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METHODS FOR THE ISOLATION AND IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS FOUND IN COMPLEX MIXTURES AND THE DETERMINATION OF THEIR POSSIBLE TOXICITY BY MEANS OF A HOST MEDIATED BIOASSAY TECHNIQUE

Progress Report
for Period June 1, 1976-February 1, 1977

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Abstract of Annual Progress Report

Techniques were developed to produce excellent high performance glass capillary columns for gas chromatographic analyses. These columns extending 100 meters or more in length and having internal diameters in the range of 0.20-0.25 mm yielded 3000-6000 + theoretical plates per meter. After surface deactivation procedures, they were most satisfactorily utilized for the analysis of a wide range of complex mixtures of organic compounds including those containing a wide array of polycyclic aromatic hydrocarbons (PAH) derived from a coal liquification process (ERDA Research Center, Morgantown, West Virginia).

Work was begun to assess the potential mutogenicity and/or carcinogencity of the various isolated PAH fractions utilizing a unique host mediated bioassay system. Preliminary results indicate that further efforts will be required to determine dose response parameters as well as suitable vehicles for the satisfactory introduction of certain PAH fractions into this particular bioassay system.

Progress Report

During the past eight months following the inauguration of our ERDA Research Contract Ey-76-S-02-2958 on June 1, 1976, two fortuitous events have occurred which have made it possible for our laboratory to make exceptional progress toward certain of our research goals in such a relatively short span of start up time. The first, as described in Section A involved the further development of advanced physical chemical techniques for isolation and identification of complex organic compounds - in this instance methods that could be broadly applied to the individual polycyclic aromatic hydrocarbons and their possible transformation products found in complex mixtures. The second event, as described in Section B, involved the development by a colleague and collaborator and availability to us - of a unique bioassay system which possesses the potential of being readily utilized in conjunction with the aforementioned advance in physical chemical methodology to eventually provide certain information concerning the possible carcinogenicity and/or mutogenicity of individually isolated (and eventually structurally identified - see renewal proposal) polycyclic hydrocarbons bound in complex mixtures under radically changing environmental conditions, for example, coal conversion processes.

Section A: Physical-Chemical Methods

The Development of Thin Film Technology to Produce High Efficiency Wall Coated Polar and Nonpolar Glass Capillary Columns for Use in Gas Chromatography.

Within a few months after the start of our contract, Dr. G. Alexander, a chemical engineer with a broad background in glass chemistry and polymer coatings, and the Principal Investigator as well as other members of our group decided (after some preliminary experiments on the construction and use of micropacked columns which highlighted certain of its disadvantages here for this particular application), to conduct further studies on some earlier observations of Alexander's in the preparation of deactivated glass capillary wall surfaces to accept thermally stable organic compounds which could act as stationary phases in the gas chromatographic process.

In the routine preparation of very high efficiency (3000-7000 theoretical plates/meter) wall coated glass capillary columns in lengths exceeding 25-30 meters two major problems have been heretofore encountered by a handful of experts scattered throughout the world. This involved, first, the poor wettability of glass by compounds used as stationary phases and second, the catalytic and/or absorptive activity of the glass wall itself, which while less then that encountered with metal tubing, is certainly high enough to cause serious difficulties when sensitive solutes, mainly of biological origin are to be analyzed.

After a number of intensive experiments in our laboratories on the use of gaseous HCl as a method 'roughening' the surface of soft glass capillary tubes the problem of routinely coating any polar stationary phase on a treated capillary tube appeared to be solved. Apparently the HCl gas reacts vigorously with the alkali ions on the glass surface thereby producing a uniform layer of NaCl

crystals. Although this reaction, which causes the tube to turn opaque, occurs almost instanteously at high temperatures (450°) we have found that exposing the soft glass capillary to HCl gas for 2 hours at 450°C optimizes the growth and uniformity of sodium chloride crystal formation. Such a layer now allows the uniform distribution on the wall of the capillary of any of the highest surface tension polar liquid phases without any 'droplet' or lens formation. Moreover, it was found that the acidic treatment did not increase the number of absorptive silanol groups present on those areas of the glass surface not covered by the NaCl crystals.

With this problem behind us, it became apparent that the usual static technic for coating capillary columns (dilute, aerated solutions of stationary phase are drawn into the usually short 20-30 meter capillary column by vacuum until filled, one end is then sealed and the other end is exposed to full vacuum for 1-2 days in order to evaporate solvent) would be limited for several reasons.

1. the procedure was laborious and time consuming (1-2 days)
2. could only be used with column lengths up to 20-30 meters x 0.25 mm i.d. since it was also difficult and time consuming to volatize off solvent from longer lengths of narrow bore tubing
3. finally, although the polar phases which were statically produced on etched columns yielded very high theoretical plate numbers, the separation factors calculated for polar solutes analyzed on certain polar phases did not coincide

with the vast literature available on 'packed' columns coated with similar phases. The reason for this phenomenon is still not apparent at this time.

Because of these as well as other reasons we therefore turned to dynamic methods of coating 0.20-0.30 mm. i.d. capillary columns in lengths exceeding 100 meters - assuming that if we were successful we could routinely have columns 100-150 meters long yielding 400,000 to 900,000 theoretical plates (4000-6000 t.p./m). Under these circumstances, we would then hopefully have a relatively easy time of realizing one of our goals by separating the many isomers of certain polycyclic aromatic hydrocarbons produced under certain conditions.

Here, again our timing was fortunate. Schomburg (1) had just published a paper describing his first experiments with a novel technique of dynamically coating long capillary columns by first filling about 5-10% of the column with the appropriate concentration of stationary phase dissolved in a solvent such as methylene chloride - then followed by filling 1-2 loops of the column with mercury. The inlet side of the capillary is then connected to a nitrogen cylinder and pressure is applied to the mercury plug which in turn moves the solute-solvent plug along the entire length of the column at a constant speed. After its exit from the column, nitrogen gas percolates through the column removing the solvent and leaving behind a uniform layer of stationary phase.

After much experimentation, we radically modified this technique, optimized the velocity of solute solvent plug as well

as the concentrations of the various different stationary phases used in gas chromatography and emerged with a procedure that could provide us with uniformly coated very high efficiency glass capillary columns of almost any desired length - at least 80 percent of the time.

To illustrate the enormous resolving power of this type of long column, Schomburg (2) claimed that by using a 300 meter long column coated with squalene, he could separate two cyclic C_8 hydrocarbons each of which contained six deuterium atoms located at different positions in the molecule. A 100 meter column was only necessary for the separation of two identical hydrocarbns that differed only by one deuterium atom.

Figures 1-10 demonstrate the wide variety of high efficiency capillary columns we now can produce in our laboratories by these procedures. (See Appendix for specific operating conditions).

Enormously encouraged by our rapid progress in this area, we moved rapidly toward some resolution of the second outstanding difficulty that still remained in the area of high efficiency glass capillary columns gas chromatography - alluded to in the earlier part of this text - that of deactivation of soft glass capillary surfaces.

It has long been recognized that the surfaces of etched or nonetched glass capillary columns coated with nonpolar phases showed significant absorptive and/or catalytic activity toward certain classes of compounds, i.e. ketones, alcohols, bases, amines etc. Although this phenomena of tailing or vanishing peaks was also observed to a lesser extent with capillary columns containing

relatively thin layers of polar phases, moderate layers of polar phases appeared to neutralize, much if not all of the adverse surface activity.

The precise chemistry involved in soft glass surface activity in this context is little understood and is worthy of a research project unto itself. Since this was not our intent, we therefore pursued the earlier observations that dilute solutions of the polar polyethylene glycol (Carbowax 20M) rendered such surfaces less active by some little understood mechanism. Experiments were conducted whereby a 1-3% solution of Carbowax 20M was coated onto on etched soft glass capillary columns by the dynamic mercury plug method followed by a regular coating of the nonpolar OV-101 phase. Indeed when certain representative compounds were chromatographed, activity was decreased (See Figs. 11-15 and Appendix). Without the Carbowax 20M undercoating, butanol would 'tail' considerably more, and pyridine would not emerge or if present - would show bizarre peak shapes. Another drawback quickly emerged with the use of Carbowax 20M. If the column temperature was increased to the 200-220°C - not an unusual temperature range for gas chromatogtaphic analysis today - the Carbowax 20M undercoating would breakdown and full surface activity of a nonpolar column would again become manifest. Along about this time, we decided to make use of an observation of Aue (3) who noted that if packed columns, coated with a thin layer of Carbowax 20M were sealed, heated to 280°C + for a given period of time and then the residual soluble layer of stationary phase was removed by passing small

quantities of solvent through the columns several times, a thin nonextractable thermally stable layer of a modified Carbowax 20M polymer remained on the surface of the columns packing - rendering it inert chromatographically (alcohols then analyzed would no longer show tails).

After much trial and error we further modified the Aue technique and made it work in conjunction with our other procedures for the preparation of high efficiency glass capillary columns. Although not altogether perfect, it has provided us with some of the best results we have seen todate in this particular sphere of endeavor (See Figures 16-19 and Appendix).

At this point (3-4 months after startup) we had the early makings of a very efficient system which could then be applied to one of the basic problems at hand - namely the separation and identification of certain polycyclic aromatic hydrocarbons in certain environments and the development of a more reliable and simplified approach toward the identification of those compounds which possessed mutagenic and/or carcinogenic activity in certain types of bioassay systems. Accordingly, we contacted John Kovach, Acting Chief of the Process Analysis Laboratory Branch at ERDA's Morgantown, West Virginia, Energy Research Center for samples obtained during certain phases of their coal liquifaction process. Our purpose at this very early point in time was two fold.

a) test out our high efficiency glass capillary columns on types of samples that we would become increasing involved with in the future and

b) obtain an early assessment of what types of problems may be encountered when such samples or selective segments of these samples were subjected to a unique bioassay technique for the assessment of mutagenicity and/or carcinogenicity (see following Section on Bioassay Techniques).

The early results of analyzing various types of process samples with this type of column system may be seen in Figures .

Several comments are in order:

1. Although there are several dozen discernible major peaks, there are probably 100-200 minor peaks present in sample.

2. The individual components of each sample could be further resolved from one another by going to still longer capillary columns, i.e. 100-200 meters.

3. Additional resolution will be obtained when sample is chromatographed on two or more 100 meter + columns containing stationary phases of different polarity.

