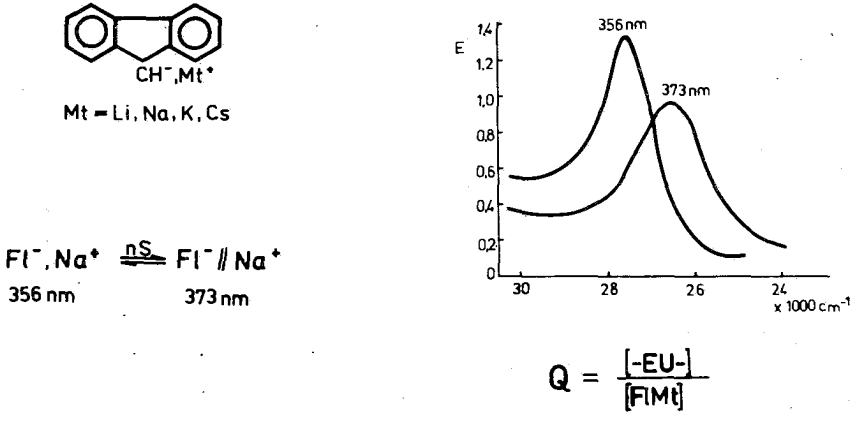


FIG. 5: *Fluorenylsodium contact ion pairs - solvent separated ion pairs equilibrium and corresponding absorption bands in the UV spectrum.*

The drawing on Fig. 6 represents the laboratory device usually used by us in studying the interaction between organometallic compounds and PE. It is a high vacuum all glass system.

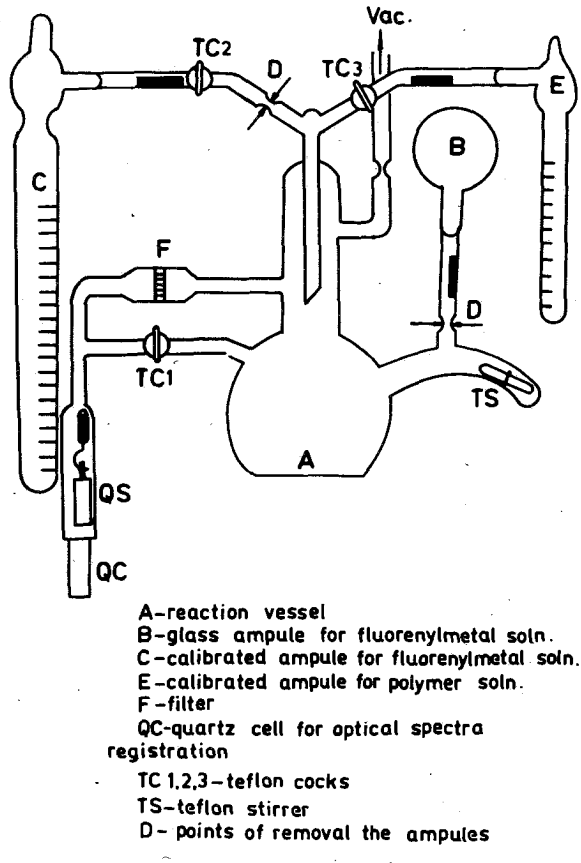


FIG. 6: *High vacuum laboratory device for spectrophotometrical studies of solvation phenomena:*
A: reaction vessel
B: glass ampule for fluorenyl metal solution
C: calibrated ampule for fluorenyl metal solution
D: points of removal the ampules
E: calibrated ampule for polymer solution
F: filter
QC: quartz cell for optical spectra registration
QS: quartz spacer
TC 1,2,3: teflon cocks
TS: teflon stirrer

When mixing a PE solution with a FlMt solution in DOX, in almost all cases an insoluble complex is formed. We were able to distinguish the following types of interaction with alkali cations (Fig. 7)[15]:

1. No interaction. PE and the metal compound did not form a complex, regardless of the ratio Q.

2. Partial binding of fluorenyl metal with PE. A precipitate of DOX insoluble complex was formed but a certain amount of unperturbed metal compound

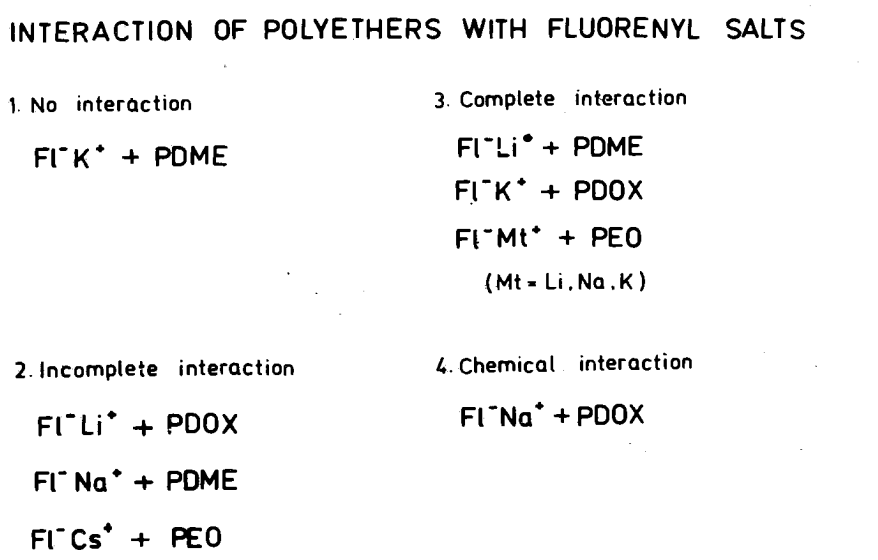


FIG. 7: *Different types of interaction of polyethers with fluorenyl salts.*

remained in all cases in the clear supernatant solution. We named this kind of interaction *incomplete* or *weak* interaction.

3. Complete binding of fluorenyl metal with PE at a definite ratio of components Q. In the supernatants above the precipitated complex no free metal compound was registered. This interaction was termed *complete* or *srong.*

4. In one case a chemical alteration of the metal compound was established. This type of interaction was named *chemical.*

I will begin with the discussion of the solvating properties of PEO. Its interaction with all counterions is complete.

When mixing solution of PEO and FlMt in DOX, i.e. two homogeneous systems, turbidity appeares which becomes more intense with increasing the PEO amount and an insoluble product is formed. Because of the fact that only DOX, FlMt and PEO are present in the reaction mixture, it should be assumed that the insoluble product results from the interaction between the polyether and the metal compound. The insoluble phase gets finely dispersed on stirring and thus it is possible to record the UV spectrum of the heterogeneous system for comparison with the spectra of the supernatant solutions after sedimentation and filtering off the precipitate. At low Q values only the characteristic absorption is registered, assigned to contact ion pairs of FlMt with the corresponding counterion, the anion concentration, however, being lower in the filtrate.

The two observations, the formation of an insoluble product and the identity of the absorption maxima of both the unperturbed compound and the precipitate can be explained by the formation of a complex as a result of

peripherical solvation of the cation by a section of the PE chain. We called it *complex A*. The formation of such species was mathematically derived from the chemical equilibrium equations of the interaction between fluorenylpotassium (FlK) and polyglycol dimethyl ethers (glymes)[16].

At higher Q values of Q the precipitate changes becoming heavier and more compact which does not allow further spectrophotometrical recording of the processes in the heterogeneous phase. On vigorous stirring it is only possible to register the formation of solvent separated ion pairs. Therefore, the initially formed complex A turns into a new entity, which we called *complex B*, where the cation is internally solvated by a section of the polymer chain. The transformation of complex A into complex B is a rapid reaction and is a function of the PEO amount in the system.

From the changes of FlMt concentration in the supernatant the apparent stoichiometry of the complexes could be evaluated presuming there is no uncomplexed polymer in the homogeneous phase:

$$S = [-EO-] : [FlMt] \text{ complex}$$

$[FlMt]_{complex}$ is the difference between the initial and the current concentration of FlMt in the supernatant. Stoichiometry slightly increases on raising Q. At complete binding of fluorenyl metal it is found to be approximately 15, regardless of the counterion (Fig. 8).

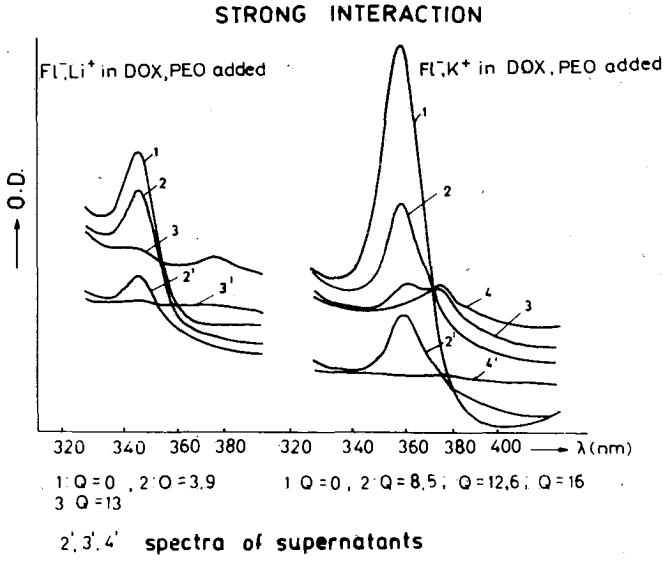


FIG. 8: *Two examples of complete interaction:*
a/ fluorenyllithium in DOX, PEO added
1: Q = 0, 2: Q = 3.9, 3: Q = 13
b/ fluorenylpotassium in DOX, PEO added
1: Q = 0, 2: Q = 8.5, 3: Q = 12.6, 4: Q = 16
2', 3', 4': spectra of supernatants.

In order to obtain more information on the interaction of PEO with FlMt we extended our investigations to THF as solvent[17]. At low Q values the formation of an insoluble complex A is similarly observed in this case. When the complex is separated and treated with pure THF, only an insignificant fraction of the initial FlMt amount is extracted. Evidently, the interaction of the metal compound with PEO is much stronger than with THF. Further addition of PEO to the system results in dissolving of complex A. Simultaneously the contact ion pairs band in the UV spectrum decreases and finally disappears while a new band at 373 nm appears, characteristic for ion pairs separated by a solvating agent (Fig. 9). This suggests that in THF the transformation of complex A into B also occurs, the latter however being soluble in THF. When FlMt solution is added to the solution of complex B, thus lowering Q, the 373 nm band decreases and a precipitate is formed. Hence complex B converts into A an there exists an equilibrium, depending on Q:

$$\text{Complex A} \underset{\text{FlMt}}{\overset{\text{PEO}}{\rightleftharpoons}} \text{Complex B}$$

The insoluble in DOX complex B obtained by addition of enough PEO to bind the total amount of the metal compound was separated and treated with THF. The result was the quantitative dissolving of the precipitate and simultaneous appearance of an intensive band at 373 nm. On mixing solutions of PEO and fluorenyllithium (FlLi) in benzene an insoluble solvation complex is formed. The interaction in this case is also complete since at high Q values the presence of FlLi is not registered spectrophotometrically in the supernatant[17].

The interaction of PEO wth metal salts was also studied by means of NMR spectroscopy. This method was previously used for studying the interaction of crown ethers and other ethereal solvating agents with metal ions where chemical shifts[18-20] of the signal for methylene protons were established. In Fig. 10 some preliminary results with metal perchlorates are shown. Chemical shifts were observed in several cases.

PEO spectrum in benzene is a singlet at 3.41 ppm due to symmetry of the molecule. On addition of potassium perchlorate this signal persists but a new, considerably more intensive signal appears at 3.48 ppm. This second spectrum could be assigned to a complex, formed between the PE and the inorganic salt. The downfield shift is due to an increased electronegativity of the oxygen atom participating in the solvation shell of the potassium cation (coordinated oxygen atom), thus corresponding to the α-protons. The signal at 3.41 ppm does not disappear in the presence of an excess of potassium perchlorate and is due to unperturbed oxygen atoms available in the complex, rather than to the presence of uncomplexed PEO. Hence, the cation is solvated by a section of the macromolecule containing both coordinated and uncoordinated oxyethylene units. Furthermore, the same spectrum recorded at fourfold expansion shows that

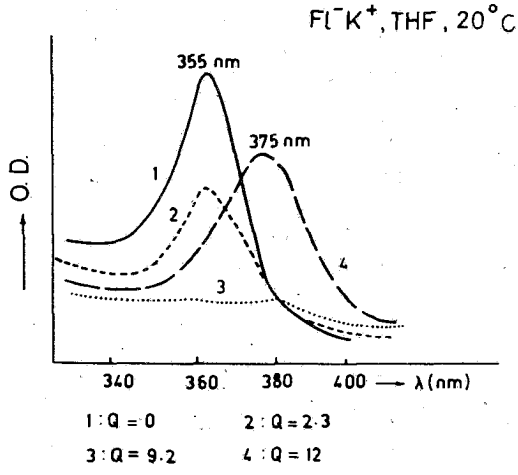


FIG. 9: *Optical spectra of fluorenylsodium in THF on PEO addition: 1: Q = 0, 2: Q = 2.3, 3: Q = 9.2, 4: Q = 12.*

the signal at 3.48 ppm is not a signlet. Its multiplet character gives evidence of nonequivalent perturbation of the solvating oxygen atoms by the electrostatic field.

Similar changes were not established in the presence of lithium perchlorate in benzene but they were observed in o-dichlorobenzene. The NMR spectra of PEO and of the complex PEO-$LiClO_4$ in o-dichlorobenzene are shown in the same Fig. 10. The molar fraction of the unperturbed oxyethylene units in the

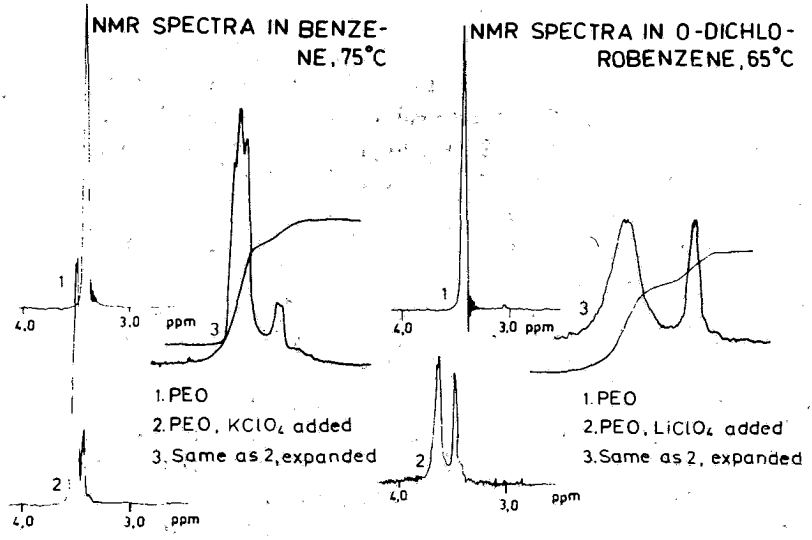


FIG. 10: *NMR spectra of PEO in:*
a/ benzene, $KClO_4$ added
b/ o-dichlorobenzene, $LiClO_4$ added.

complex with lithium counterion is larger, as can be seen from the integrals of the expanded spectra. This would mean that the lithium cation is solvated by a smaller number of oxygen atoms than the potassium cation, assuming equal stoichiometry of the complexes as suggested by the electronic spectra. The α-protons signal does not exhibit multiplet character but is only broadened.

The interaction of PDME with FlLi (Fig. 11) and of PDOX with FlK (Fig. 12) is complete. The stoichiometry of both complexes at complete binding the metal compound is 15 monomer units (with totally 30 oxygen atoms) per cation. The polymers form two types of complexes with the respective counterion. According to the nature of FlMt ionic species they correspond to solvation complexes A and B of PEO and a similar equilibrium exists between them depending on Q. All four products are insoluble in DOX.

The interaction of PDOX with FlLi (Fig. 13) and of PDME with FlNa is incomplete. The molar fraction of the solvated compound increases exponentially when increasing the amount of polymer in the system. There exists a limiting value which cannot be exceeded regardless of the excess of polyether. It is higher for FlLi, PDOX (ca. 75% of the initial FlLi solvated) than for FlNa, PDME (only ca. 10% of the initial FlNa complexed). The apparent stoichiometry of the complex between PDOX and FlLi was determined at the saturation point, by back titration of the complex (excess of polymer) with FlLi solution. It was found to be 16 dioxene units per lithium cation. PDME and FlNa form only solvation complex A while in the case of PDOX and FlLi complex A partially converts to form small amounts of complex B.

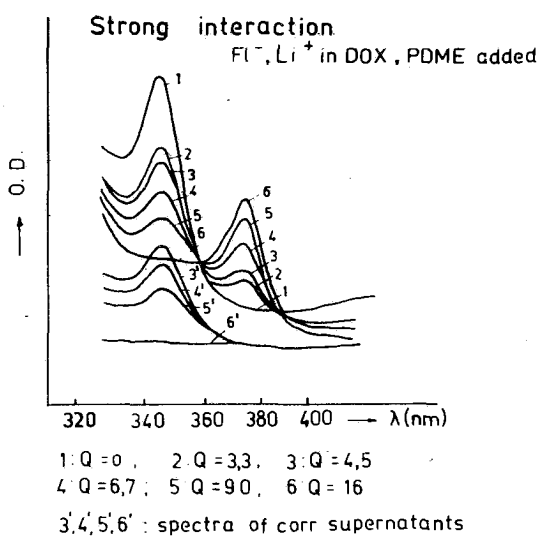


FIG. 11: *Optical spectra of fluorenyllithium in DOX on PDME addition (complete interaction): 1: Q = 0, 2: Q = 3.3, 3: Q = 4.5, 4: Q = 6.7, 5: Q = 9.0, 6: Q = 16 3', 4', 5', 6': spectra of corresponding supernatants.*

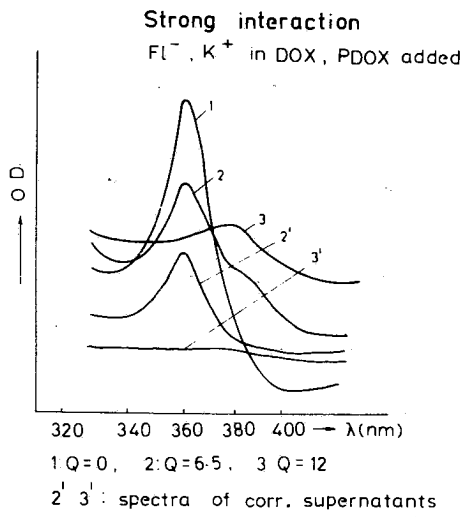


FIG. 12: *Optical spectra of fluorenylpotassium in DOX on PDOX addition (complete interaction):*
1: Q = 0, 2: Q = 6.5, 3: Q = 12
2′, 3′: spectra of corresponding supernatants.

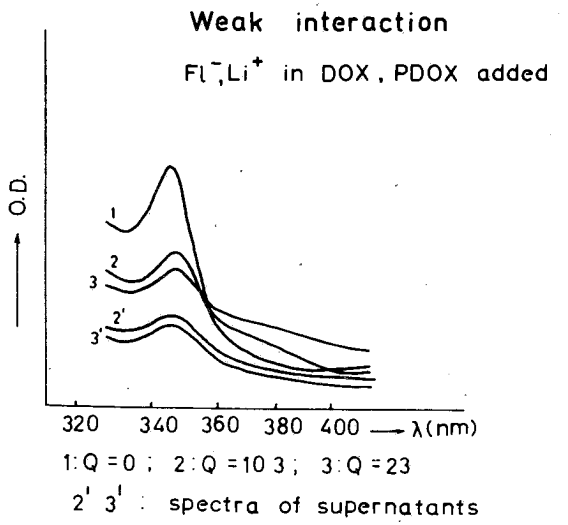


FIG. 13: *Optical spectra of fluorenyllithium in DOX on PDOX addition (incomplete interaction):*
1: Q = 0, 2: Q = 10.3, 3: Q = 23
2′, 3′: spectra of supernatants.

FlK does not interact with PDME in THF or DOX.

FlNa forms a stable complex of type A with PDOX. The apparent stoichiometry at small Q is close to that of FlK, PDOX. At Q = 3 however, a side reaction takes place which becomes the main process at Q values higher than 8. The initial sodium compound is quantitatively transformed into products the nature of which was not determined. The absorption at 356 nm characteristic for FlNa contact ion pairs decreases and finally disappears, and simultaneously a couple of new bands at 562 and 641 nm develops. The system remains heterogeneous, the supernatant absorbing at 562 nm only while both bands are present in the spectrum of precipitate (Fig. 14). The system is not paramagnetic. Besides, the chemical change is not accompanied by consumption or liberation of fluorene.

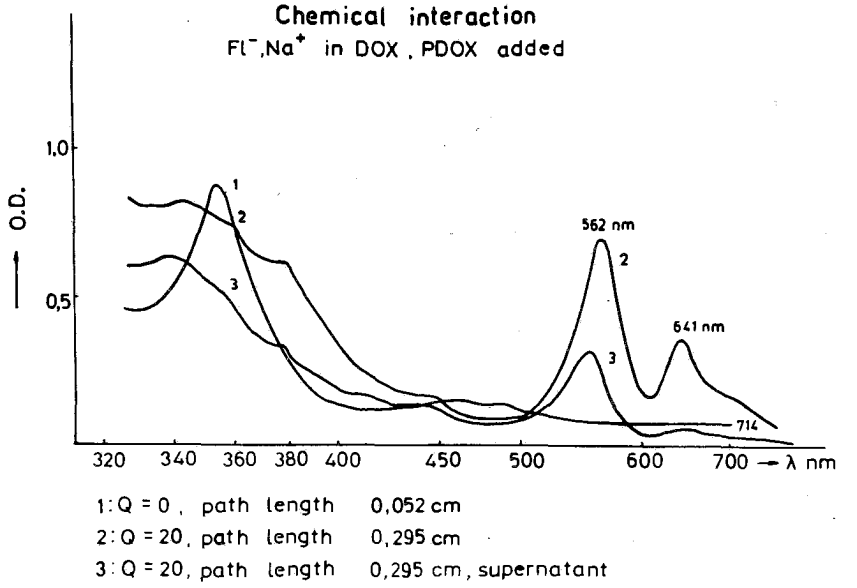


FIG. 14: *Optical spectra of fluorenylsodium in DOX on PDOX addition (chemical interaction):*
1: Q = 0, path length 0.052 cm,
2: Q = 20, path length 0.295 cm,
3: Q = 20, path length 0.295 cm, supernatant.

The interaction of polyethers with fluorenyl metal compounds represents a rather complicated picture which could schematically be expressed in the following way (Fig. 15). The chemical equations depict the solvation of a molecule of FlMt by a macromolecule of PE, the precipitation of complex A and its transformation into complex B, the latter being soluble or not depending on the solvent. According to the results obtained a molecule of PEO complexes, when PEO has a molecular weight of 20.000, actually carries 30 or

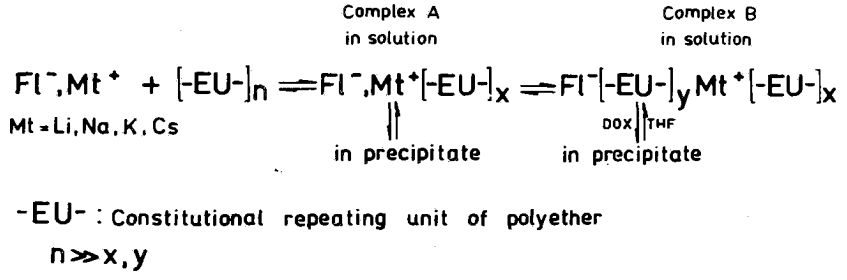
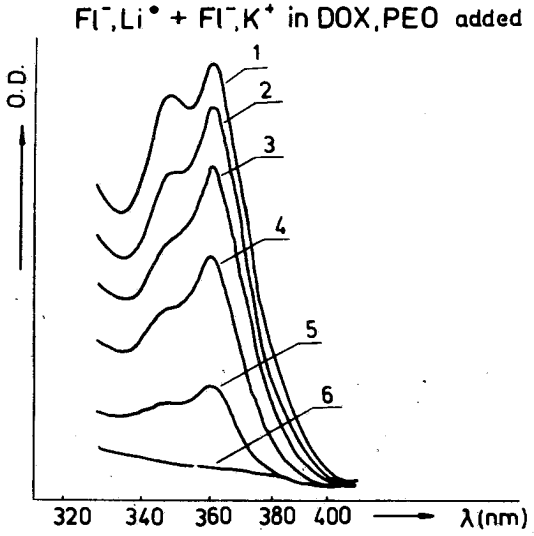


FIG. 15: *Complex A and complex B formation between polyethers and fluorenyl salts in ethereal solvents.*

more molecules of FlMt. This unusual stoichiometry as well as the presence of two distinct complexes in two phases, makes difficult to derive equations for depicting the equilibria and to evaluate the stability constants of complexes A and B and the equilibrium constant of their mutual transformation.

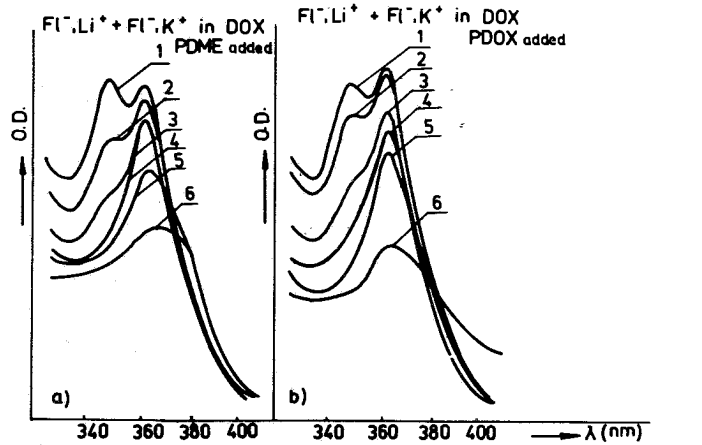
All three polyethers are able to form complexes with practically all cations studied. As seen from the results presented, PEO proves to be an universal (nonspecific) solvating agent for the cations of lithium, sodium and potassium. In contrast to this, with PDME and PDOX selective complexing ability was demonstrated. The best approach to study the selectivity in cation binding is to treat (to titrate) a mixed solution containing two metal derivatives, e.g. FlLi and FlK, with a PE solution. The mixture exhibits two sharp absorption bands at 348 and 361.5 nm in the UV spectrum corresponding to the contact ion pairs of FlLi and FlK, respectively. After PE addition characteristic changes are really registered. Fig. 16 shows the spectral changes in the supernatant solution when PEO is added. An insoluble complex is formed with both cations (disappearance of the characteristic maxima) with a certain preference to the Li cation. This proves the universal solvating power of PEO. In the next Fig. 17 two cases of pronounced selectivity of the other two polyethers are shown. The concentration of FlK in the supernatant solution remains constant in both cases (absorption at 361.5 nm) untill practically the total amount of the lithium derivative (absorption at 348 nm) is captured in the precipitate. Curiously enough, the potassium cations are also solvated by PDME after adding new portions of PE.

The different behaviour of PDME with respect to the different cations is most probably due to both steric and conformation reasons. The polymer macromolecule has compact structure because of the large number of comparatively bulky pendant methoxy groups. The coordinating sites, the ethereal oxygen atoms, are sterically hindered in spite of their position in the pendant groups and the predominantly syndiotactic configuration. Consequently, the tiniest cation, i.e. lithium should be best solvated (it is about four times smaller than sodium cation and more than ten times smaller than potassium cation). Furthermore, optimal solvation of lithium cations requires four oxygen atoms,



Optical spectra of fluorenyllithium and fluorenylpotassium mixed solution in dioxane on titration with poly(ethylene oxide)

FIG. 16: *Optical spectra of mixed fluorenyllithium and fluorenylpotassium solution in DOX on PEO addition.*



Optical spectra of fluorenyllithium and fluorenylpotassium mixed solutions in dioxane on titration with:
a) polydimethoxyethylene (PDME)
b) polydioxene (PDOX)

FIG. 17: *Optical spectra of mixed fluorenyllithium and fluorenylpotassium solution in DOX on addition of:*
a/ poly(dimethoxyethylene)
b/ poly(p-dioxene).

for sodium cations they are five or six, while in the case of potassium cations the minimum number of oxygen atoms reaches seven[16]. It is conceivable that conformations of PDME macromolecules allowing favourable cooperative arrangement of more than four oxygen atoms are less probable.

In contrast to PDME, PDOX solvates potassium cations. This can be explained in terms of enhanced rigidness of the polymer molecules. The C-C bond in the monomer unit is fixed being a part of the dioxene ring thus PDOX having less (nearly twice) kinetic units per unity length along the main polymer chain than PDME. The reduced segmental mobility could result in larger dimensions of the induced favourable conformation. Coordination with four or even five oxygen atoms may not be sufficient to cover the length of the segment closing the ring shaped solvating cage with the oxygens facing its interior. As a concequence, the lithium cation should be peripherically solvated and thus complex B cannot be formed with a lithium salt. On the other hand, the titration of the FlLi and FlK mixture with PDOX clearly shows that the stability constant of complex A (peripherical solvation) has higher values for FlLi than for FlK. This is to be expected since the surface charge density of the small lithium cation is larger. This will result in a stronger coordination of the oxygen electron pairs.

The results of the experiments presented till now allow us to compare the solvating properties of the examined polyethers and their monomeric analogues. The conclusion to be made for PDOX is immediate since experiments were carried out in dioxane. The low molecular ether cannot compete with the small amounts of PDOX added. Therefore, the polymer exceeds its monomeric analogue in cation binding ability.

Small segments of PDME and PEO macromolecule chains are represented in Fig. 18. It is seen that the oxygen atoms in PDME are connected by an aliphatic sequence of three carbon atoms which is known to be unfavourable for solvation as compared with the grouping $-OCH_2CH_2O-$ in DME and PEO. Thus, if we assume that the macromolecule is a non-elastic rod, PDME should exhibit weaker solvating properties than its monomeric analogue DME. As for PEO macromolecules, according to the same consideration they could not demonstrate their polydentate character. Consequently, in spite of the effective ethereal grouping, the cation binding ability of the polymer should likewise be inferior to that of DME or, at least, should not exceed it. On the other hand, taking into account the flexibility of the polymer chain and the possibility a section of it to rearrange into a favourable conformation, the polymers can exced the monomeric ethers, i.e. a «polymer effect» can be registered.