4. The columns should be further conditioned prior to use so that base line shift during temperature programming is held to a minimum.

5. Since so many compounds are present that may be potentially harmful, some initial shortcuts are in order in an effort to assess the synergistic relationship that two or more structurally related compounds may have on biological systems (namely, in this instance, the bioassay system).

Within the next few weeks, appropriate columns will become available in order to accomplish that which was mentioned under 2, 3 and 4. John Kovatch of ERDA's Research Branch in Morgantown, West Virginia, mentioned that companion samples will also be forwarded to us from experiments with different process conditions.

Section B: A Host Mediated Bioassay Method

In this section, I have requested a colleague, Dr. Robert Capizzi, an active worker in the field of chemical mutagenesis utilizing a particular type of biological detection system, to describe his views and experiences todate with this technique. Dr. Capizzi has collaborated with us in preliminary studies to delineate those parameters that are essential to the successful deployment of this method with the aforementioned chromatographic techniques for the screening of chemicals for 'toxic' biological effects. Since the results from this type of bioassay system are usually not known for several weeks after startup, in selective instances we intend to use this method in conjunction with the Ames system (see Renewal Proposal) in an effort to gain first hand experience at correlating results obtained-under specific laboratory conditions- with a mammalian and a bacterial system.

References

1. Schomberg, G., Proc. of First International Symposium on Glass Capillary Chromatography, Hindelang, Germany 1975, p. 61

2. Schomberg, G., Hussmann, H. and Weeke, F. J. of Chromatography 99 (1974) 63

3. Aue, W.A., Hastings, C.R. and Kapila, S. J. of Chromatography 77 (1973) 299

Appendix

Figures 1-6

Demonstrate the capability of coating glass capillary columns with a wide variety of stationary phases of differing polarity (OV 101-methyl silicone, OV 17-phenyl methyl silicone, CarboWax 20 M-polyethylene glycol, FFAP-polyethylene glycol + terephthalic acid, Silar 10C-cyanopropyl silicone, OV 275-cyanosilicone) which yield efficiencies ranging from 3500 to about 7000 theoretical plates per meter !!!

Figures 7-10

Depict the use of these high efficiency glass capillary columns in separating certain arbitrarily selected complex mixtures containing widely differing classes of compounds

Figures 11-15

Demonstrate considerable glass surface deactivation when the glass tubing is pretreated with solutions of CarboWax 20M followed by a thermal 'baking in' period. The desired stationary phase is then applied to the surface of the precoated column as described in the text. Note the significant improvement in the shapes of the alcohol (butanol) and pyridine peaks (decreased tailing) in contrast to that obtained without the pretreatment procedure.

Figures 16-19

Depict the chromatographic analysis of products obtained from the ERDA Morgantown, W.VA. coal liquefaction process. Samples FB-39SPT and Z-E represent fractions normally obtained by solvent extraction.

Column: OV-101, #925, 29 meters
Temp: 150°
Pressure: 20 psi
Att: 1x8, Chart Speed 0.5"/min.
Sample: 0.4 µl Methyl Esters of Fatty Acids, C-10, C-11, C-12

Comment: Peak 3 yielded 4563 theoretical plates/meter

FIGURE I

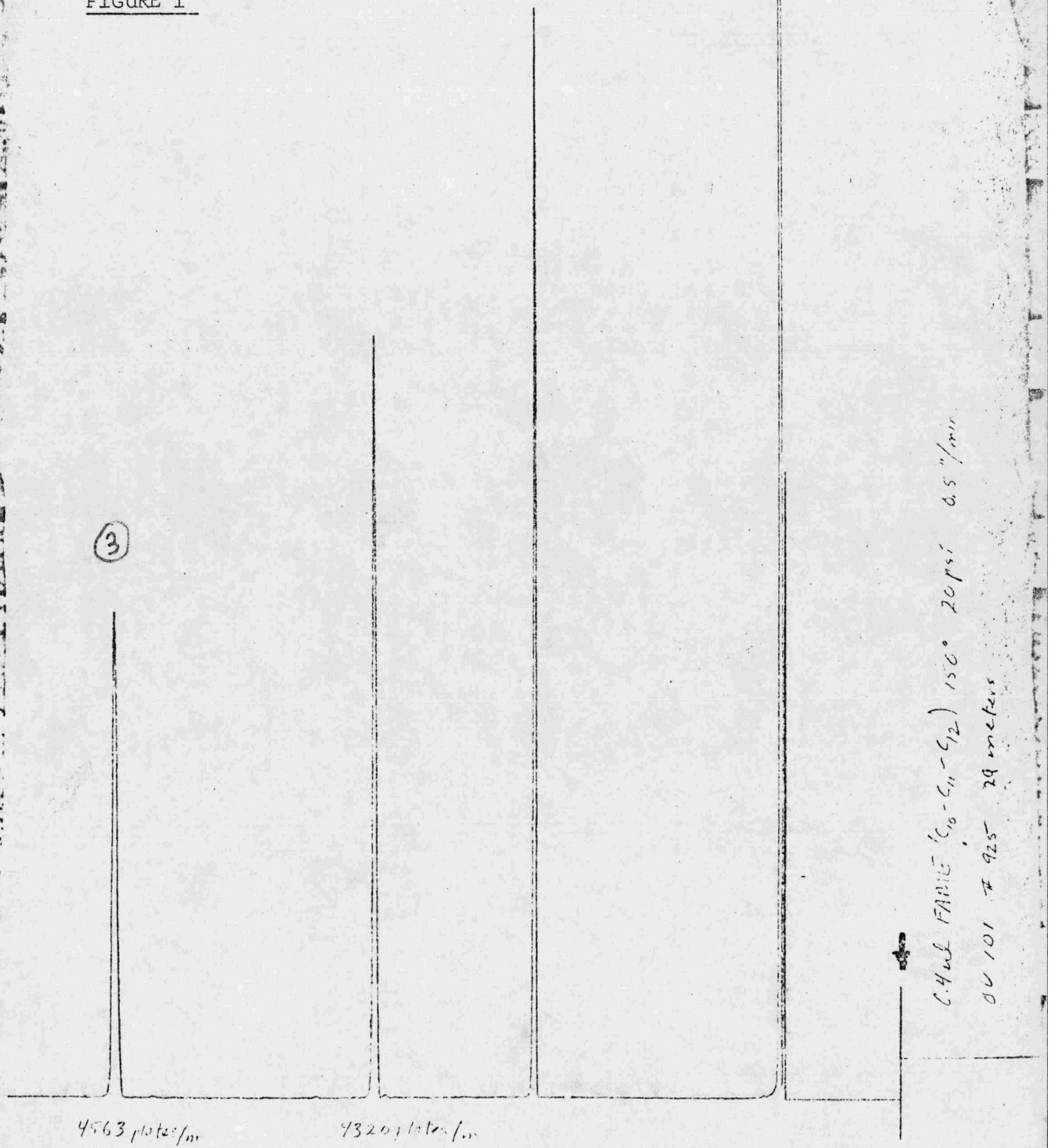


FIGURE II

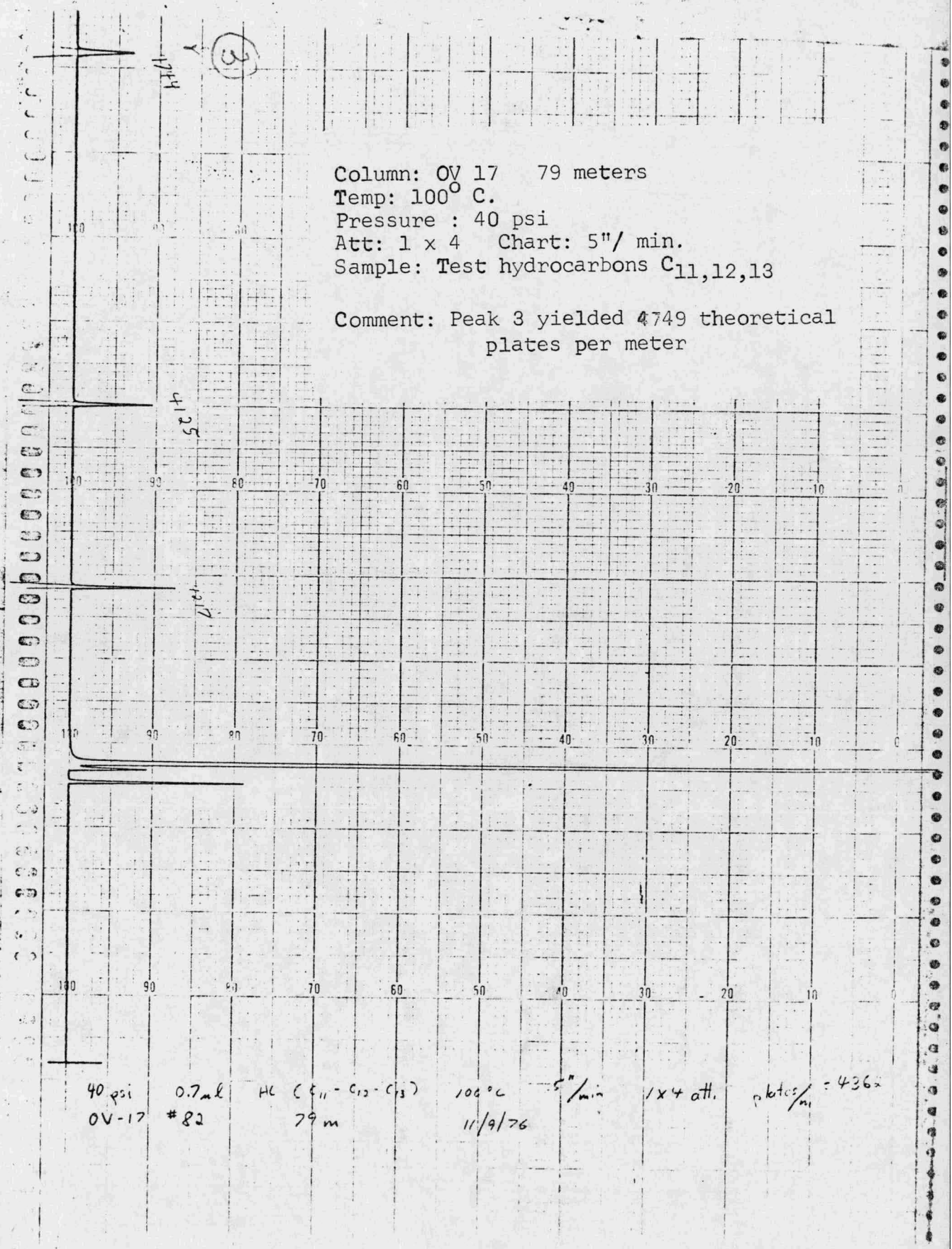

Column: OV 17 79 meters
Temp: 100° C.
Pressure : 40 psi
Att: 1 x 4 Chart: 5"/ min.
Sample: Test hydrocarbons $C_{11,12,13}$
Comment: Peak 3 yielded 4749 theoretical plates per meter
4749
4135
4017
100 90 80 70 60 50 40 30 20 10 0
40 psi 0.7 mℓ HC (C_{11} - C_{12} - C_{13}) 100°C 5"/min 1x4 att. plates/m = 436
OV-17 #82 79 m 11/9/76