The comparison of PDME and PEO with dimethoxyethane has to be carried out indirectly. Investigations on complex formation of glymes with fluorenyl compounds were performed by Smid et al. under the same conditions and data of the equilibrium constant K for DME are available[12]:

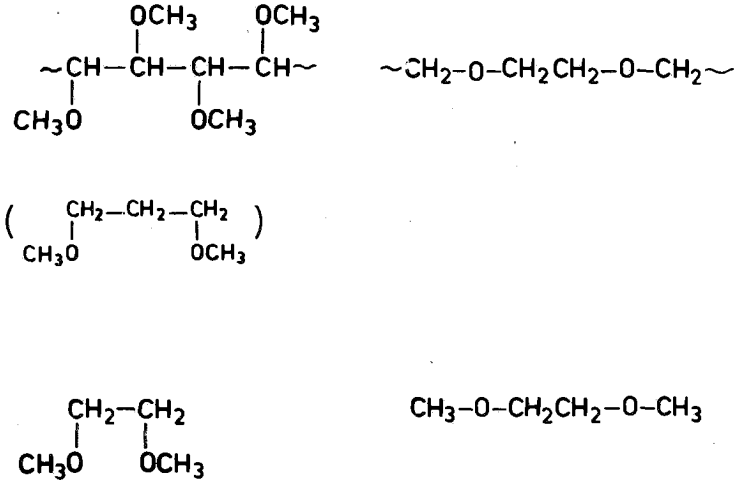


FIG. 18: *Molecular structure of low and high molecular ethers.*

$$Fl^-, Li^+ + n\,g\,G \underset{}{\overset{K}{\rightleftharpoons}} Fl^-, G_n, Li^+$$

$$K = \frac{1}{[G]^n} \frac{[Fl/\ /Li]}{[Fl,\ Li]} = \frac{1}{[G]^n} R$$

R is the molar fraction ratio of complexed to uncomplexed metal compound:

$$R = [Fl/\ /Li] : [Fl,\ Li]$$

If the total amount of FlLi in the system is fixed, R is a simple function of the glyme concentration. The total concentration of dimethoxyethane in DOX necessary for obtaining values of R = 1 and R = 10 for a 10^{-3} mole/l FlLi solution, is calculated from the respective values of the equilibrium constant K and the stoichiometry n and is given in Table I. The concentrations of PDME and PEO added to the same FlLi solution to reach the same R values of polymer complexed and uncomplexed metal compound, expressed in the corresponding ethereal units, are also included in the Table for comparison.

It is seen that both polyethers exceed their low molecular analogue, dimethoxyethane, which is a manifestation of the «polymer effect» in complex formation with FlLi. The same is true also for sodium and potassium cations solvation by PEO.

Can we use the complex forming properties of the polyethers studied for practical purposes? Yes, we can, at least in perspective. I will show you some examples.

TABLE I: *«Polymer effect» in the solvation of lithium cations by polyethers: a comparison of the complexing power of DME and its polymeric analogues PDME and PEO.*

Ether	[G] × 10^3 mole/l	
	R = 1	R = 10
DME	3320	8740
(–DME–)	9.6	15.8
$(–EO–)_2$	2.0	4.8

[FlLi] = 10^{-3} mole/l

We just saw that when treating a mixture of lithium and potassium salts with PDOX or PDME, lithium ions are first bound as an insoluble complex. This fact demonstrates the principle possibility for separating or removing different ions from their mixtures by distribution in different phases and simple filtration.

An interesting application of PEO for solubilization of inorganic salts in organic solvents was recently shown by Hirao et al.[3]. They found that potassium permanganate can be dissolved in benzene in the presence of PEO. The benzene permanganate solution was further used for oxidation of transtilbene. This is a good example for using the polymer in the phase-transfer catalysis.

Polythers can also be used for acceleration of nucleophilic reactions. A possibility in this respect was demonstrated simultaneously by Hirao et al.[3] and by us in one case of the Williamson reaction, namely in the preparation of butylphenyl ether:

$$\text{PhOMt} + \text{n-BuBr} \xrightarrow{\kappa'} \text{n-BuOPh} + \text{MtBr}$$

The values of the pseudomonomelecular rate constant k′ (tenfold excess of n-BuBr) obtained in our Laboratory are collected in Table II. As seen, in the presence of PEO they are with two orders of magnitude larger than in its absence.

The results obtasined in our Laboratory clearly show that polyethers possess strong solvating ability with respect to alkali cations and exhibit a certain selectivity in the complex formation with them. The observed phenomena cannot be explained only by the commonly accepted concepts of coordination of positive charges with ethereal oxygen atoms and the «polymer effect» was found to be responsible for the increased cation binding power of polythers as

TABLE II: *Accelerating effect of PEO in Williamson reaction: changes of pseudomonomolecular rate constant k′ on DME and PEO addition.*

Mt	Additive	[—EU—] : [Mt^+]	k [min^{-1}]
Na	—	0	8.7×10^{-6}
Na	DME	6.2	9.1×10^{-6}
Na	PEO*	7.5	8.2×10^{-4}
K	—	0	4.2×10^{-4}
K	DME	9.4	4.8×10^{-4}
K	PEO*	8.3	3.0×10^{-2}

DOX, 45°C, tenfold excess of n-BuBr.
* Molecular weight 20 000.

compared with their monomeric analogues. Therefore, polyethers are considered promising as complexing agents and further investigations are now being undertaken especially in the field of their influence on the kinetics of nucleophilic reactions.

Extensive summary

The pronounced cation binding properties of macrocyclic polyethers are known and already find practical application. Poly(dimethoxyethylene), PDME, poly(p-dioxene), PDOX, and especially poly(ethylene oxide), PEO, are cheap and available, thus being very attractive as solvating agents. In the present paper the results are given of the studies on complex formation between the lithium, sodium and potassium derivatives of fluorene with these macromolecular polyethers in low polarity media. The methods employed are electronic and NMR spectroscopy. The formation of two types of complexes is observed corresponding to different solvation states of the cations, peripherical and internal. PEO exhibits universal (nonspecific) cation binding properties, while PDME and PDOX interact in a different manner with cations studied. Direct evidence is found of selective complex formation in mixed fluorenyllithium and fluorenylpotassium solutions.

Polyethers can be considered as high molecular analogues of ethereal solvents and polyglycol dimethyl ethers (glymes). On the basis of literature data for the latter and experimental results for the polymers, a comparison is made of their complexing ability. The solvating properties of polymeric ethers are superior to those of their monomeric analogues which is explained by the «polymer effect». Possible applications of the cation binding properties are briefly outlined.

References

1. Bhattacharyya D.N., Smid J., and Szwarc M., J. Phys. Chem., **69,** 624 (1965).
2. Shinohara M., Smid J., and Szwarc M., J. Am. Chem. Soc., **90,** 2175 (1968).
3. Hirao A., Nakahama S., Takahashi M., and Yamazaki N., Makromol. Chem., **179,** 915, 1735 (1978).
4. Cheng T.S. and Halasa A.F., J. Polym. Sci. Polym. Chem. Ed., **14,** 583 (1976).
5. Uryu T., Seki T., Kawamata T., Funamoto A., and Matsuzaki K., J. Polym. Sci. Polym. Chem. Ed., **14,** 3035 (1976).
6. Chao Y. and Cram D.J., J. Am. Chem. Soc., **98,** 1015 (1976).
7. Hogen-Esch T.E. and Smid J., J. Am. Chem. Soc., **89,** 2764 (1967).
8. Lassalle D., Bioleau S., and Sigwalt P., Eur. Polym. J., **13,** 599 (1977).
9. Pedersen C.J., J.Am. Chem. Soc., **89,** 7017 (1967).
10. Panayotov I.M., Tsvetanov Ch. B., Berlinova I.V., and Velichkova R.S., Makromol. Chem. **134,** 313 (1970).
11. Panayotov I.M., Petrova D.T., and Tsvetanov Ch. B., Makromol. Chem., **176,** 815 (1975).
12. Smid J.: «Spectrophotometric studies on ion-pair equilibria» in Szwarc M., Ions and Ion Pairs in Organic Reactions, Vol 1., pp. 96-136, Wiley-Interscience, New York, 1972.
13. Hogen-Esch T.E. and Smid J., J. Am. Chem. Soc., **87,** 669 (1965).
14. Hogen-Esch T.E. and Smid J., J. Am. Chem. Soc., **88,** 307 (1966).
15. Panayotov I.M., Dimov D.K., Tsvetanov Ch. B., Skorokhodov S.S., and Stepanov V.V., J. Polym. Sci. Polym. Chem. Ed., in press.
16. Chan L.L., Wong K.H., and Smid J., J. Am. Chem. Soc., **92,** 1955 (1970).
17. Panayotov I.M., Tsvetanov Ch. B., and Dimov D.K., Makromol. Chem., **177,** 279 (1976).
18. De Boer E. and Grotens A.M., J. Am. Chem. Soc., **92,** 4742 (1970).
19. Dixon J.A., Gwinner P.A., and Lini D.C., J. Am. Chem. Soc., **87,** 1379 (1965).
20. Chan L.L. and Smid J., J. Am. Chem. Soc., **89,** 4547 (1967).

Chimica Chronika, New Series, 9, 325-335 (1980)

A NOVEL SYNTHETIC APPROACH TO STEROID ALKALOIDS OF SOLANIDINE TYPE

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A number of partial syntheses of the aglycon part of solanidine alkaloids has been described in the chemical literature. However, starting materials used in these syntheses were mainly natural steroid compounds containing the same number of carbon atoms in the molecule, identical or quite similar carbon skeleton and corresponding number and nature of all chiral centers as are found in synthesized solanidine derivatives. Thus, Uhle and Jacobs[1] achieved the first successful partial synthesis of a solanidine derivative by converting sarsasapogenin (I) into the *5beta*-solanidan- *3beta*-ol (II). In a much shorter and a more straightforward way rubijervine (III)[2] and isorubijervine (IV)[3,4,5] were converted into solanidine (V), while leptinidine (VI) was transformed into *5alpha*-solanidan-*3beta*-ol (VII)[6].

By converting tomatidine (VIII) and dihydrosolásodine (IX) into four C-22, C-25 epimeric solanidanones (X, XI, XII, XIII) the absolute configurations of the chiral centers at C-20, C-22 and C-25 were finally resolved[7,8].

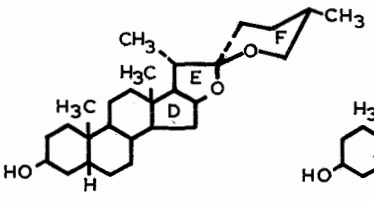
Scheme 1. summarizes only the structures of the C-27 steroid starting materials and the final products in the above mentioned partial syntheses of solanidine derivatives.

If the starting material is a synthetic C-21-steroid precursor, instead of a natural C-27- steroid outlined in the scheme 1., all three chiral centers at C-20, C-22 and C-25 have to be synthetically created. Scheme 2. presents two rather recent syntheses of demisidine (VII) (*5alpha*-solanidan-*3beta*-ol)[9,10], and of solanidine (V)[11], starting with pregnane derivatives (XIV and XVI).

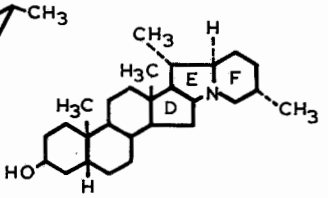
In both synthetic routes (*A* and *B*, scheme 2). there are several major drawbacks. Namely. the third step in the synthetic route *A* was even non-stereoselective giving the solanidine precursor XV *e* only as side-product. The procedure also involved a tedious chromatographic separation.

In the route *B*, starting material (XVI) had to be chromatographically separated from its trans-isomer (in respect to the double bond 17, 20), the chiral nitro-ester had to be prepared by resolving the corresponding racemic mixture, while the Michael addition in the step 1. gave two epimers at C-22. in none of the syntheses described in the scheme 2. It was possible to control stereospecifically the formation of the chiral center at C-22. In both cases C-20 had the right stereochemistry (20 S) due to its thermodynamic stability. The chiral center at C-25 had to be pre-synthesized.

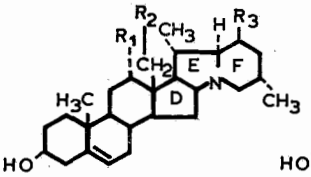
SCHEME 1.



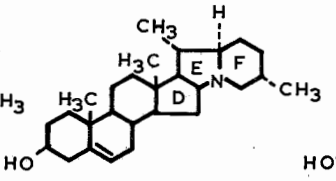
I



II

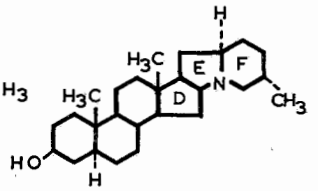


III (R_1 = OH, R_2 = R_3 = H)



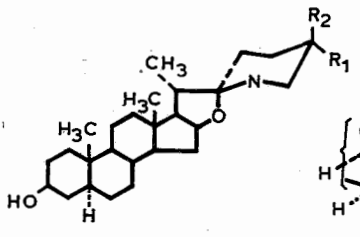
V

IV (R_1 = R_3 = H, R_2 = OH)

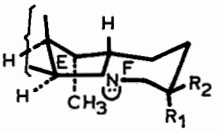


VII

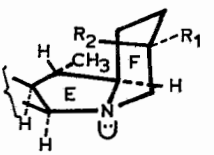
VI (R_1 = R_2 = H, R_3 = OH)



VIII (R_1 = CH_3, R_2 = H, 20S, 25S) IX (R_1 = H, R_2 = CH_3, 20S, 25R)



X (R_1 = CH_3, R_2 = H) XI (R_1 = H, R_2 = CH_3)

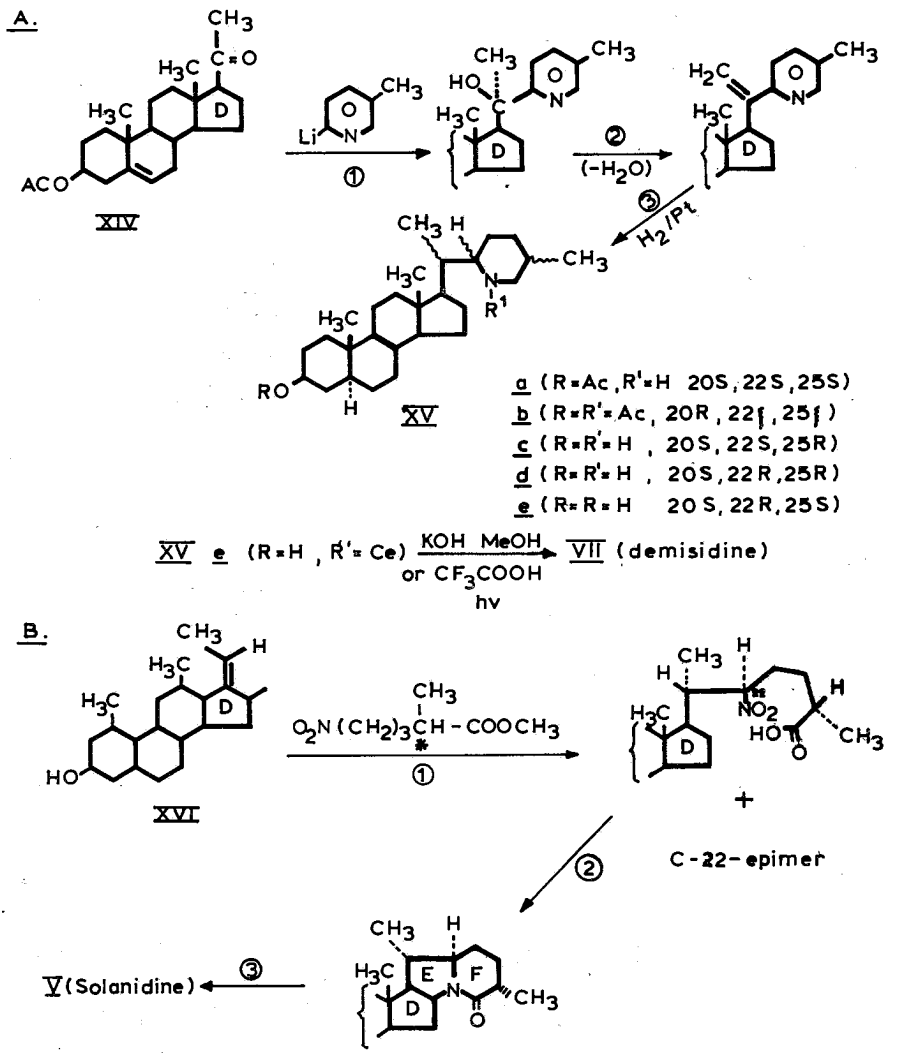


XII (R_1 = CH_3, R_2 = H) XIII (R_1 = H, R_2 = CH_3)

Our work in this field has been mainly concerned with finging out a new, possibly efficient method to synthesize steroid indolizidine system (equivalent to condensed ring E and F system of solanidine derivatives) and with examining possibilities of creating the chiral center at C-22 stereospecifically.

The present work was based on our previous discovery[12,13] of a cyclization reaction of 16-picolinylidene-17-oxo-steroids to the indolizidene derivatives, under conditions of catalytic hydrogenation. This reaction is summarized in the scheme 3.

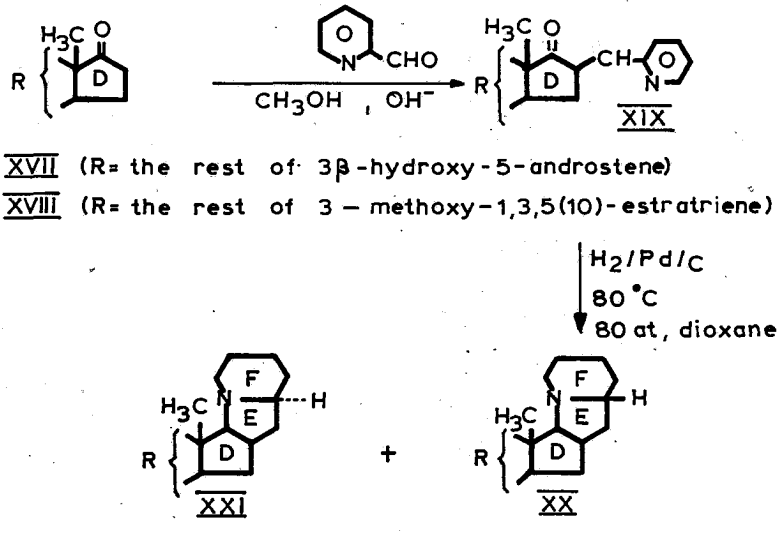
SCHEME 2.



The only, thus the key intermediate, 16-picolinylidene- 17-oxo-compound XIX was readily available by a straightforward Claisen-Schmidt reaction of 17-oxo-compound XVII or XVIII with pyridine-2-aldehyde. However, the formed indolizidene system of XX and XXI was unnatural with a tertiary N-atom attached to C-17 (in natural series this N-atom is bound to C-16), and in addition a mixture of two possible epimers at C-22 was obtained.

In order to accomplish a partial synthesis of steroid alkaloids of the natural solanidine type, by using the mentioned reductive cyclization reaction, we had

SCHEME 3.



to work out new convinient synthetic pathways to the key-intermediates (with reversed positions of functional groups at C-16 and C-17 in respect to our first discovery[12,13]) -17-picolinylidene-16-oxo-steroids.

For that purpose, starting with 16-oxo-steroids and carrying out Claisen-Schmidt reaction with pyridine-2-aldehyde, as previously described, seemed to us incovinient due to the following reasons:

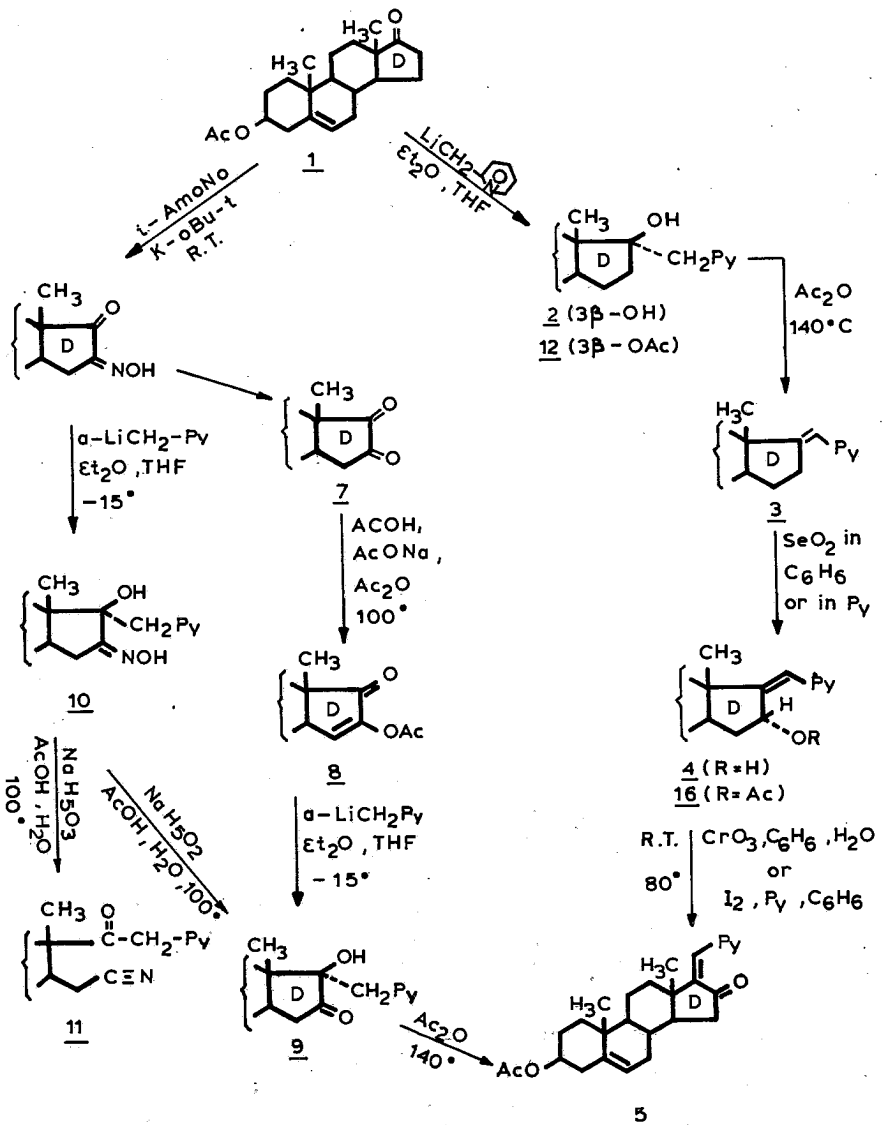
α) 16-oxo-steroids are only obtainable from 17-oxo-steroids in a three step synthesis;

b) there are potentially two active methylene groups (at C-15 and C-17) which might undergo Claisen-Schmidt condensation; in such cases formation of bis-condensation product is observed even when equimolar amounts of aldehyde and ketone components are used.

For the synthesis of 17-picolinylidene-16-oxo-5-androsten- 3*beta*-ol-acetate (*5*) we worked out three independant synthetic routes which are outlined in the scheme 4.

The synthetic route *1 — 2 — 3 — 4 — 5* was achieved in an overall yield of about 20%. In this sequence of reactions the step of allylic oxidation of C-16, in our hands, was only possible by using SeO_2, although the yields were rather poor (up to 40%). Other oxidation reagents gave complex reaction mixtures. Thus, CrO_3 in AcOH — H_2O at 10°, for example, gave 7-keto-compound *13* and 7,17-di-oxo-compound *14*, scheme 5.

SCHEME 4.

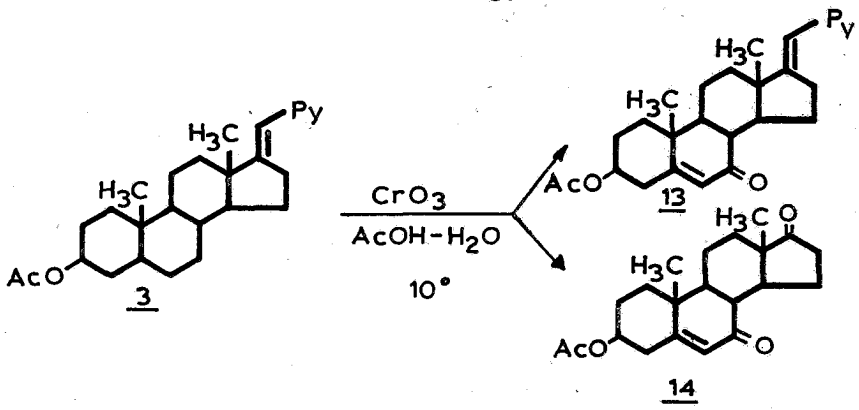


The other two routes, over 16-oximino-17-oxo-intermediate *6*, gave overall yields of only 2%. Therefore, these synthetic pathways are of no practical importance, but they proved independently introduction of 16-oxo-function via SeO_2 oxidation. The main observed difficulties in these two routes were:

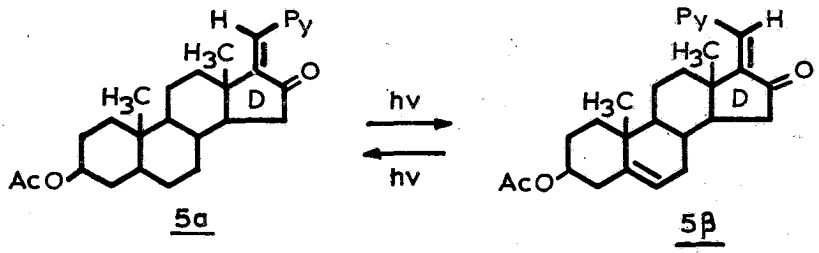
a) instability of the compound *10* which underwent Beckmann fragmentation reaction, giving secco-cyano-compound *11*, even in AcOH at 100°;

b) Instability and water solubility of diketone *7* in the presence of $NaHSO_3$.

SCHEME 5.



SCHEME 6.

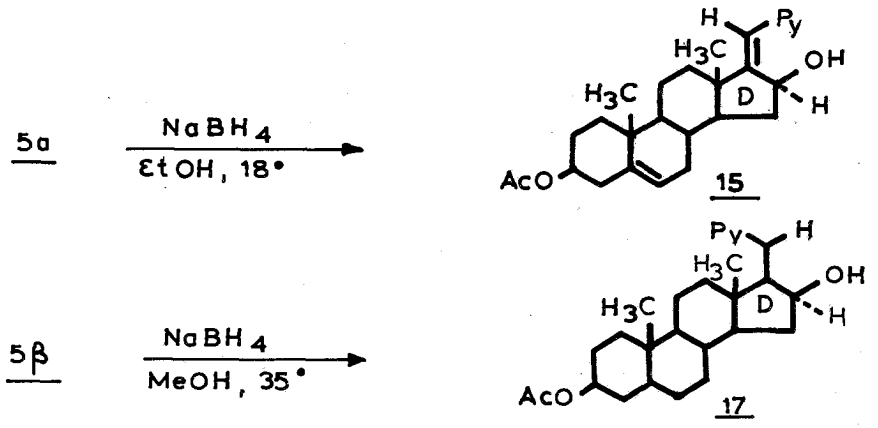


Having achieved the synthesis of the crucial intermediate *5*, we established that it was a mixture of the two geometrical isomers. We also observed that by standing in benzene solution at room temperature, exposed to daylight, isomeric ketones *5a* and *5b* are mutually interconvertable, scheme 6.

The ketones *5a* and *5b* were separated and obtained in pure forms only if all laboratory operations (chromatography, evaporations and crystallizations) had been performed in the absence of light (wrapping columns and vessels in Al-foil was practically sufficient).

The structures of the geometrical isomeres *5a* and *5b* were determined on the basis of the NMR-signals of the C(20)- *H* atom (in the case of *5a* it appears as a singlet at 6.6 ppm, while in the case of *5b* at 7.35 ppm). This is in a good agreement with known examples in the chemical literature[14]. The stereochemistry of the important intermediate *4* (the allylic oxidation product, scheme 4) was established indirectly by preparing both possible 16-*beta*-analogues of *4*, namely the alcohols *15* and *17*, according to the sheme 7.

SCHEME 7.



The crucial intermediate ketones *5a* and *5b* were separately subjected to the catalytic hydrogenation in dioxane, with Pd/C as catalyst, whereupon a rather surprising result was observed. Namely, the *cis*-isomer (*5a*, Py-rest and 16-oxo group are *cis*) gave exclusively the 21-R-stereoisomer (*18* or *19*, corresponding to the natural solanidine derivatives), while the *trans*-isomer *5b*) afforded as the only isolated product the 21-S-stereoisomer (*20*, corresponding to the 22-iso-solanidine series). In the synthetically obtained 21, 27-bis-nor-*5alpha*-solanidan-*3beta*-ol-acetates (*18* and *20*) the positions C (21) correspond to the positions of C (22) in solanidine series.

The scheme 8. summarizes the reductive cyclization reactions mentioned above.