FIGURE III

Column: CarboWax 20M 20 meters
Temp: 85°C.
Pressure: 13 psi He
Att: 1 x 4 Chart: 0.5" per minute
Sample: Test methyl esters of fatty acids, C-10,11,12

Comment: Peak 3 yielded 4700 theoretical plates per meter

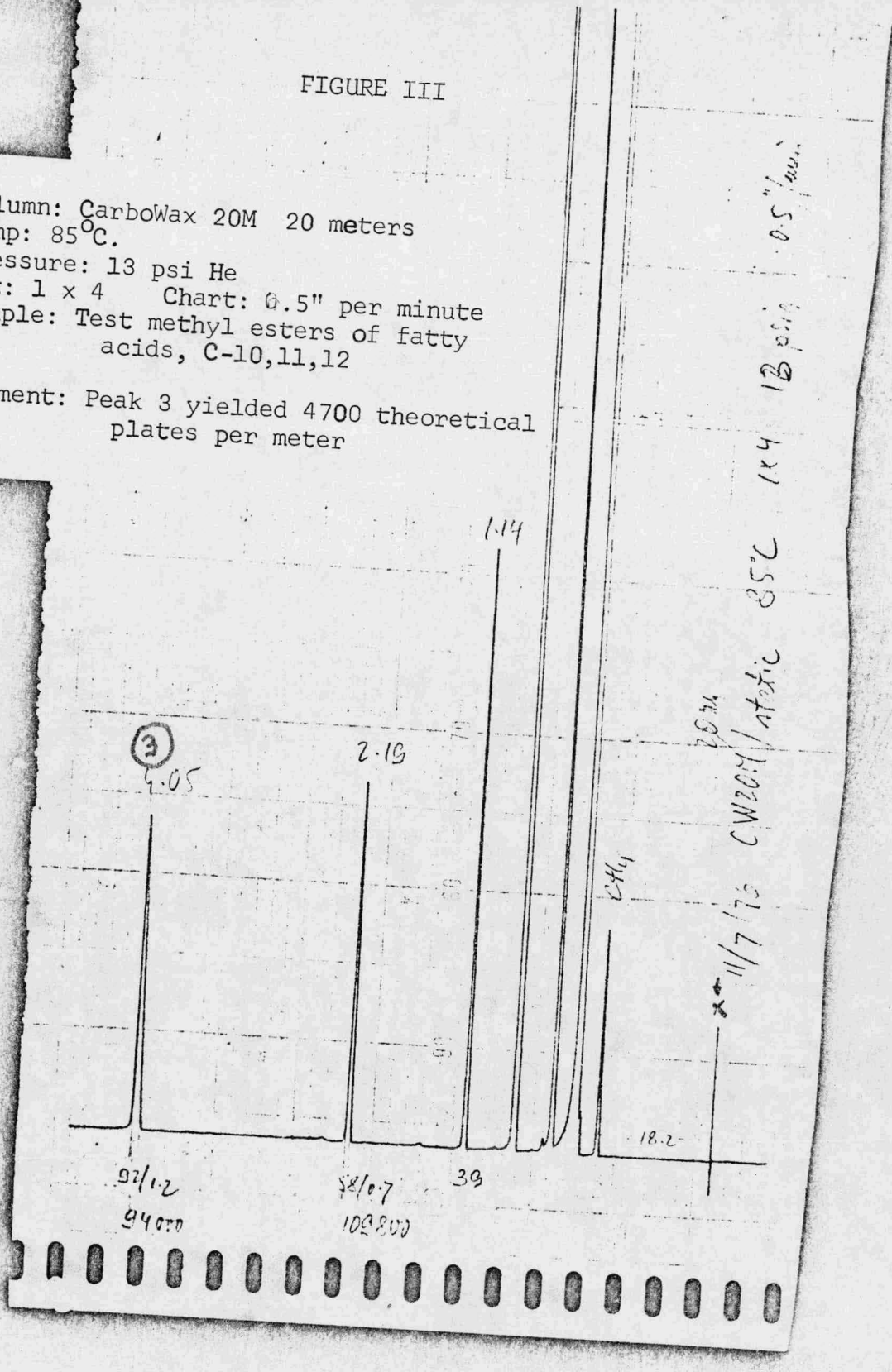
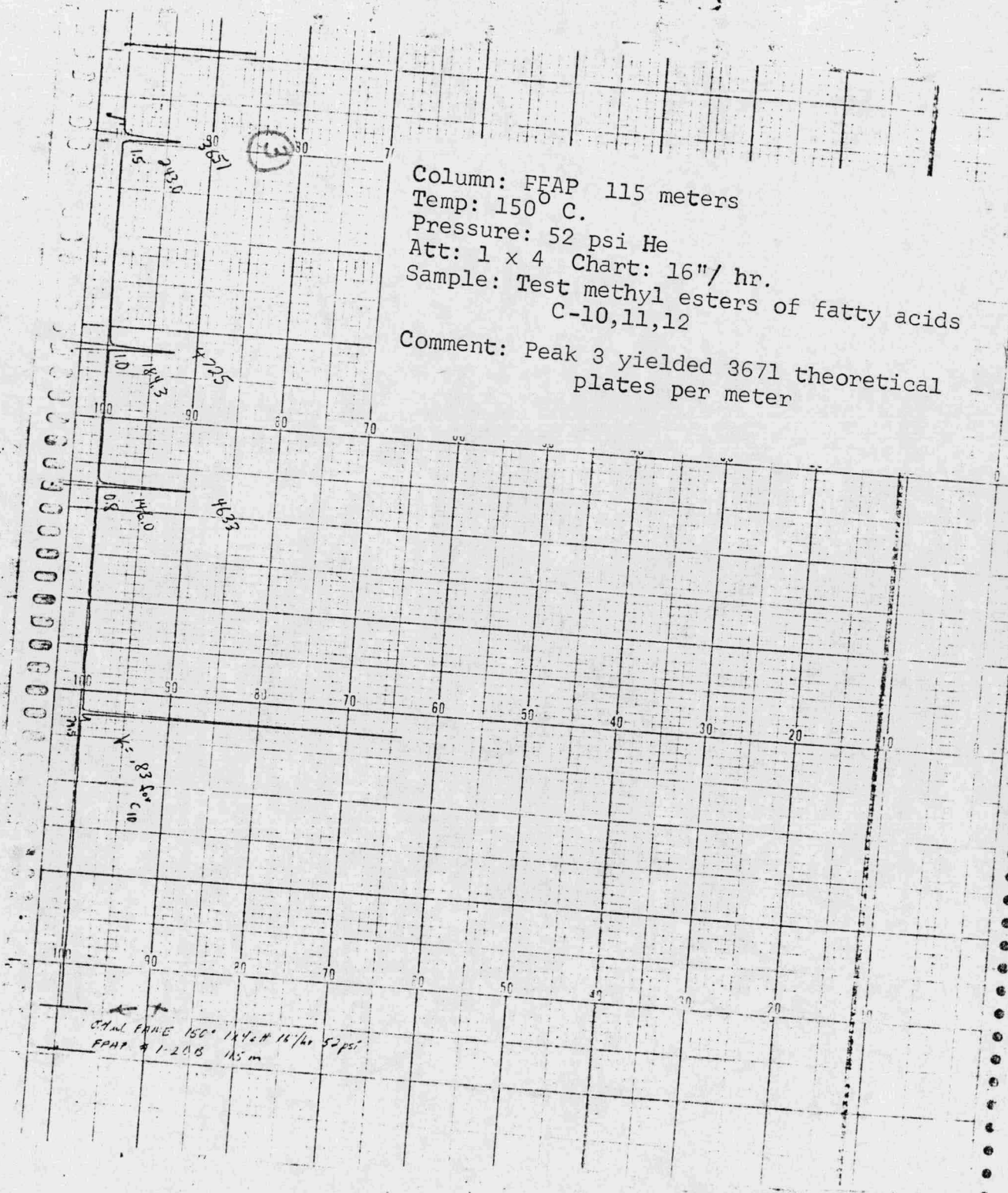


FIGURE IV



Column: FFAP 115 meters
Temp: 150° C.
Pressure: 52 psi He
Att: 1 x 4 Chart: 16"/ hr.
Sample: Test methyl esters of fatty acids C-10,11,12