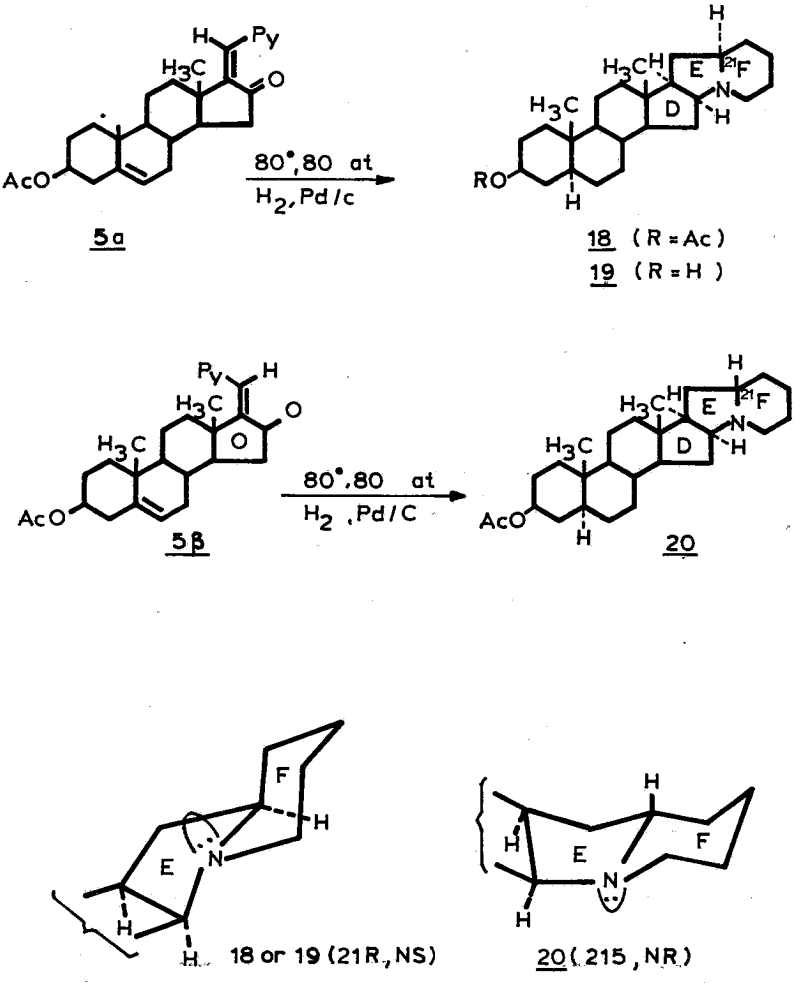
To propose a plausible mechanism for the stereospecific reductive cyclization process, we made the following assumptions:

a) spectral data (IR and NMR) indicate that the 16-oxo-17- picolinylidene-system of *5a* and *5b* is conjugated; therefore, it should be as planar as possible. Now, from all possible planar conformations (*5a, 5a', 5b* and *5b'*) we assume (according to Stuart models) *5a* and *5b* as much more stable due to very serious steric interactions between C(22)-H either with C(16)=0 group, as in the case of *5a'*, or with C(12)-H, as in the case of *5b'*;

b) the successive addition of hydrogen from the catalyst surface occurs from the *alpha*-side, during the whole process of hydrogenation, because the *beta*-side is sterically hindered by C(18) -angular methyl group;

c) the formation of cyclized immonium hydroxide intermediate of the type *18''* is assumed requesting the proximity of the =NH- and C(16)=0 groups, like in the case of *18'*. In the case of *5b* this assumption has to involve the transformation of the conformation *20'* into the conformation *20''* by free rotation around C(17)-C(20) bond, in which case 21-H reverses from *alpha* to *beta* configuration.

SCHEME 8.



Based on the above considerations, a possible mechanism of these rather unexpected stereospecific reductive cyclizations is outlined in the scheme 9.

The table 1. summarizes melting points, solvents for recrystallizations, and spectral data (IR, UV, NMR and MS) for all compounds which are not described in the chemical literature.

SCHEME 9.

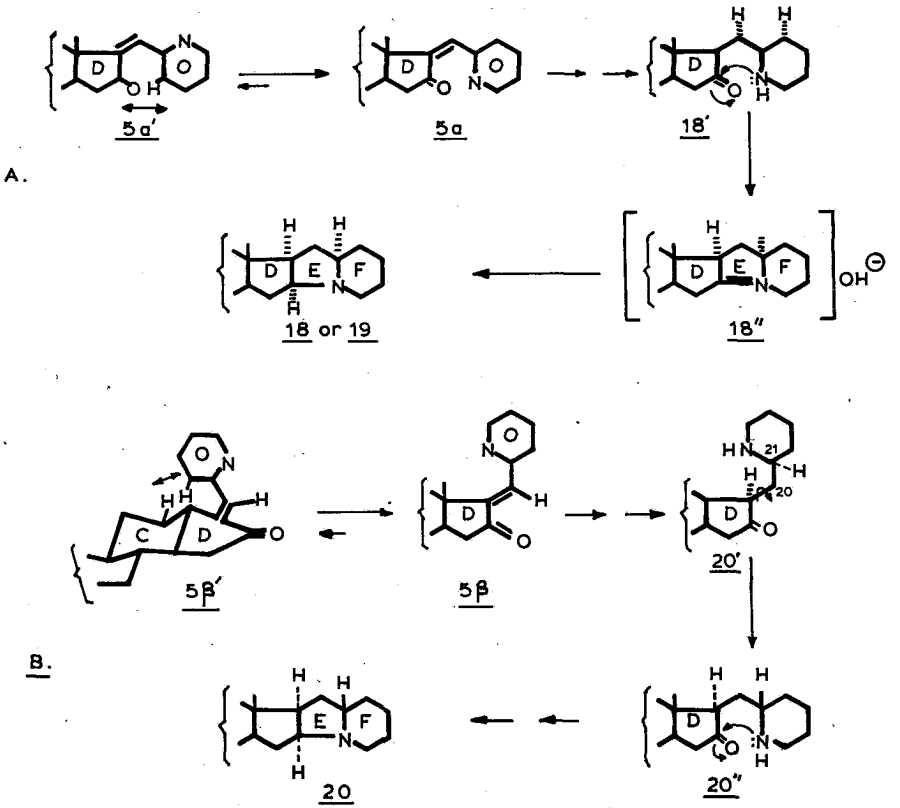


TABLE 1

2: M.p. 178° (from ethylacetate); IR: 3420, 3320, 3050, 1600, 1030, 750 cm^{-1}; NMR ($CDCl_3$): 0.9 (s, 3H), 1.0 (s, ^3H), ^{2,9} (^{1}AB-quartet, 2H), 3.3-3.8 (1H), 5.2 (d, 1H), 6.8-8.3 (m, 4H);
3: M.p. 205° (from methanol); IR: 3050, 1730, 1655, 1585, 1245, 775, 750, 740 cm^{-1}; NMR ($CDCl_3$): 0.9 (s, 3H), 1.1 (s, ^3H), ^{2,1} (^{1}s, 3H), 5.4 (d, 1H), 6.3 (t, 1H), 6.9-7.8 (m, 4H);
4: M.p. 235° (from ethylacetate); IR: 3240, 1730, 1650, 1595, 1255, 1030cm^{-1}; NMR ($CDCl_3$): 0.9 (s, 3H), 1.1 (s, 3H), 2.0 (s, 3H), 4.3-4.9 (1H), 5.0(1H), 5.45 (d, 1H), 6.25 (d, 1H), 7.5 (s, 1H), 7.0-8.7 (m, 4H); MS (m/e): 421 (66.8%), 406 (18.1%), 148 (19.5%), 147 (100%), 146 (35.6%), 119 (42.1%);
5a: M.p. 196-200 (from methanol); IR: 3090, 3040, 1725, 1705, 1615, 1580, 1560, 780, 740 cm^{-1}; UV: lambda (max) 215 and 294 nm; NMR: 1.1 (s, 6H), 2.05 (s, 3H), 4.2-5.1 (1H), 5.4 (d, 1H), 6.6 (s, 1H), 7.0-8.7 (m, 4H); MS (m/e): 419 (30.3%), 404 (8.2%), 360 (26.4%), 359 (93.4%), 344 (16.8%), 186 (15.1%), 173 (53.6%), 159 (66.8%), 145 (100%), 144 (64.3%);
5b: M.P. 201-205° (from methanol); IR: 3050, 1725, 1715, 1645, 1580, 1560, 770, 750 cm^{-1}; UV (ethanol): lambda(max) 212, 266 and 294 nm; NMR ($CDCl_3$): 1.1 (s, ^3H), 1.3 (s, 3H), 2.0 (s, 3H), 4.2-5.0 (1H), 5.4 (d, 1H), 7.35 (s, 1H), 7.0-8.6 (m, 4H); MS (^{1}m/e): 419 (58.8%), 404 (16%), 360 (28.1%), 359 (100%), 344 (21.5%), 173 (16.5%), 159 (13.9%), 145 (19.2%), 144 (44.6%);

7: M.p. 185-190° (from acetone/hexane); IR: 3400, 1750, 1050 cm^{-1}; NMR ($CDCl_3$): 1.05 (2s, 6H), 3.2-3.9 (1H), 5.4 (d, 1H); MS (m/e): 302 (33.3%), 232 (100%), 214 (40.7%), 199 (35%), 105 (29.6%); UV (EtOH, EtONa): 311-314 nm;
8: M.p. 162° (hexane/acetone); IR: 3075, 1770, 1725, 1620, 1250, 1200, 1030, 1018 cm^{-1}; NMR ($CDCl_3$): 1.1 (s, 3H), 1.2 (s, 3H), 2.0 (s, 3H), 2.25 (s, 3H), 4.3-4.9 (1H), 5.5 (d, 1H), 7.1 (d, 1H); MS (m/e): 327 (45.3%), 326 (89.5%), 311 (30.0%), 284 (73.2%), 160 (41.3%), 145 (76%), 43 (100%); UV (EtOH) 239 nm;
9: M.p. 194° (from ethylacetate); IR: 3400, 3220, 1745, 1595, 1090, 1040, 770, 748 cm^{-1}; NMR ($CDCl_3$): 0.9 (s, 3H), 1.05 (s, 3H), 3.0 (s, 2H), 3.3-3.8 (1H), 5.3 (d, 1H), 5.6 (1H), 6.9-7.7 (m, 4H); MS (m/e): 395 (12%), 367 (44%), 352 (16%), 334 (6%), 162 (24%), 149 (20%), 135 (28%), 120 (60%), 93 (100%);
11: an oil; IR: 3380-2400, 1700, 1630, 1595 cm^{-1}; NMR ($CDCl_3$): 1.05 (s, 6H), 1.25 (s, 3H), 1.40 (s, 3H), 4.05 (s, 2H), 3.1-3.8 (1H), 5.2 (d, 1H), 5.55 (1H), 6.8-8.7 (m, 4H); MS (m/e): 392 (14.3%), 279 (42.8%), 167 (53.6%), 149 (100%), 120 (25.0%), 83 (42.8%);
13: M.P. 165° (from methanol); IR: 3060, 1730, 1665, 1650, 1630, 1585, 1245 cm^{-1}; UV (EtOH): 244 and 286 nm; NMR ($CDCl_3$): 0,9 (s, 3H), 1.25 (s, 3H), 2.05 (s, 3H), 4.4-5.2 (broad signal, 1H), 5.8 (s, 1H), 6.3 (t, 1H), 6.9-8.6 (m, 4H); MS (m/e): 419 (100%), 404 (17.5%), 359 (5.4%), 344 (12.2%), 184 (9.4%), 170 (10.8%), 144 (12.2%), 130 (24.3%), 93 (27%);
14: M.P. 200-201° (n-hexane); IR: 1740, 1730, 1670, 1625, 1585, 780, 750, 740 cm^{-1}; UV (EtOH): 235 nm; NMR ($CDCl_3$): 0.9 (s, 3H), 1.25 (s, 3H), 2.05 (s, 3H), 4.4-5.2 (1H), 5.8 (s, 1H); MS (m/e): 344 (4.2%), 285 (21.2%), 284 (100%), 283 (42.5%), 269 (8.5%), 256 (31.9%);
15: M.P. 244-245° (from methanol); IR: 3320, 1730, 1660, 1585, 1245 cm^{-1}; UV: 250 and 294 nm; NMR ($CDCl_3$): 1.09 (s, 6H), 2.0 (s, 3H), 4.3-5.0 (1H), 5.45 (d, 1H), 6.2 (d, 1H), 7.0-8.5 (m, 5H); MS (m/e): 421 (81.8%), 406 (21.8%), 388 (5%), 361 (6.2%), 346 (7.5%), 147 (100%), 146 (41.8%), 133 (21.8%), 119 (49.1%), 106 (14.5%), 93 (27.2%);
16: M.P. 179-180° (from ether-hexane); IR: 1740, 1725, 1650, 1580, 1240 cm^{-1}; UV (EtOH): 247, 253, 282 and 298 nm; NMR ($CDCl_3$): 0.95 (s, 3H), 1.1 (s, 3H), 1.85 (s, 3H), 2.05 (s, 3H), 4.2-5 (1H), 5.4 (d, 1H), 6.9-8.5 (m, 4H); MS (m/e): 463 (25%), 448 (3.6%), 420 (100%), 403 (42.8%), 388 (14.3%), 184 (35.7%);
17: 248-249° (from methanol); IR: 3510, 3320, 1725, 1655, 1580, 1240 cm^{-1}; UV (EtOH): 247 and 274 nm; NMR ($CDCl_3$): 1.0 (s, 3H), 1.3 (s, 3H), 2.0 (s, 3H), 3.15 (s, 1H), 4.2-4.9 (1H), 5.4 (d, 1H), 6.7 (d, 1H), 7.0-8.6 (m, 4H); MS (m/e): 421 (100%), 406 (23.3%), 403 (10.3%), 388 (16.5%), 361 (4.5%), 346 (3.5%), 170 (11.9%), 157 (29.1%), 147 26.5%), 131 (12.7%), 119 (26.6%), 93 (45.2%), 80 (79.1%);
18: M.P. 180-181° (from methanol); IR: 2935, 2915, 2890, 2860, 2840, 2820, 2770, 2750, 2720 (Bohlmann bands), 1725, 1245 cm^{-1}; NMR ($CDCl_3$): 0.8 (s, 6H), 2.0 (s, 3H), 4.4-5.2 (1H); MS (m/e): 413 (63.8%), 398 (10%), 177 (14.8%), 176 (100%), 163 (11.4%), 150 (9.5%), 123 (18.4%), 122 (93.6%), 84 (7.9%), 81 (7.2%);
19: M.P. 186-187° (from methanol); IR: 2925, 2900, 2845, 2830, 2790, 2770, 2750, 2715 (Bohlmann bands) cm^{-1}; MS (m/e): 371 (54.8%), 356 (15.6%), 355 37.8%), 177 (13.5%), 176 (88.9%), 123 (19.3%), 122 (100%);
20: M.P. 178° (from methanol); IR: 2935, 2915, 2890, 2860, 2840, 2820, 2810, 2770, 2750, 2710, 1730, 1245 cm^{-1}; MS (m/e): 413 (92.6%), 398 (11.4%), 177 (19.6%), 176 (100%), 163 (15.2%), 150 (13.4%), 123 (27.8%), 122 (99.96%), 84 (12.8%), 81 (10%);

Remark: Compounds *1, 6, 10* and *12* are known from our published previous work; compounds *2* and *3* are known in the chemical literature[15,16], but the spectral data are missing; for all compounds given in the table 1. satisfactory elemental microanalises were obtained.

References

1. F.C. Uhle, W.A. Jacobs, J. Biol. Chem., **160,** 243 (1945).
2. Y. Sato, W.A. Jacobs, J. Biol. Chem., **179,** 623 (1949).
3. S.W. Pelletier, W.A. Jacobs, J. Amer. Chem. Soc., **75,** 4442 (1953).
4. D. Burn, W. Rigby, J. Chem. Soc., 963 (1953).
5. F.L. Weisenborn, D. Burn, J. Amer. Chem. Soc., **75,** 259 (1953).
6. R. Kuhn, I. Löw, Chem. Ber., **94,** 1096 (1961).
7. Y. Sato, H.G. Latham, J. Amer. Chem. Soc., **78,** 3146 (1956).
8. Y. Sato, N. Ikekawa, J. Org. Chem., **26,** 1945 (1961).
9. K. Schreiber, G. Adam, Tetrahedron, **20,** 1707 (1964).
10. G. Adam, K. Schreiber, Tetrahedron, **20,** 1719 (1964).
11. S. V. Kessar, A.L. Rampal, S.S. Gandhi, R.K. Mahajan, Tetrahedron, **27,** 2153 (1971).
12. M. Stefanovič, I.V. Mićović, D. Jeremić, D. Milkjković, Tetrahedron, **26,** 2609 (1970).
13. I.V. Mićović, D. Miljković, M. Jaredić, M. Stefanović, Glasnik Hemijskog Društva, Beograd (Bull. Chem. Soc., Beograd), **38,** 287 (1973).
14. L.M. Jackman and S. Sternhell: «Applications of NMR Spectroscopy in Organic Chemistry», Second Edition, Pergamon Press, **1969.,** p. 223, table 3-8-1.
15. J. Heer, K. Hoffmann, Helv. Chim. Acta, **39,** 1814 (1956).
16. W. Gustowski, M. Kocor, J. Michalski, Bull. Acad. Pol. Sci., (Ser. Sci. Chim.), **21,** 887 (1973).

Acknowedgments: The authors are grateful to Scientific Fund of Vojvodina for supporting this work and to the Association of Greek Chemists for giving us an unexpected opportunity to deliver this lecture.

Summary

The short survey of the known methods for syntheses of the steroid alkaloids of solanidine type has been given.

Our own research in this field concerns with a synthetic work directed to obtaining 21,27-bis-nor-solanidine derivatives by using a reductive cyclization reaction of 16-oxo-17-picolinylidene-C-19-steroids. Three independant synthetic routes have been worked out for obtaining these crucial intermediates. Special efforts have been successfuly made to control stereospecifically the creation of C-21 chiral center of newly formed indolizidene systems.

Chimica Chronika, New Series, 9, 337-352 (1980)

ENZYMATIC AND MICROBIAL CONVERSION OF CELLULOSIC AGRICULTURAL BYPRODUCTS FOR THE PRODUCTION OF ANIMAL FEED, ETHANOL AND CHEMICALS

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Introduction

The use of agricultural and other cellulosic wastes to produce various useful products with microorganisms has become very important during the last ten years. Although some of these byproducts are harvested and used, most are disposed of. They can be incorporated into the soil where they are converted to humus via microbial and climatic action, a very slow process. As an alternative, open field burning is practiced in many places including Greece, which causes air pollution.

The problem of agricultural wastes is a universal one since the annual quantities produced are immense. Straw alone amounts to 600 million tons, produced in the world every year. Greece is among the principal straw producing countries where pulp production is practical[1].

About half of the dry matter of most agricultural wastes consists of cellulose. The rest is lignin, nitrogenous compounds and ash (mostly silica). Table 1 shows as an example the composition of untreated ryegrass straw[2].

TABLE 1: *Composition of untreated rygrass straw (%).*

Crude protein	3.1
Crude fat	0.4
Cell soluble matter	32.7
Cellulose	35.1
Hemicellulose	21.7
Lignin	5.9
Ash	1.1

The Greek Situation

Greece is importing animal feed of a total value of the order of $ 200.000.000 every year. This is comprised of mainly corn ($ 150.000.000), soya beans ($ 30.000.000), barley and some other grains. Greece imported in 1978, 7.561.000 tn of crude oil and other fuel of a total value of $ 900.000.000. This was raised in 1979 to $ 1.400.000.000 and for 1980 to

about $ 2.400.000.000. The above expenses amount to about over 20% of our national budget. The fuel cost alone is equal to the total amount in currency of all imports. Thus, the utilization of agricultural cellulosics for animal feed and energy is of vital importance.

The lignocellulosic byproducts of the annual agricultural production in Greece can be bioconverted, in high biological value animal feed, ethanol and other valuable chemicals, (amino acids, vitamins, antibiotics, etc.) that today are exclusively imported. It is well known that ethanol can replace up to 20% the gasoline without any modification in automobile engines.

The use of microorganisms for the conversion of cellulosic wastes, of agriculture and industry, for the production of animal feed and/or alcohol seems to be the best way of bioconversion. Special strains of fungi can break down cellulose and lignin in their native state. The action of microorganisms is not climate dependent, has great productivity and can be controlled. The construction of a plant for the production of animal feed and/or alcohol does not require large land surface and this kind of technology does not pollute the environment.

However, even if agricultural wastes are potentially excellent sources of energy, there are problems for their utilization in their natural state. For example, the main shortcomings of straw as animal feed are its (a) low digestibility, (b) low protein content, (c) poor palatability, and (d) bulkiness. The nutritive value of straw depends partly on the availability of nutrients in straw for the animals. Chemical factors such as lignification, silification, and crystallinity of cellulose play an important role in the availability of the nutrients. This is shown by the fact that the digestibility of rice straw is only about 30% while the digestibility of dehydrated alfalfa is more than 50%[3].

Also, the production of ethanol from cellulose requires the hydrolysis of cellulose to glucose for the subsequent fermentation. Generally, the conversion of glucose to ethanol is 50%.

For Greece the interest from the Government and private sector will be shown when there will be technicoeconomical studies to a pilot plant scale and the evidence for the benefit and applicability of such technology for the Greek situation.

Among the cellulosic, agricultural byproducts of Greece those with economic interest from the stand-point of fermentations are:

1. Cereal straw
2. Corn stalks
3. Cotton stalks
4. Olive pip residues

1. *Cereal straw:* The annual production of Greece in wheat, barley and straw, according to the Ministry of Agriculture, is as follows:

TABLE 2: *Annual production in thousands tons.*

Year	Wheat	Barley	Straw
1975	2.078	924	1.501
1976	2.351	955	1.653
1977	1.715	706	1.210
1978	2.659	956	1.807
1979	2.410	869	1.639

Thus, the annual straw production of wheat and barley which are the two most important Greek cereals are about 1.500.000 tn. The way the stem is cut in Greece produces about 120 kg/stremma, so the rest is left in the fields and it is either ploughed or burned which is the most common. The straw production could be doubled very easily (220 kg/stremma) if the combine would cut the stem nearer to the ground (in about 10 cm). Macedonia and Thessalia are the major straw producing places in Greece producing 40% and 23% of the total production respectively. The rest is produced dispersed in the rest of the country. Thus, about 350.000 tn of straw is produced every year in Thessalia and about 600.000 tn in Macedonia. A paper industry in Thessalia is using 80.000 tn leaving a great amount still without use. Even if the price of straw at the place of production is minimal, the cost of collection and transportation comes to about $ 55/tn.

2. *Corn stalks:* The annual production of corn stalks, according to calculations of the Ministry of Agriculture is about 1,000,000 tn. In general, a small portion of the total production is cut with the grain and is silaged. However, the largest portion of the stalks, after fruit harvesting is woodified, and is not apt for animal feed, without any treatment.

The annual production of corn is estimated to 800.000 tn and the stalks to fruit ratio is 1.16. At present the corn stalks are left in the field. For systematic harvest of most stalk remains, the stems should be cut at 10 cm from the ground, and they should be transferred to a vehicle which comes along the combine.

3. *Cotton stems:* The annual production of cotton (with the seeds) in Greece according to calculations of the Cotton Institute is 400.000 tn. If we estimate the weight of the stems 80% of the cotton weight, then the annual production of cotton stems is about 320.000 tn. Today, the stems are not utilized and are incorporated in the ground with the plough. Nevertheless, there are machines that can eradicate the whole plants from the ground. This should be the practice for Greece for two main reasons. First, because it will protect the plantation from mycetological diseases and second the utilization of the whole plant biomass will be possible, for bioconversions.

4. *Olive pips residue:* The annual production of olive pips residue is about 250.000 tn according to the Ministry of Agriculture. The residue comes from 400.000 tn of olive seeds which are produced annually in Greece, after they are pressed for the removal of seed oil. Today, the olive pips residue is used

only as fuel for heating purposes. It has a potential animal feed value, because of the rich nutritional substances that it contains. However, it is poorly palatable in its natural state because of the high percentage of the woody tissue which it contains. We believe that it can be converted to a high quality fodder via microbial fermentation and that this is the best approach for its bioconversion.

Background Information

a) Lignocellulose Chemistry

Cellulose is a linear polymer of D-glucose residues linked through β (1→4) linkage. The difference between starch and cellulose, both polymers of D-glucose, is that cellulose has β (1→4) linkages and starch α (1→4) (Fig. 1).

The β (1→4) linkage gives cellulose such physical strength and rigidity that enzymes secreted in the digestive tract of most mammals cannot attack it. Ruminants, however, can utilize cellulose, as many microorganisms in the rumen form the enzyme cellulase.

The number of glucose units per molecule of cellulose (DP: degree of polymerization) ranges from a low of 15 to a high of 15,000 glucose molecules[4].

Alpha cellulose has been defined arbitrarily a cellulose insoluble in 17.5% NaOH (DP > 200).

Neutralization with acids of the soluble portion gives a precipitate which is the beta cellulose containing shorter chains (DP = 15-200) (β glucans) together with associated mannans and pentosans.

Finally, gamma cellulose is the material remaining in solution (DP < 15); Cellulose in plant cell walls usually forms fibrils surrounding the cell in parallel arrays, often in criss-cross layers forming an intricate, strong network.

Hemicelluloses are polymers of pentoses. Most commongly found are D-xylans, polymers of D-xylose in (1→4) linkages with side chains of arabinose and other sugars. Polymers of uronic (sugar acid) acids are also included among hemicelluloses.

In nature cellulose is usually found together with lignin and in order for cellulose degradation to occur, enzymes that break down lignin should be present. Lignin is a three dimensional aromatic polymer of phydroxy cinnamomic alcohols that acts as cement in and among the cellulose fibrils (Fig. 2).

For this reason, lignocellulolytic organisms are of primary importance when we discus degradation of native cellulose. Various kinds of mushrooms *(Pleurotus, Volvariella, Lentinus, Phanerochaete)* are capable of breaking down lignocellulose. Thus, they deserve more investigation. The microbial degradation of lignin can be tested by $^{14}CO_2$ evolution from lignin substrate labeled with ^{14}C[5]. According to this method the actinomycete *Thermonospora fuska* did not break down lignin while the fungi *Polyporus versicolor* and *Phanerochaete chrysosporium* resulted in large amounts of $^{14}CO_2$ evolution[6].

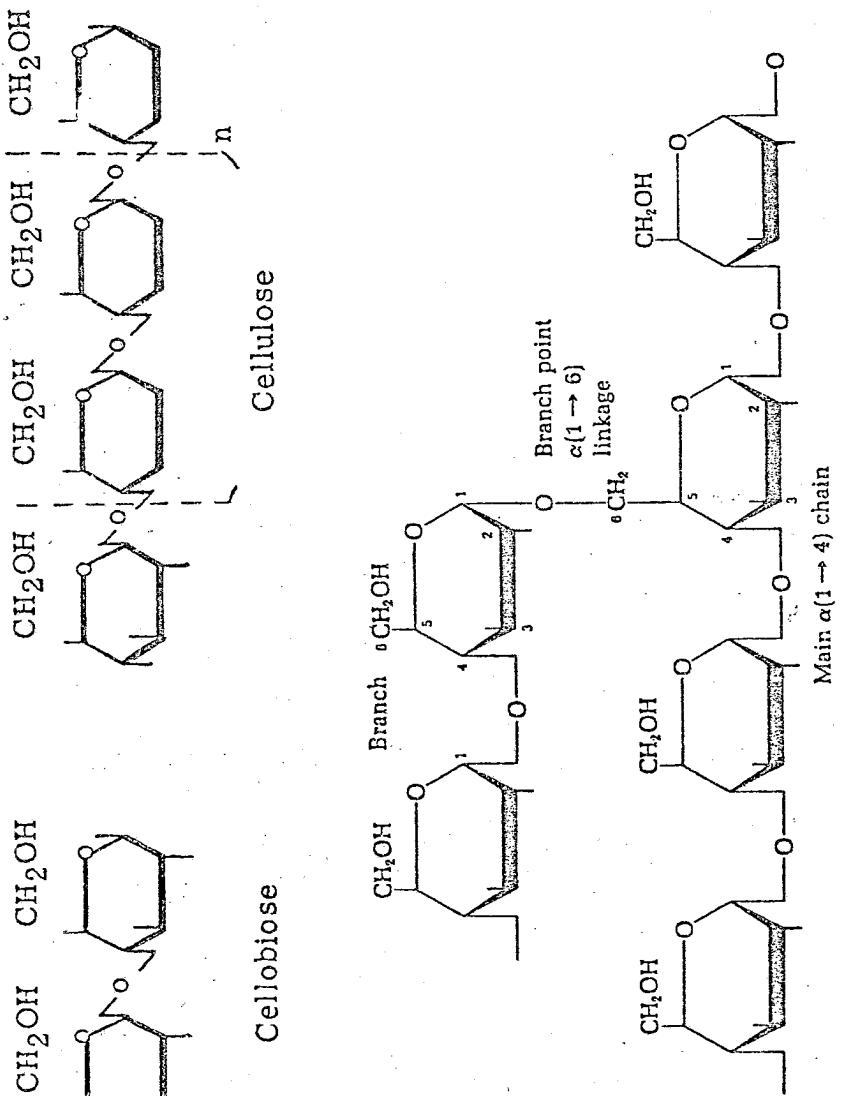

FIG. 1: *Structure of cellulose and starch.*

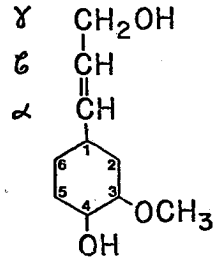

FIG. 2: *Lignin monomer*

b) Microbial degradation of cellulose

Recently[7] various enzyme mechanisms have been dicovered for cellulose degradation and their extracellular regulation in the fungus *Sporotrichum pulverulentum,* the perfect stage of which is *Phanerochaete chrysosporium.* The hydrolytic enzymes involved include 1) five endo-1,4-b-glucanases, 2) one exo-1,4-b glucanase (it was calculated that the ratio of activity between the 4 endo-glucanases T_1, T_{2a}, T_{2b}, T_{3a} and T_{3b} in the culture solutions was 4:1:1:1:1). The weight ratio of endoglucanase protein to exoglucanase protein is approdimately 1:1. Small but significant differences in the amino acid compositions of the different endoglucanases were found, 3) one or several 1,4-b-glucosidases, 4) an oxidative enzyme of importance in *in vitro* degradation seems to be a cellobiose oxidase. *In vitro* degradation of cotton with *S. pulverulentum* occurred twice as fast in O_2, compared to N_2, atmosphere. The oxidizing enzyme has been purified and it is a hemoprotein. It has been shown to oxidize cellobiose to cellobionic acid, 5) a cellobiose: quinone oxidoreductase is of importance both in cellulose and lignin degradation. One of three isoenzymes that have been purified is a flavoprotein with FAD as the prosthetic group. In the case of *Trichoderma viride,* similar enzymes have been found with the exception of the oxidative enzyme.

c) Mode of Action

Cellulose and lignin degradation is happening concurrently in nature. Lignin is not degraded unless there is at least one of the polysacharides present, degraded simultaneously with lignin. One may suggest that the role of the oxidizing enzyme is to oxidize cellulose by inserting uronic acid moities and thus breaking hydrogen bonds between cellulose chains. This causes swelling of the cellulose and makes the crystalline parts more accessible. This is in accordance with the old C_1-C_χ theory[8] (Fig. 3) that the cellulose is first activated so that subsequent hydrolytic enzymes can act. The oxidizing enzyme has been purified and is a hemoprotein. It has been found to oxidize cellobiose to cellobionic acid (Fig. 4). The enzyme may also oxidize the reducing end group formed when a β-1,4-glucosidic bond is plit through the action of the endoglucanases. This is at present only a speculation. Neither endo- or exo-glucanases can act alone[6].