Comment: Peak 3 yielded 3671 theoretical plates per meter

FIGURE V

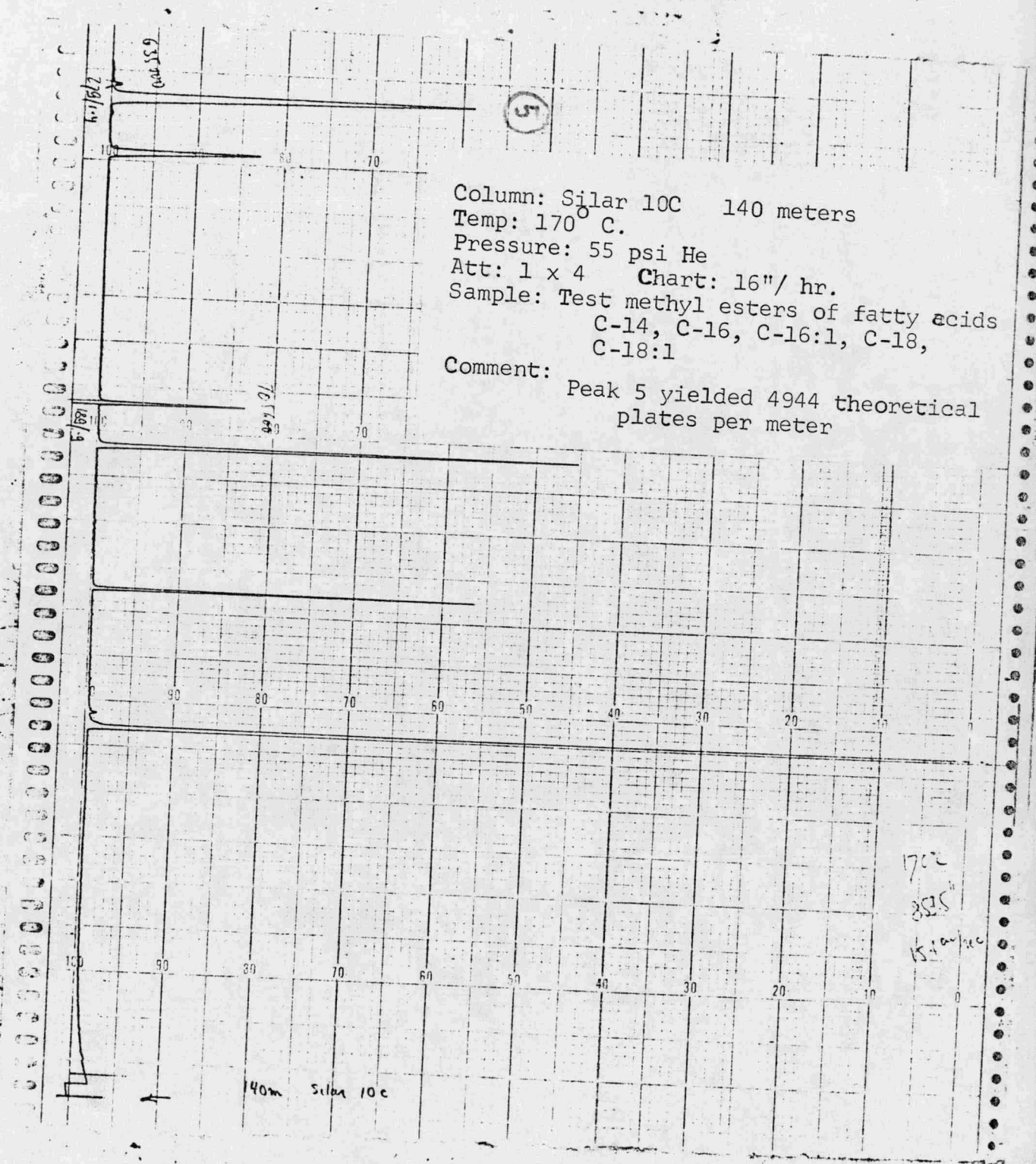
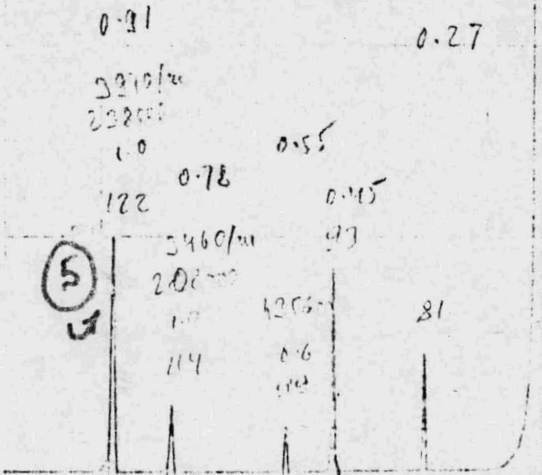

Column: Silar 10C 140 meters
Temp: 170° C.
Pressure: 55 psi He
Att: 1 x 4 Chart: 16"/ hr.
Sample: Test methyl esters of fatty acids
C-14, C-16, C-16:1, C-18,
C-18:1
Comment:
Peak 5 yielded 4944 theoretical
plates per meter
140m Silar 10c

FIGURE VI

Column: OV 275 60 meters
Temp: 180° C.
Pressure: 32 psi He
Att: 1 x 4 Chart: 0.5"/ min.
Sample: Test methyl esters of fatty acids
C-14, C-16, C-16:1, C-18, C-18:1

Comment: Peak 5 yielded 3970 theoretical plates per meter



180°C
32 psi He
0.5"/min
1 x 4
OV-275
60m

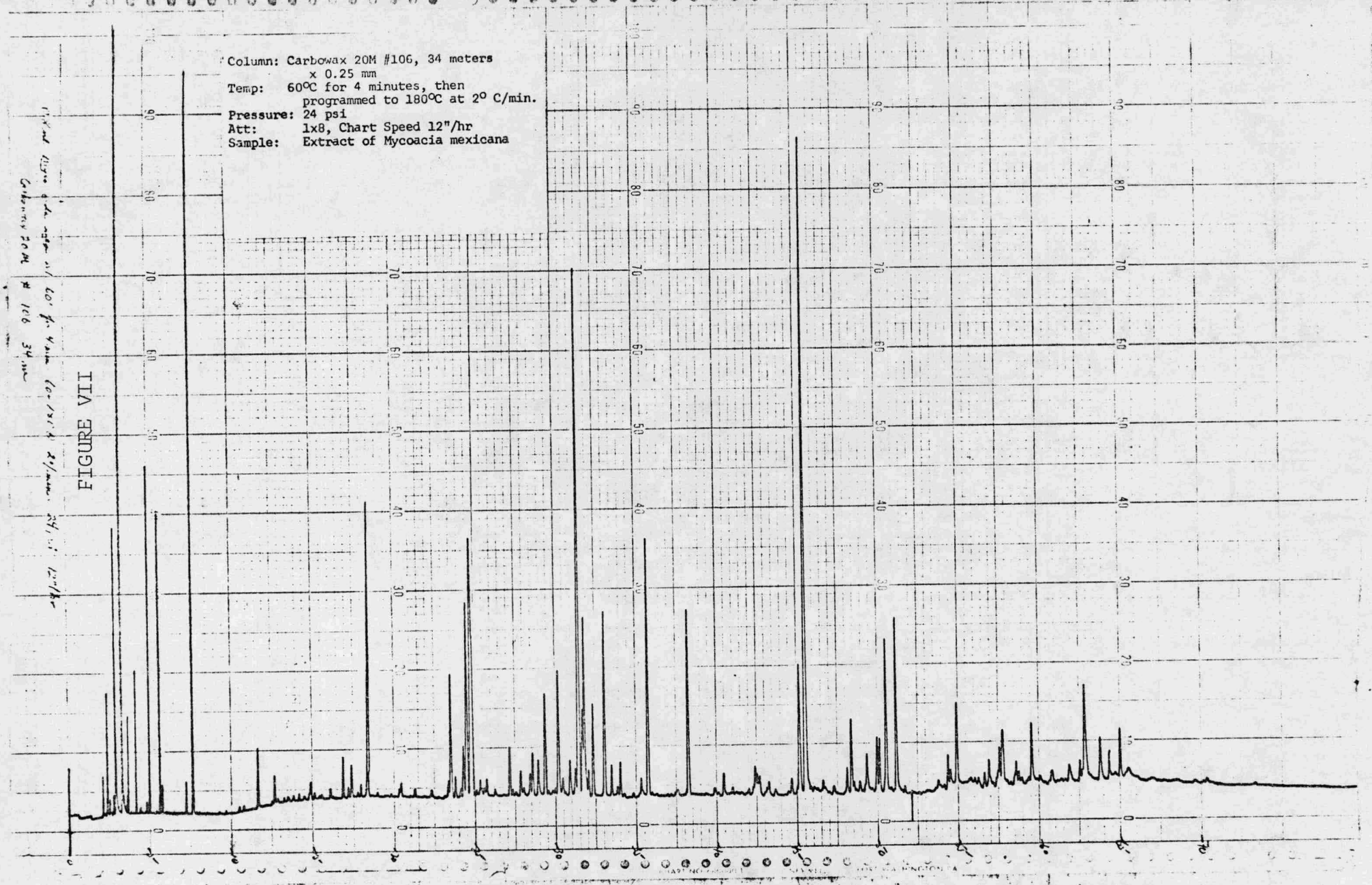

Column: Carbowax 20M #106, 34 meters
x 0.25 mm
Temp: 60°C for 4 minutes, then
programmed to 180°C at 2° C/min.
Pressure: 24 psi
Att: 1x8, Chart Speed 12"/hr
Sample: Extract of Mycoacia mexicana

FIGURE VII

FIGURE VIII

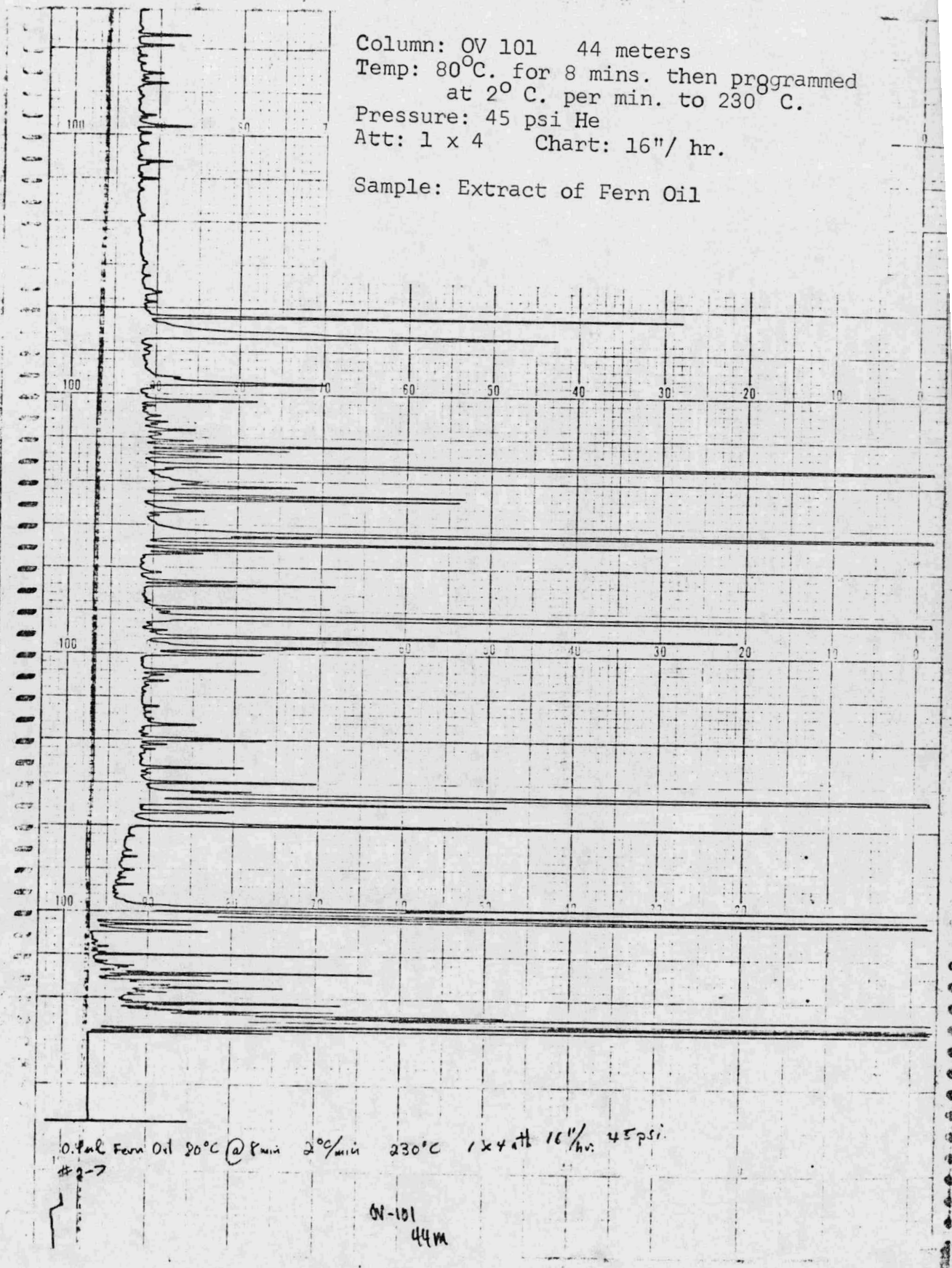
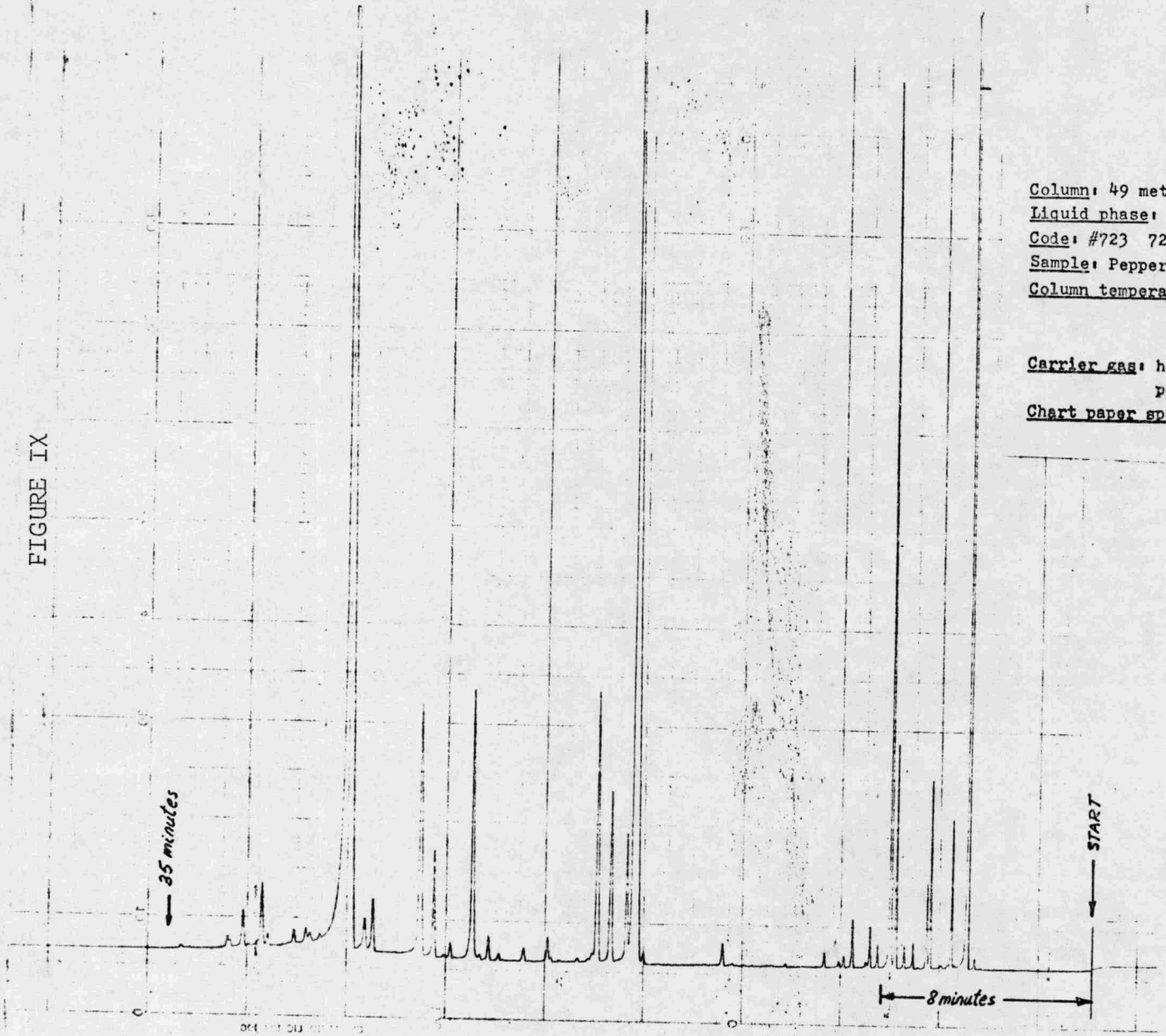

Column: OV 101 44 meters
Temp: 80°C. for 8 mins. then programmed at 2° C. per min. to 230° C.
Pressure: 45 psi He
Att: 1 x 4 Chart: 16"/ hr.
Sample: Extract of Fern Oil
100
80
70
60
50
40
30
20
10
0
0.1ml Fern Oil 80°C @ 8min 2°C/min 230°C 1x4 att 16"/hr. 45psi
#2-7
OV-101
44m

FIGURE IX



Column: 49 meters x 0.25 mm ID
Liquid phase: Carbowax 20M
Code: #723 726
Sample: Peppermint oil
Column temperature: 80°C for 9 minutes then programmed at 2°C/min to 150°C
Carrier gas: helium, 30 psig inlet pressure
Chart paper speed: 16 in./hour

FIGURE X

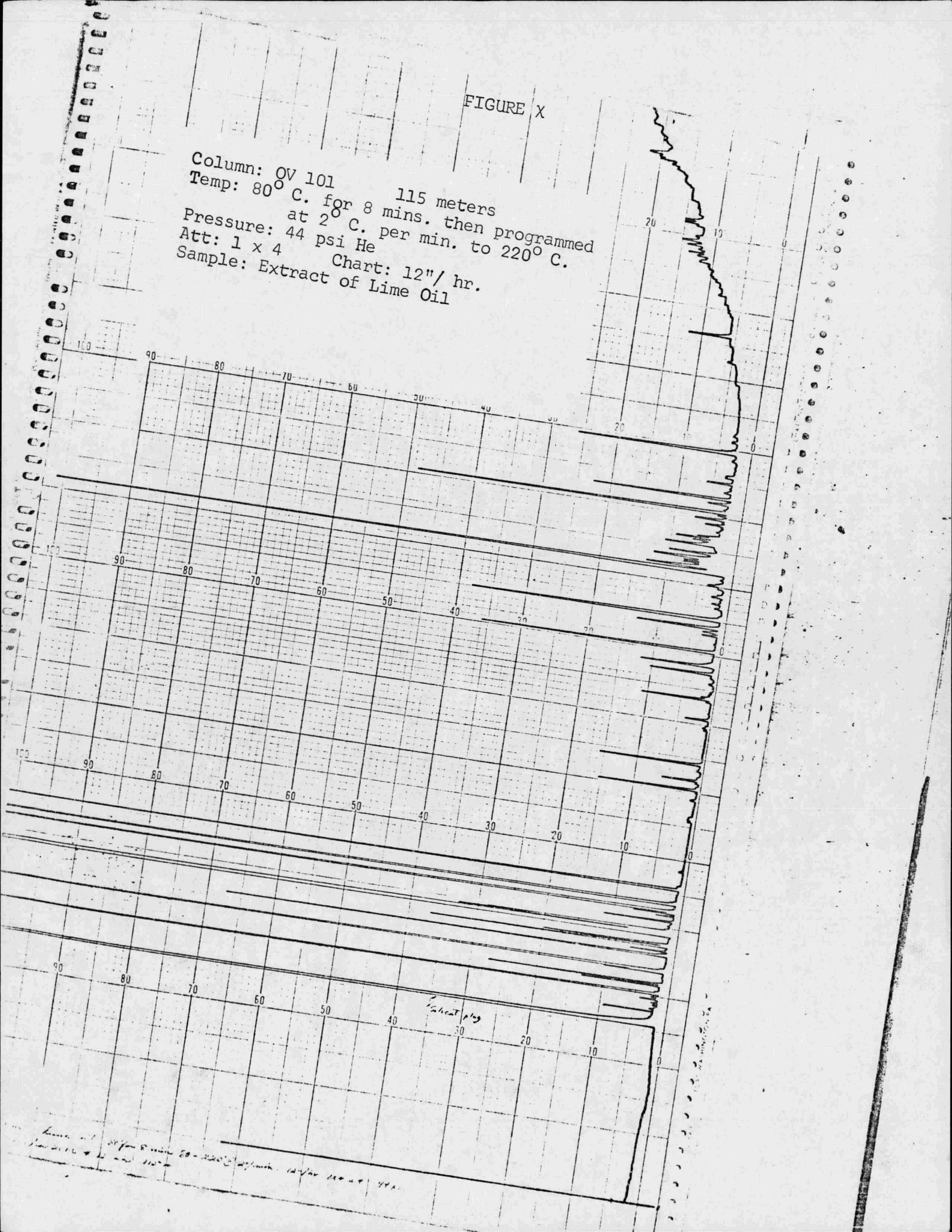

Column: OV 101 115 meters
Temp: 80° C. for 8 mins. then programmed at 2° C. per min. to 220° C.
Pressure: 44 psi He
Att: 1 x 4 Chart: 12"/ hr.
Sample: Extract of Lime Oil