A strong synergistic response was observed between the endo-glucanases and the exo-glucanases on de-waxed cotton. If de-waxed cotton was pretreated with endo- 1,4-β-glucanases, the exo-1,4- glucanases released much more degradation products than from untreated cotton. This strongly supports the theory that the enco-1,4,-β-glucanases, acting randomly over the cellulose chain, go in first and open up chain ends where the exo-enzyme can act[8].

Treatments to improve the feed value of cellulose

a) Alkali treatments

Sodium hydroxide treatment was widely used to upgrade the feed value of agricultural cellulosic wastes for many years. The old Beckman process[10] involved soaking chopped straw in a 1.5% NaOH solution at atmospheric temperature and pressure, after which the straw was drained and washed free of alkali. The treated straw increased in digestibility about twofold. NaOH treatments have been used to alter the lignocellulose of plants in order to improve the digestibility for ruminant feed. Some of the more comprehensive studies are by Donefer in 1973[11].

Donefer suggested that NaOH levels greater than 8 to 10 g/100 g straw resulted in a decreased rate of cellulose digestion. Also higher water volumes increased the amount of cellulose digested, probably due to better wetting of straw.

The «dry» process is the treatment of straw with final NaOH concentration of 2-4% (wt/wt) of straw. This treatment was applied to rygrass straw in Oregon, USA and some was commercially exported to Japan for animal feed[12].

In general, previous alkali treatment seems to enhance the effects of high pressure and temperature, microbial and enzymatic digestion and acid hydrolysis of cellulosic materials[13].

The lignin content of the substrate seems to be the limiting factor for the effectiveness of alkali treatment. Feist et al. showed a decrease in IVRD (In Vitro Rumen Digestibility) when the lignin content increased for NaOH treated hardwoods.

Increases in straw digestibility have been reported with the use of aqueous ammonia[15]. As is the case of NaOH, ammonia is believed to hydrolyze glucuronic acid ester cross links, thereby providing ready access to structural carbohydrates by rumen microorganisms. This results in an increased IVRD; on the other hand when ammonia acts on the straw, acetyl groups are separated and ammonium acetate is formed[16]. Thus, an extra benefit of ammonia treatment is the supply of organic nitrogen.

Other alkalis, KOH and $CA(OH)_2$ have been used, but their efficiency was generally somewhat less than that of NaOH[17].

b) Acid hydrolysis

Acid hydrolysis followed by microbial fermentation has been studied by various workers[18-23]. When polymeric carbohydrates are treated with an a-

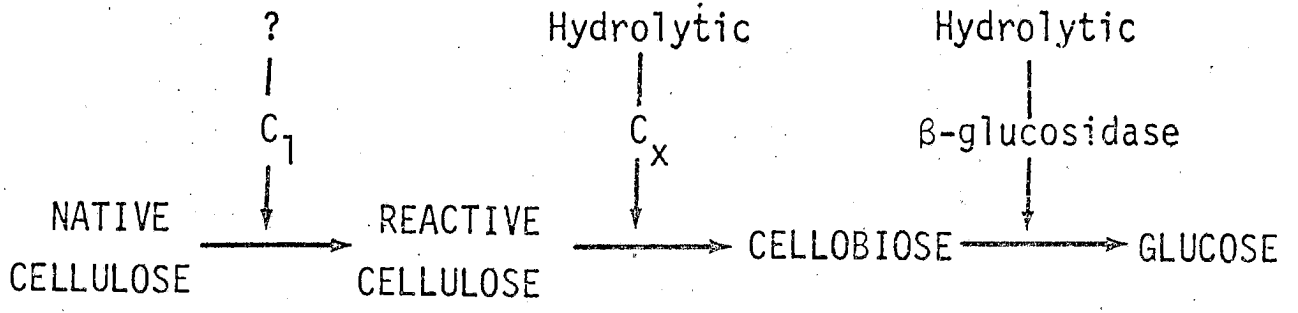

FIG. 3. *Mode of cellulose degradation by cellulase.*
Reece **et al.,** 1950.

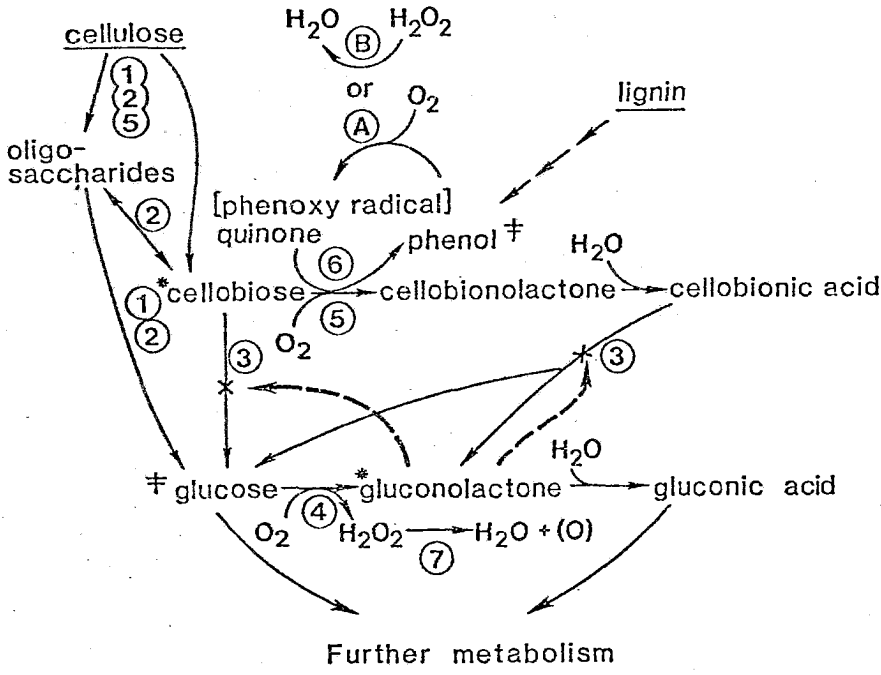

FIG. 4: *Enzyme mechanisms for cellulose degradation and their extra-cellular regulation in* ***S. pulverulentum.*** *Enzymes involved in cellulose degradation: 1, endo-1,4-β-glucanases; 2, exo-1,4-β-glucanase; 3, β-glucosidase; 4, glucose oxidase; 5, cellobiose oxidase; 6, cellobiose: quinone oxidoreductase; 7, catalase. Enzymes involved in lignin degradation: A. laccase; B, peroxidase.*
** denotes products regulating enzyme activity: gluconolactone inhibits 3; cellobiose increases transglycosylations.*
∓ denotes products regulating enzyme synthesis: glucose, gluconic acid- → catabolite repression; phenols → repression of glucanases.

queous acidic reagent, two major reactions take place: glucosidic hydrolysis and dehydration[24]. The glucosidic hydrolysis starts with initial protonation of both the glycosidic oxygen and the ring oxygen. The resulting hemiacetal hydrolyzes rapidly to free sugar. The hydrolysis rate is controlled by the rigidity of the glycon ring (Fig. 5).

The dehydration involves the reaction of carbohydrates through many intermediates to the formation of aldehydes. The sugars with an unstable ring will degrade more rapidly. These degradation products occur mostly in very drastic hydrolytic conditions (high temperature and pressure) and are known to be toxic to microorganisms[25-27].

Han and Callihan, 1974, showed[28] that by pretreatment with dilute acids and heat, cellulosic materials could be made more soluble. The variety of water soluble products formed have been found to be suitable for yeast propagation[21]. The dilute acid hydrolyzes the hemicellulose and, possibly, the amorphous cellulose, leaving the alpha cellulose and lignin intact.

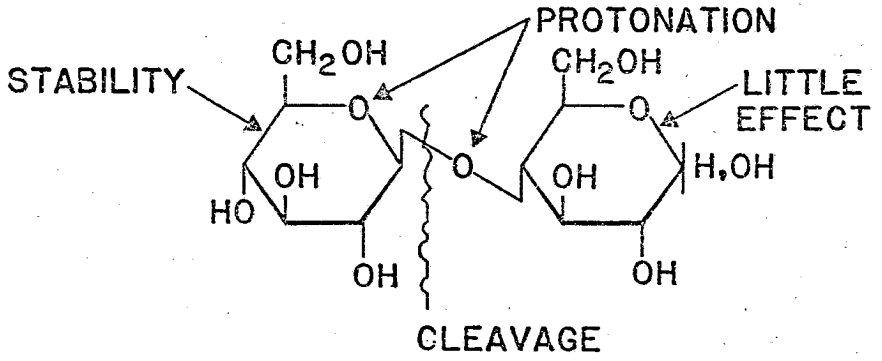

FIG. 5. *Factors in glycoside hydrolysis.*

Digestibility, protein level and palatability of straw and other cellulosic wastes is enhanced by treating them with dilute aqueous solution of HCl and H_3PO_4, ammoniating the acid treated substrate, and fermenting it with a yeast such as *Aureobasidium pullulans*. The so-treated straw is useful as a feed for ruminants and other animals[29].

c) Other Chemical Treatments

Oxidizing agents like periodates and nitrigen dioxide have been used to degrade the parts of cellulose as an effort to increase digestibility. According to another report the crystallinity of cellulose was reduced with oxidizing agents, but the change in crystallinity did not correlate with digestibility[28].

Lignin oxidizing agents like $NaClO_3$ have been used to disrupt the lignocellulose complex and improve the digestibility of cellulose complex and cellulose material[30]. The digestibility of straw was increased but the product was poor in palatability because of the high levels of NaCl.

Also, gaseous SO_2 was used to treat the lignocellulose complex *in situ* in plant residue[31]. Experiments with sawdust showed an increase in the digestibility of the treated material while there was a decrease of the lignin content. This suggests that lignin is depolymerized by this treatment.

d) Physical treatments

Various kinds of physical treatments were used to disrupt lignocellulosic complex. Among these were moist heat expansion (extrusion)[32], dry heat expansion[33] and even steam explosion[34]. These methods serve as a pretreatment for increasing the feed efficiency or for bioconversion of the treated material to single cell protein.

It seems that these treatments alone without any subsequent alkali or acid treatment are ineffective for the growth of various microorganisms[28]. Besides,

these treatments require high temperatures which produce further degradation of hydrolyzed monomeric sugars and lignins which are harmful to microbial growth. Such products are furfural, formaldehyde and formic acid[25].

The effect of particle size of the substrate on processes like enzymatic digestion or fermentability is self- evident because of the direct physical contact between the enzyme and the substrate. Investigations have shown that the smaller the size of the substrate the higher the enzyme digestion and the fermentability, which is to be expected[35,36]. However, these kinds of treatments do not seem to be economical on an industrial scale[28]. There is always a degree of grinding depending on the extent of digestion of the material, the need for high or low animal production, and the cost of grinding. Other types of physical treatments include irradiation by gamma rays, high velocity electrons, and UV radiation[37-39]. These are too expensive for practical purposes.

Developments in bioconversion

a) Enzymatic treatments

In general reports on the increase in digestibility of lignocellulosic material by microbial and enzymatic treatments were variable and inconclusive[40-41].

The low digestibility of straw is not caused by the cellulose alone but mainly by the complex formed between lignin and cellulose. Thus, by distrupting the lignin-cellulose complex we would expect to improve the digestibility of straw. One approach might be treating the straw with simultaneous applications of cellulase and lignase. In nature two types of gungi belonging to Basidiomycetes possess such enzymes. One type of wood rot fungi, the «brown rot», preferentially attack cellulose and hemicellulose, leaving lignin intact. Thus, the decaying residue turns brown in color. White rot fungi, on the other hand, attack lignin, causing the decaying residue to turn white.

There is a special reaction called Bavendams reaction[42] used to classify wood-rotting fungi into lignin and non-lignin decomposers. The enzyme responsible for lignin degradation was found to be a laccase (polyphenol-oxidase) excreted by white rot fungi on lignin decomposition[43,44]. Digestibility of straws was shown to relate inversely to lignin content[40].

Ralston et al. 1962 studied[45] the effect of protease, amylase and pectinase on the digestibility of low quality roughage. The digestibility of either extract of a ratio subjected to a commercial preparation of proteolytic and cellulolytic enzymes was significantly reduced, whereas fungal protease and fungal amylase gave a significant increase.

The bioconversion of cellulose includes at the first stage its hydrolysis to glucose and subsequently the glucose conversion to alcohol and other useful products. For the saccharification of cellulosic substrates there should be a pretreatment of the substrate aiming to the delignification and the reduction of crystallinity of cellulose[46]. This stage is the most energy intensive and the most expensive of the whole procedure[47].

At Natick Research Laboratory, MA, U.S.A. the preferred method of pretreatment is pot milling[48]. Using newspaper print as a cellulosic substrate, they have shown that pot milling resulted in the best saccharification (70%) in 48 hr.

The first step involves growing the fungus *Trihoderma viride* in culture medium containing shredded cellulose and various nutrient salts. Following its growth culture is filtered and the solids discarded. The clear straw colored filtrate is the enzyme solution, that is pH adjusted to 4.8 and introduced to the reactor. Milled cellulose is then introduced in the reactor, and the enzyme broth is allowed to react with cellulase to produce glucose sugar. There is also a recycle of enzyme and unreacted cellulose back into the reactor. The crude glucose syrup is filtered for further use, in alcohol production, single cell protein and other fermentation products. With this method 1/2 ton of glucose was produced from one ton of waste paper which can be fermented to produce 68 gallons of ethanol or in other words a 25% conversion from paper to ethanol.

Other investigators[49,50] favor chemical methods as a pretreatment of cellulosics for saccharification. According to these studies rice straw and begasse, were delignified when boiled in 1% NaOH for 3 hours. In general hardly hydrolyzable cellulose such as rice straw produced strong cellulase (higher Filter Paper Degrading activity), while delignified, easily hydrolyzable cellulose produced weak cellulase with Trichoderma viride strains. For optimum saccharification it was shown that an enzyme concentration of 3% and a substrate concentration 10% is required. Autosaccharification of rice straw and bagasse was exhibited but the sugars produced inhibited the cellulase activity via feedback mechanisms.

Generally, the enzymatic activity in the fungus *T. viride* is quickly suppressed when glucose, xylose or galactose are below 5%. Addition of glucose or cellobiose in cultures of *T. viride* grown on cellulose resulted in reduced activity[51]. The same phenomenon of enzyme activity inhibition was shown and with strains QM 9414 and MCG 77 of *T. viride* in 2% cellulose fermentation[52].