FIGURE XI

Column: OV 101 25 meters
Temp: 80° C.
Pressure: 14 psi
Att: 1 x 32 Chart: 0.5"/min.
Sample: a) benzene & pyridine
b) butanol & benzene
Comment: no pretreatment of glass surface, pyridine and butanol 'tail' significantly

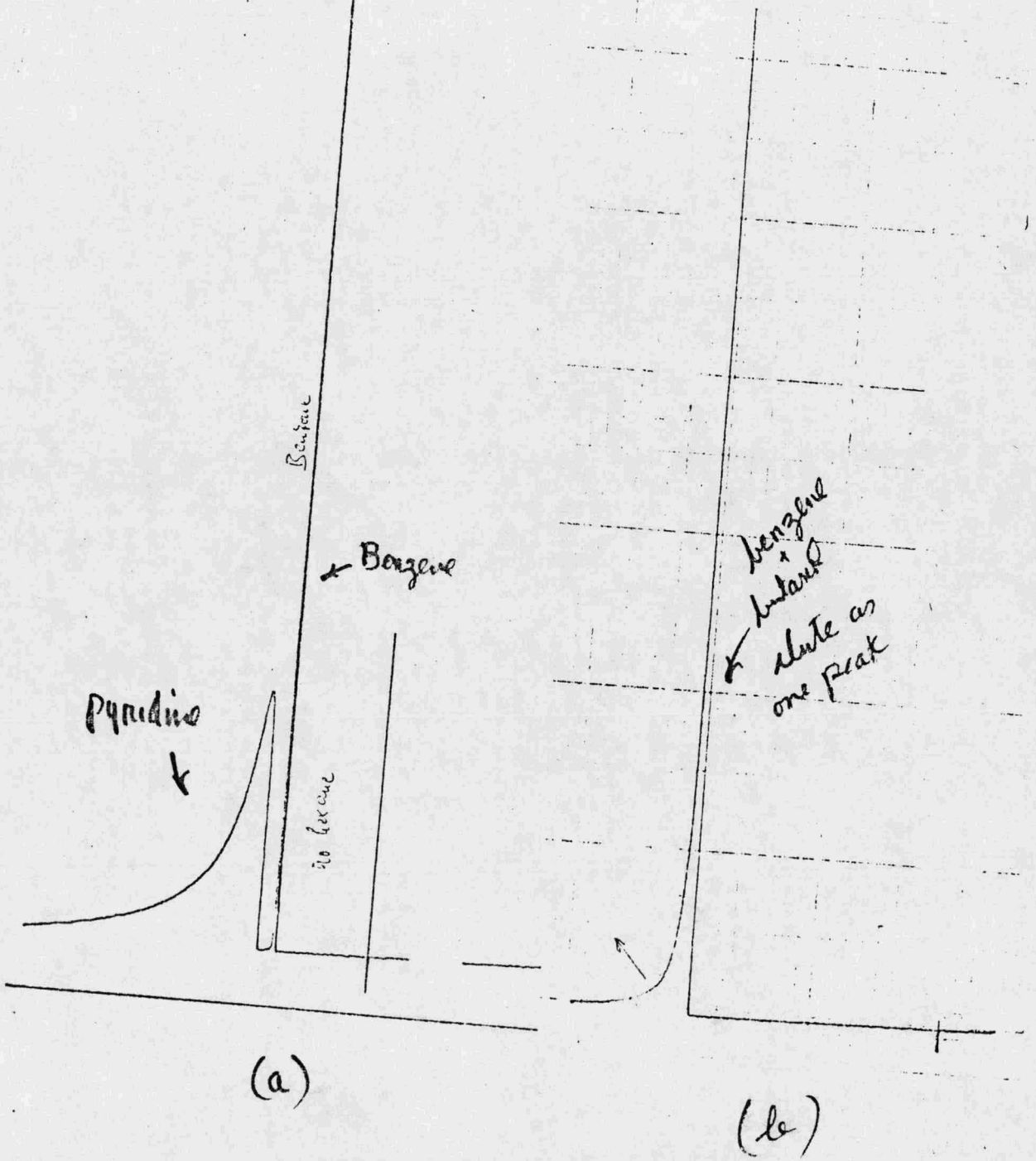


FIGURE XII

Column: OV 101 45 meters
Temp: 80° C.
Press: 28 psi He
Att: 1 x 32 Chart: 0.5"/min.
Sample: McReynold's Test Mixture
1)benzene 2) 2-pentanone
3)butanol 4) nitropropane
5) pyridine
Comment: glass pretreated. With a very non-polar coating such as OV 101, pyrid. & butanolshow sig. decrease in tailing

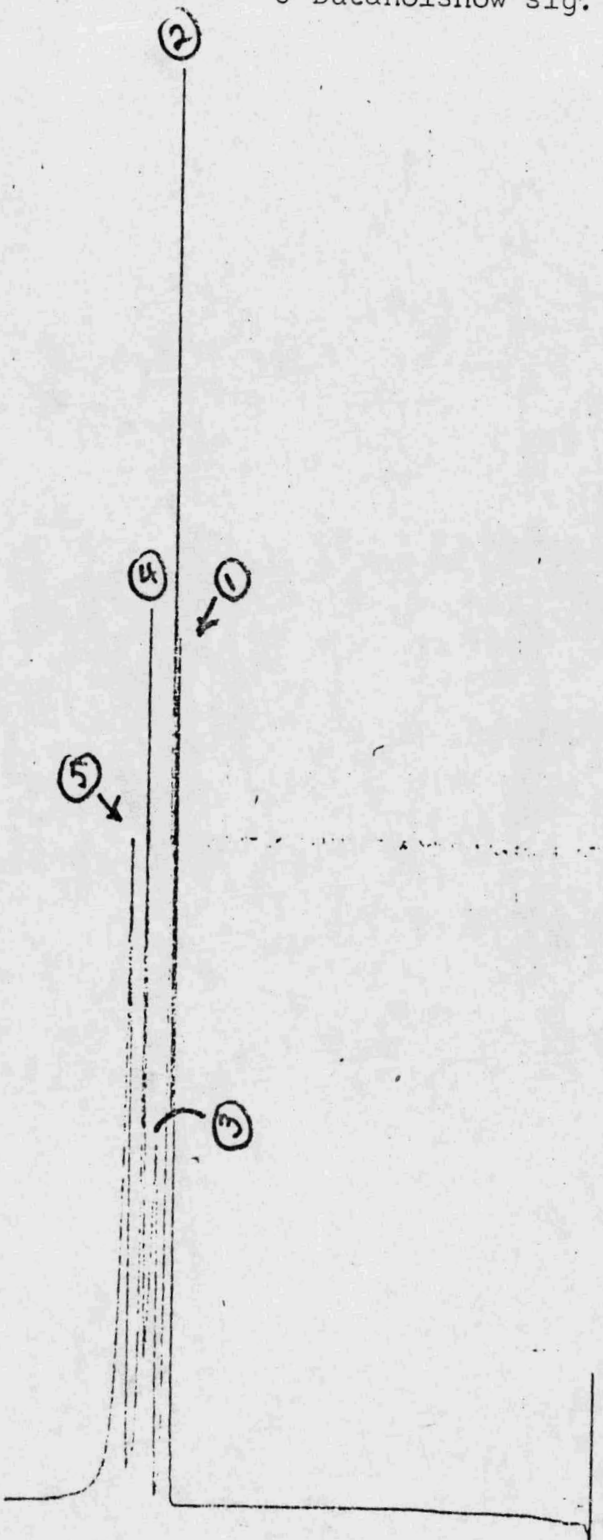


FIGURE XIII

Column: OV 17 78 meters (NON POLAR)
Temp: 80° C. moderate
Pressure: 34 psi He
Att: 1 x 32 Chart: 16"/hr.
Sample: McReynold's Test Mixture
1)benzene 2) 2-pentanone
3) butanol 4) nitropropane
5) pyridine
Comment: successfully pretreated glass surface then coated with non polar phase

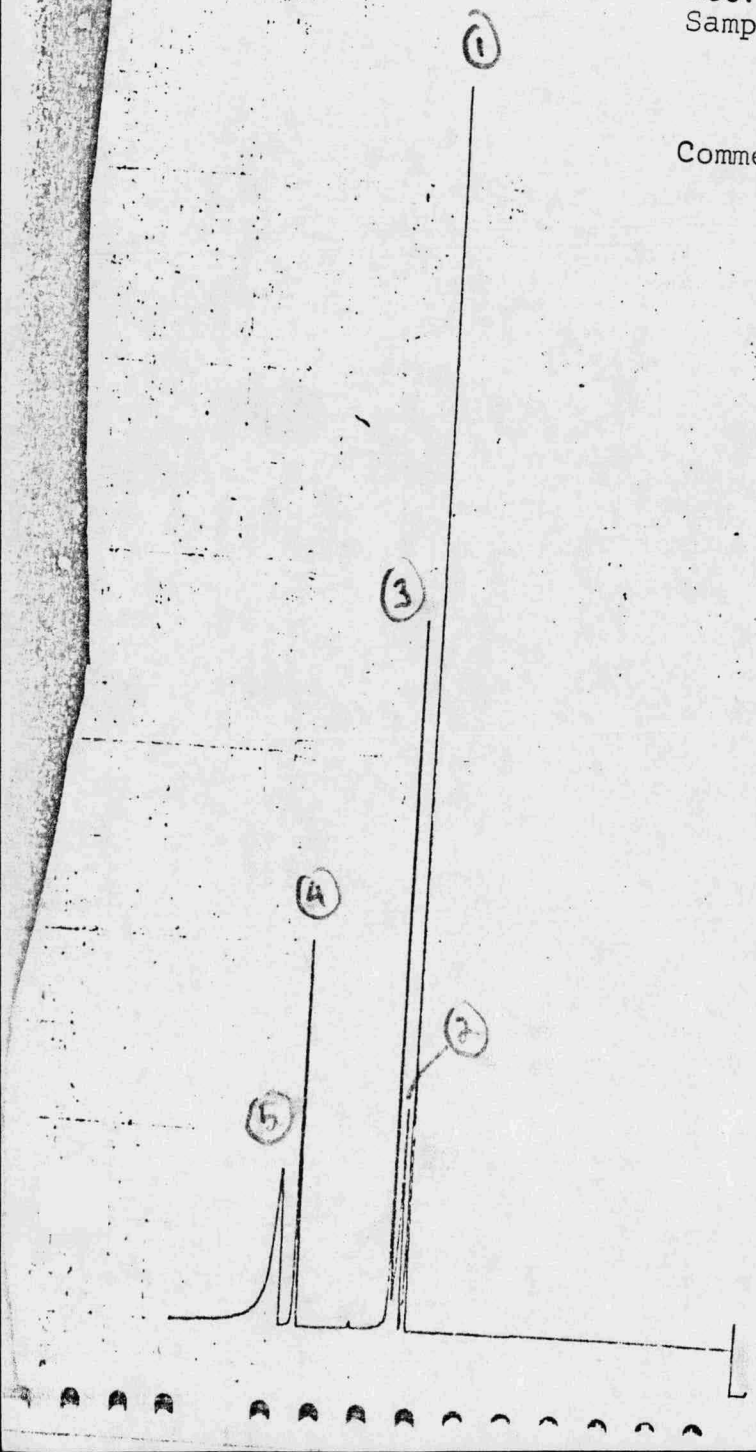


FIGURE XIV

Column: FFAP (POLAR PHASE) 43 meters
Temp: 80° C.
Pressure: 22 psi He
Att: 1 x 32 Chart: 30"/hr.
Sample: McReynold's Test Mixture
1) benzene 2) 2-pentanone
3) butanol 4)nitropropane
5) pyridine
Comment: no pretreatment of glass surface
alcohol and pyridine 'tail' badly

FIGURE XV

Column: CarboWax 20 M 25 meters
Temp: 90° C. (POLAR PHASE)
Pressure: 14 psi
Att: 1 x 32 Chart: 1"/ 2 mins.
Sample: McReynol's Test Mixture
1) benzene 2) 2-pentanone
3) butanol 4)nitropropane
5) pyridine
Comment: Glass surface pretreated - excellent alcohol & pyridine peaks

12/9/76 CW20M Coe SC- McR-mix 90°C

FIGURE XVI

Column: SE 30 44 meters
Temp: 110-250° C. at 2° per min.
Press: 30 psi
Att: 1 x 8 Chart: 12"/hr.
Sample: Fraction ZE from ERDA, Morgantown
W.Va. coal liquefaction proces

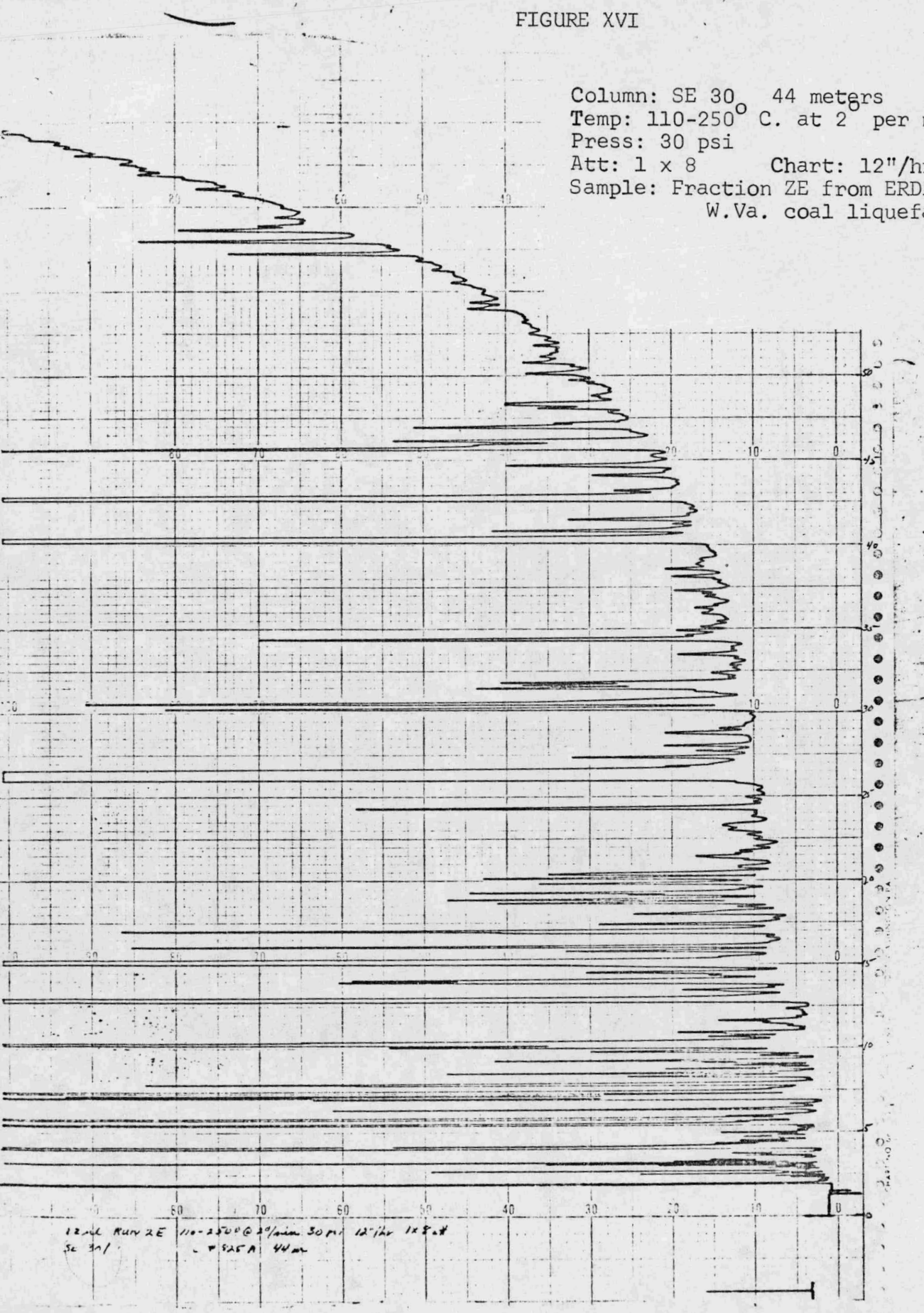


FIGURE XVII

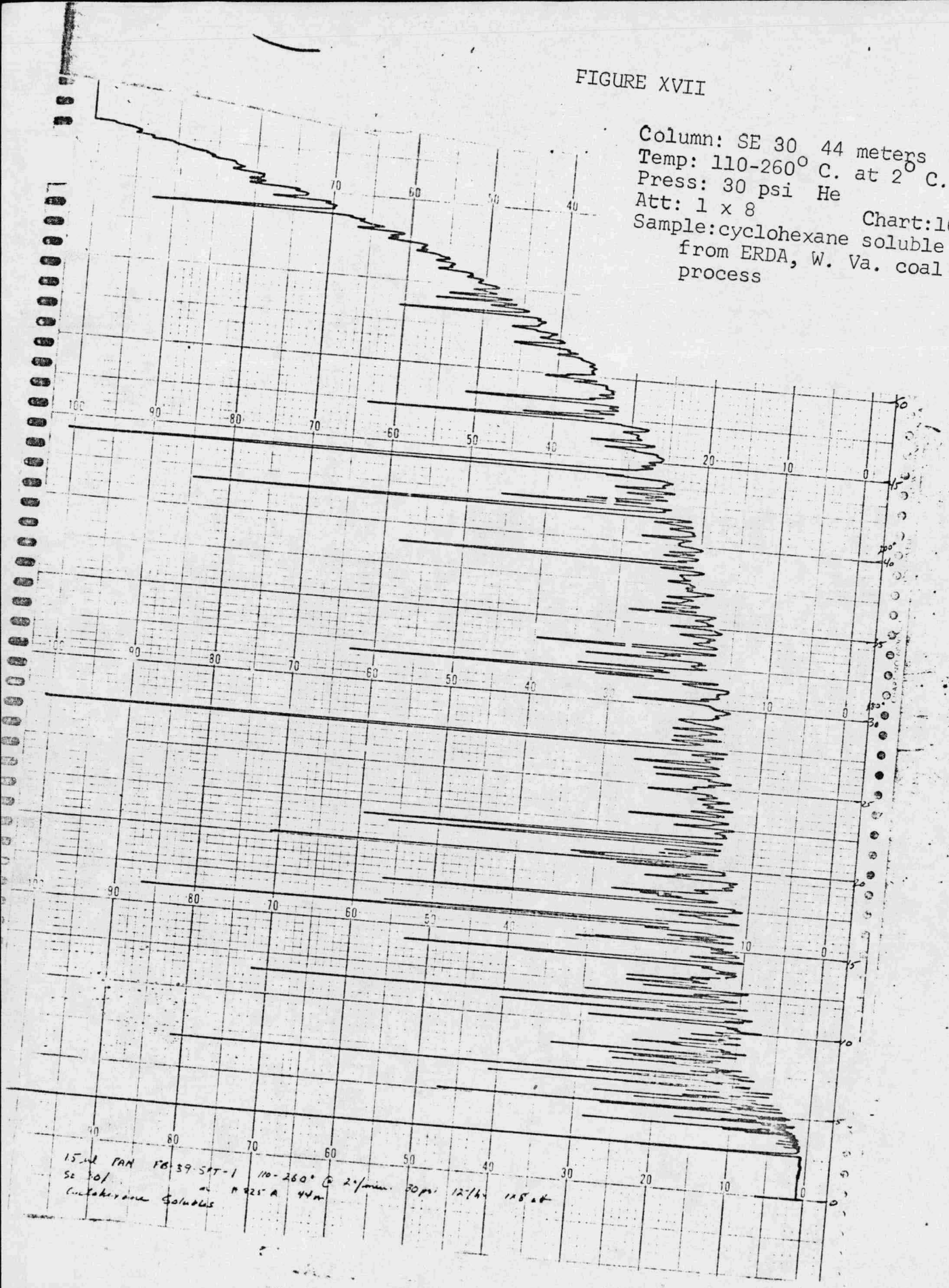

Column: SE 30 44 meters
Temp: 110-260° C. at 2° C. / min.
Press: 30 psi He
Att: 1 x 8
Chart:16"/hr.
Sample:cyclohexane soluble fraction
from ERDA, W. Va. coal liquefaction
process