Trichoderma produces only low amounts of extracellular glucosidase. Thus cellobiose accounts for over one-half of the soluble sugar[51]. Cellobiase activity secreted by *T. viride* and its mutants is suboptimal for conversion of cellulose to glucose in the *T. viride* system[51,53].

Studies by Shewale @ Sadana, 1978 and 1979[54,55] propose the enzymatic hydrolysis of cellulosics with the fungus *Sclerotium rolfsii.* This fungus produces comparable amounts of total sugar with *T. viride* yet, most of it was glucose with little or no cellobiose, probably due to the high cellobiase content in the culture filtrate. This is the main advantage in hydrolysis of cellulosics by *S. rolfsii* for sugar production.

b) Thermophilic microorganisms

For the degradation of lignocellulosics, thermophilic microorganisms were also used. The advantage in this case is that you can avoid contamination because their optimum temperature requirements ranges between 50°—60°C.

The actinomycete *Thermonospora curvata* was isolated from numicipal refuse[56] and it has been shown[57] that it has both C_1 and C_χ types of enzymes, for cellulose degradation. The presence of soluble reducing sugars inhibited cellulase production, as in the case of *T.viride*. The actinomycetes *Thermonospora fuska* and *Streptomyces thermodiastaticus* were shown to be able to degrade cellulose but they do not have cellobiase for the degradation of cellobiose[58].

Simultaneous degradation of cellulose to glucose and fermentation to ethanol in one step, was described by Cooney et al., 1978, and Gordon et al. 1978, using the anaerobic bacteria *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*[59-60]. Cellulose is eventually converted to ethanol and acetic acid. However, the final ethanol concentration produced by this method was not over 5%. Mixed cultures of *C. thermocellum* and *Methanobacterium thermoautotrophicum* produced more H_2, acetic acid and CO_2 and less ethanol[61]. Mixed cultures of *C. thermocellum* and *C. thermohydrosulfuricum* increased the rate of cellulose degradation and produced 1.4 moles of ethanol per mole of cellulose, which is a 38.8% conversion[62].

Research from Gulf Oil in pilot plant scale at Kansas and Hawaii of the U.S., have shown that mixed cultures from *Trichoderma reesei* and the yeats *Saccharomyces cereviciae, S. carlsbergensis* and *Candida brasicae,* increased the ethanol production 40%, due to the direct conversion of glucose to ethanol.

c) Technology of alcoholic fermentation

Rapid ethanol fermentation of cellulose hydrolysate was studied by Ghose and Tyagi, 1979[63]. According to the study using yeast concentration of *Saccharomyces cerevisiae* 23.6 g/L, they were able to produce an ethanol concentration of 9.7% in a period of six hours. The extract also contained 6-7% reducing sugars, among which 70% was glucose and 30% other sugars mainly xylose, cellobiose and cellodextrins.

Glucose concentration was increased up to the level (10-20% (w/v)) with a rotary vacuum evaporator. The maximum fermentor productivity of ethanol obtained in continuous culture employing cell recycle and a 0.127 v/v/m air flow rate was 32.0 g/liter/hr, which is almost 7.5 times higher than the normal continuous process without cell recycle and air sparging. The viability of the yeasts remained above 6% in the fermentor with no need for oxygen increase or unsaturated fatty acids and sterols. Yeast growth was limited by either glucose or ethanol[64].

Due to the fact that ethanol productivity decreases linearly with ethanol concentration, for higher productivity ethanol should be removed as soon as it is formed. One system is to vacuum with cell recycle[65]. With this system ethanol production increased 12 fold compared to the usual fermentation with cell recycle. The main drawback however is the very high energy requirements for cooling and for pumping out the CO_2 to atmospheric pressure[64].

d) Use of immobilized whole yeast cells

A different approach of the subject of bioconversion is the use of immobilized enzymes and cells. The substrate moves upward into the base of the columns, through the gel. The enzymes in the yeast cells convert the glucose to ethanol and carbon dioxide[66,67]. Such columns can produce 14-15% alcohol in 2-8 hr, with a productivity of 20-80 g/L/hr. These productivity rates are comparable to the most satisfactory fermentation systems that are available today. Ethanol could be continuously and efficiently produced for long periods of time (more than three months) without the loss of ethanol-producing activity. The yeast immobilization involves mixing of the yeast with the support medium and incubation in a nutritional medium on a rotary shaker to increase the number of living cells. Using Kappa carrageenan (a kind of polysaccharide used as food additive) and the yeast *Saccharomyces carls bergensis* a final population of 5.4×10^9 cells/ml of gel was reached[68].

Economic considerations

a) Cellulosics for animal feed

The current price of alfalfa is 5 dr/kg and that of cereal grain 5.5-6 dr/kg. The fermented product could compete for animal feed if its price will be formed to 5 dr/kg or less. It is known that with the Greek enrollment in the Common Market the prices of cereal grain will reach the international levels, i.e. 8-10 dr/kg. Thus for the future the proposed product, when its nutritional value will be proved to be adequate for animal feed, it will be highly competitive due to the low cost of the initial material.

b) Cellulosics for ethanol

The use of alcohol as automobile fuel is known from the thirties. However, its production was not generalized due to the low cost of hydrocarbons. In the last 10 years due to the increase of the fuel proces, alcohol production was enhanced in many countries. The United Nations University organized a Conference on the bioconversion of Organic Residues[69] and in March 1979 the U.N. Industrial Development Organization held a Workshop on Fermentation Alcohol for use as fuel and Chemical Feedstock in developing countries, in Vienna, Austria[70]. Some of the main points of the workshop can be summarized as follows:

1) Blend of 10% alcohol with gas increased the mileage (miles/gallon) by 5.6%.
2. The same blend increased the F-1 octane number by 4 units.
3. There is a positive volume change of mixing alcohol with unleaded gas with a maximum in 12.5% of alcohol volume.
4. Blending alcohol (10%) with gas causes fewer pollutants in the engine exhaust. Specifically GASOHOL fuel produces less CO and about equal amounts of NO_χ and unburned hydrocarbons. The total emission for Gasohol are somewhat less than for unleaded gasolin.

Many countries have already adopted the use of gasohol for fuel. In the U.S. 28 states are selling gasohol while in Brazil many cars are burning exclusively alcohol. If eventually a 15% ethanol blend with 85% unleaded gas will be established for use in automobiles, this blend should sell from the state at the price of super gasoline. The current price of regular gasoline is 29 dr/1 and of super is 33 dr/1. Therefore, the selling price of blending fuel should be 33 dr/1. Thus, the price of ethanol in the blend can be calculated as follows:

(price of regular/1) $\times$ 0.85 + K $\times$ 0.15 = (price of super/1) where K = price of ethanol/1.

By solving the equation we find that K = 55.6 dr/1. The current ex-factory prices of 95% ethanol is about 20 dr/1. Hence, the profit from selling the ethanol as fuel will be 55.6 - 20 = 35.6 dr/1. This profit could be used from the government for various purposes.

References

1. Rydholm, S.A. Pulping Processes. Interscience (John Wiley and Sons, Inc.), p. 676, New York (1967).
2. Han, Y.W., Yu P.L., and S.K. Smith, Biotech. and Bioeng. 20, 1015 (1978).
3. Han, Y.W. and A.W. Anderson. Economic Botany 28, 338 (1974).
4. Lehninger, A.L. «Biochemistry 2nd ed.», pp. 267, 729-742 Worth Publishers Inc., New York (1975).
5. Crawford, L.D. and R.L. Crawford. Appl. Envir. Microbiol. 31, 714 (1975).
6. Eriksson, K.E., Pettersson B. and V. Westermark. FEBS Letters 49, 282 (1974).
7. Eriksson, K.E. Biotechnol. Bioeng. 20, 317 (1978).
8. Reese, E.T. «Cellulose decomposition: Fungi in marine boring and fouling organisms», ed. by D.L. Ray, Univ. Wash. Press, Seattle (1959).
9. Streamer, M., Eriksson K.E. and B. Pettersson. Eur. J. Biochem. 59, 607 (1976).
10. Beckmann, E. Chem. Abst. 16, 765 (1922).
11. Donefer, E. «Effect of processing on the nutritional value of feeds», National Academy of Science, Washington, D.C. (1973).
12. Odell, F.G. and T.R. Miles. Consulting engineer's report to the Oregon Field Burning Committee, Salem, Oregon, pp. 41 (1974).
13. Guggloz, J., Kohler G.O, and T. Klopenstein. J. Anim. Sci. 33, 131 (1971).
14. Feist, W.C., Baker A.J., and H. Tarkow, J. Animal Sci. 30, (5), 832 (1970).
15. Wais. A.C., Jr., Guggloz J., Konler G.O., H.G. Walker, Jr. and W.N. Garret. J. Animal Sci. 35 (2)[?] 109 (1972).
16. Laguta, A.F. Zhivotnovodsvo 23, 86 (1961).
17. Anderson, D.C. and A.T. Ralston. J. Anim. Sci. 37, 148 (1973).
18. Eklund, E., Hatakka, A., Mustranta, A. and P. Nyberg, Eur. J. Appl. Microbiol. 2 (3), 143 (1976).
19. Grant, G.A., Han Y.W. and A.W. Anderson. Appl. Envir. Microbiol. 35, 549 (1978).
20. Han, Y.W., Pence J.W., and A.W. Anderson, Appl. Microbiol. 29, 708 (1975).
21. Nagy, G.M., Vaiilant K. and A. Sos Balint. Biotechnol. Bioeng. 17, 1023 (1975).
22. Tsao, G.T. ERDA report No. 18, E(11-1) 2755, p. 41 (1976).
23. Israilides, C.J., Grant G.A. and Y.W. Han. Appl. Envir. Microb. 36 (1); 43 (1978).
24. Harris, J.F. Proc. of the 8th Cellulose Conference, Appl. Polymer Symp. 28, 131 (1975).
25. Frey, K.L. M.S. Thesis, Oregon State University (1973).
26. Harris, E.E., Hajny G.J., Hannan M. and S.C. Rogers, Ind. and Eng. Chem. 37, 390 (1946).
27. Saeman, J.F. Ind. Eng. Chem. 37, 43 (1945).
28. Han, Y.W. and C.D. Callihan. Appl. Microbiol. 27, 159 (1974).

29. Israilides, C.J., Grant G.A. and Y.W. Han, Dev. Ind. Microb. 20, 603 (1978).
30. Green, J.W. Methods in Carbohydrate chemistry, vol. III, Cellulose, p. 12, Academic Press (1963).
31. Millett, M.A., Baker A.J. and L.D. Satter. Seminar on cellulose as a chemical and energy resource at U.S. Berkeley, CA (1974).
32. Weast, R.C. «Handbook of Chemistry and Physics», vol. 47, 3206. The Chemical Rubber Company, Cleveland, Ohio (1966).
33. Williams, M.A. and Baer. Feedstuffs, 36, 48 (1964).
34. Jurasek, L. Dev. Ind. Microb. 20, 177 (1978).
35. Chose, T.K. and S.A. Kostick. Advances in Chem. Sero. 95, 415 (1969).
36. Han, Y.W. and D.W. Chen, Biotech. and Bioeng. Vol. XX, 567 (1978).
37. Millett, M.A., Baker A.J., Feist W.C., Mellenberger R.W. and L.D. Satter. J. Animal Sci. 31 (4), 781 (1970).
38. Klein, D.A., Rockhill R.C., Eldridge J.P., and J.E. Park, Tappi 53, 1469 (1970).
39. Pritchard, G.I., Pidgen W.J. , and D.J. Minson. Canc. J. Animal Sci. 42, 215 (1962).
40. Kirk, T.K. and W.E. Moore. Wood and Fiber 4, 72 (1972).
41. Ronatschuk, H. (Oy Tampella Ab, Tampere, Finland) S.R. Tannenbaum and D.I. Wang (eds.), «Single Cell Protein II», MIT Press, Cambrigde, Mass. (1975).
42. Bavendamm, W. Z. Pflanzenkrankh. Pflanzenchutx. 38, 257 (1928).
43. Fahraeus, G. and G. Lindeberf. Physiol. Plantarum 6, 150 (1953).
44. Higuchi, T. J. Japan Forestry Sac. 36, 22 (1954).
45. Ralston, A.T., Church D.C. and J.E. Oldfield. J. Anim. Sci. 21, 306 (1962).
46. Millet, M.A., Backer A.J., and L.D. Satter, Biotechnol. @ Bioeng. Symp. No. 6, 125-153 (1976).
47. Nystron, J. Biotechnol. @ Bioeng. Symp. No. 5, 221-224 (1975).
48. Spano, A.L., Medeiros J. and M. Mandels, Proc. Conference Washington, D.C., pp. 541-566 (1976).
49. Toyama, N. and K. Ogawa. Biotechnol. @ Bioeng. Symp. No. 5, 225-244 (1975).
50. Toyama, N., Biotechnol. @ Bioeng. Symp. No. 6, 207-219 (1976).
51. Sternberg, D. Biotechnol. @ Bioeng. Symp. No. 6, 35-53 (1976).
52. Gallo, B.J., Andreotti R., Roche C., Ryu D. and M. Mandels. Biotechnol. and Bioeng. Symp. No. 8, 89 (1978).
53. Yamanaka, Y., and C.R. Eilke. AIChe 81 st. Ann. Meet. pp. 11-14 Kansas City (1976).
54. Sheawale, G.I. and J.C. Sadana, Can. J. Microbiol. 24, 1204 (1978).
55. Shewale, J.G. and J.C. Sadana. Can. J. Microbiol. 25, 773 (1979).
56. Stutzenberger, J.F., Kaufman A.J. and R.D. Lossin. Can. J. Microbiol. 16, 553 (1969).
57. Stutzenberger, J.F. Appl. Microbiol. 22, 147 (1971).
58. Crawford, L.D., and E. McCoy. Appl. Microbiol. 24, 150 (1972).
59. Cooney, C.L., Wang I.C., Sy-Dar Wang, Gordon J. and M. Jiminer. Biotechnol. and Bioeng. Symp. No. 8, 103-114 (1978).
60. Gordon, J., M. Jiminer, Cooney C.l. and Daniel I. C. Wang. The AlCh.E. Symp. No. 181, vol. 74: 91-97 (1978).
61. Weimer, P.J. and J.G. Zeikus. Appl. and Envir. Microbiol. 33, 289 (1976).
62. O'Sullivan, A.D. C @ EN Sept. 17, pp. 27-28 (1979).
63. Chose, K.T. and R.D. Tyagi. Biotechnol. @ Bioeng. XXI, 1387 (1979a).
64. Ghose, K.T. and R.D. Tyagi. Biotechnol. @ Bioeng. XXI, 1401 (1979b).
65. Cysewski, G.R. and C.W. Wilke. Biotechnol. @ Bioeng. XIX, 1143 (1977).
66. Gaddy. J.L. and O.C. Sitton, First European Congress of Biotechnology, Interlaken (1978).
67. Mavitura, F. and C.G. Sinclair, European Congress on Biotechnology, Interlaken (1978).
68. Chibata, I. and T. Tosa. TIBS 5, 88 (1980).
69. The U.N. University Bioconversion of Organic Residues for Rural Communities. Food and Nutrition Bulletin Supplement 2 (1979).
70. U.N. Industrial Development Organization Workshop on Fermentation Alcohol for Use as Fuel and Chemical Feedstock in Developing Countries. Vienna, Austria (1979).