FIGURE XVIII

Column: SE 30 44 meters
Temperature: 110^0 -260^0 C. programmed at $2^oC.$ / min/
Pressure: 30 psi He
Att: 1 x 8 Chart: 12"/ hr.
Sample: 'product oil', #FB 39 SPT from ERDA Morgantown, W.Va. coal liquefaction process

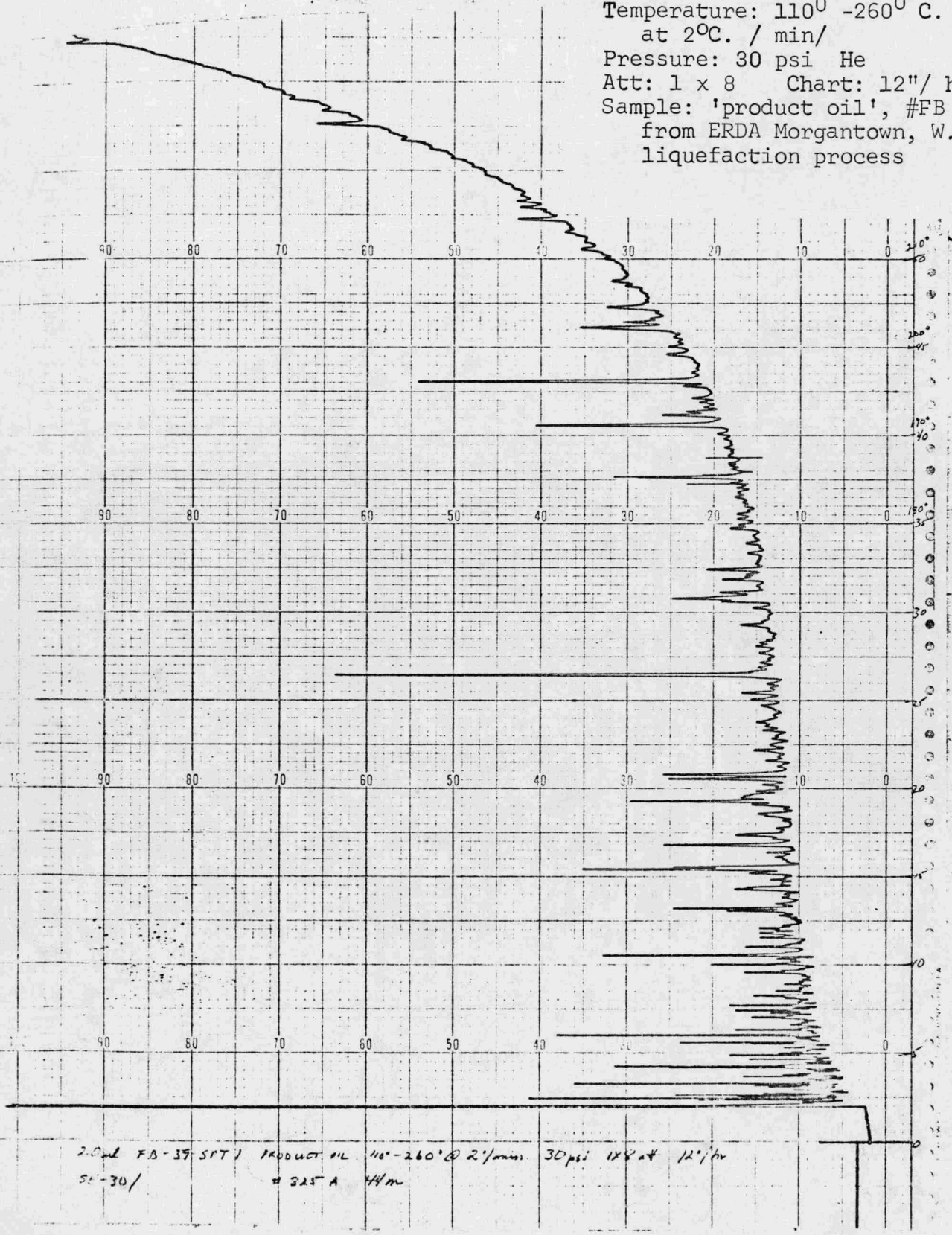
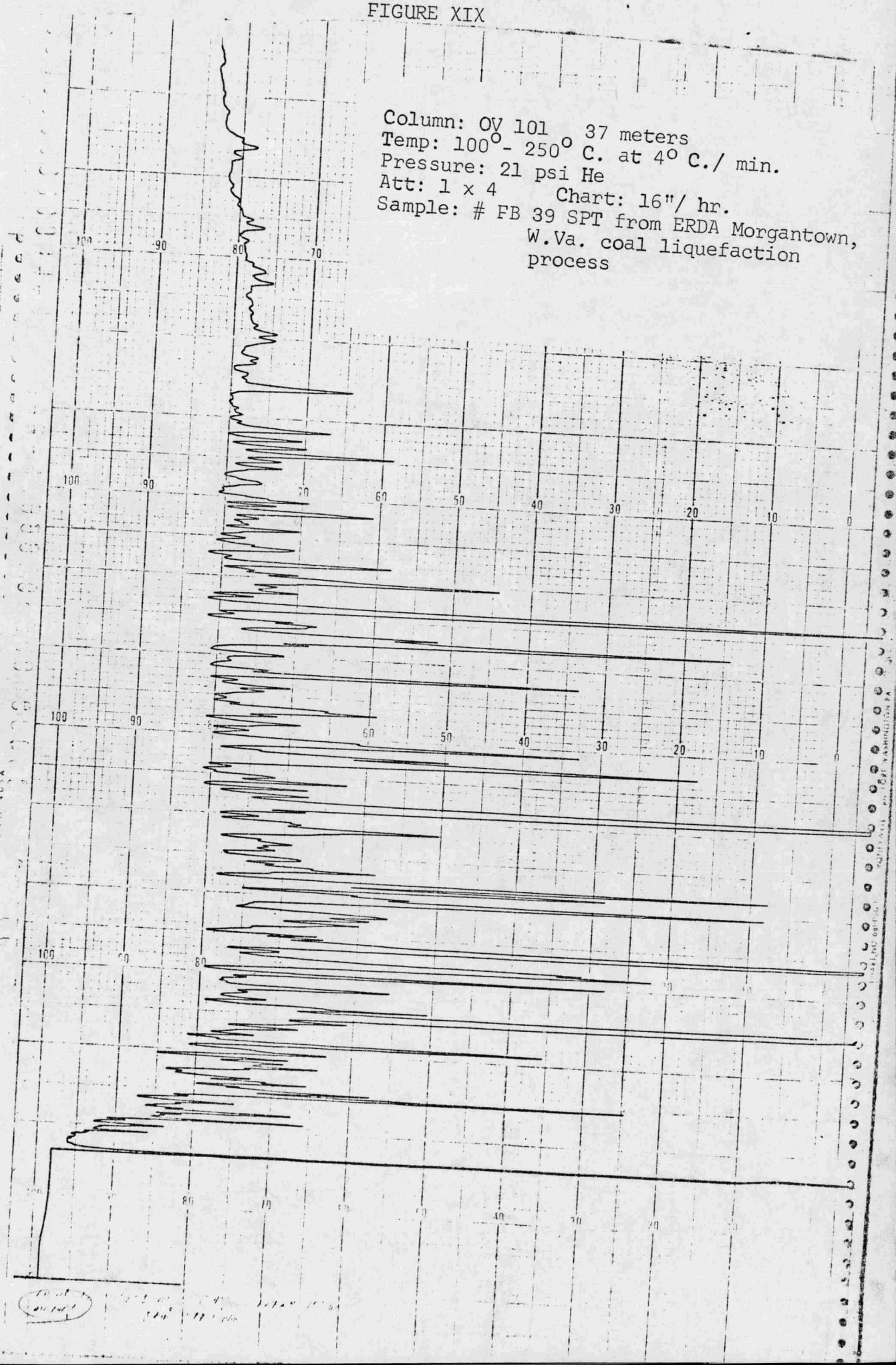


FIGURE XIX


Column: OV 101 37 meters
Temp: 100° - 250° C. at 4° C./ min.
Pressure: 21 psi He
Att: 1 x 4 Chart: 16"/ hr.
Sample: # FB 39 SPT from ERDA Morgantown, W.Va. coal liquefaction process

SECTION B:

A HOST-MEDIATED ASSAY FOR THE DETECTION OF CHEMICAL MUTAGENS USING THE L5178Y/ASN$^-$ MURINE LEUKEMIA

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I. Introduction

The most commonly used procedures for the detection of chemical mutagens employ prokaryotic and eukaryotic systems ranging from phage to mammalian cells in culture (1-3). The use of any one of these tests in an attempt to establish the human genetic health hazard from chemical mutagens, has as one major limitation its inability to consider host biotransformation of the chemical. This is a very essential feature since many agents are known to require metabolic activation in order to exert their mutagenic or carcinogenic effects. Conversely, a chemical which is a very potent mutagen *in vitro* may not present a significant hazard *in vivo* due to its lack of bioavailability to the target cells stemming from rapid metabolic deactivation or excretion.

Attempts to overcome this drawback include the exposure of bacterial tester strains to various tissue homogenates of animals which had been exposed to a mutagen (4-6) and activation of the mutagen *in vitro* by the use of drug metabolizing fractions derived from liver homogenates (5). Other approaches include murine host-mediated assays which employ bacteria (7), yeasts (8,9) or fungi (10) as indicator organisms. While indicating mutagenically active compounds which are inactive *in vitro*, limitations of these methods include the short duration of in-animal exposure to the chemical, lack of comparative cytogenetics and perhaps varied sensitivity as a result of differences in cell biology, e.g., transport, repair mechanisms, etc. compared to mammalian indicator cells.

Much thought has been devoted to attempts to place the above studies in proper perspective with regards to the safety evaluation of chemicals from a genetic standpoint. Recent proposals to governmental regulatory agencies in the U.S. and Europe have recognized that no one test to date can fully establish the genetic safety of chemicals (11,12). Consequently a battery of tests have been recommended in the overall screening procedure. Mutational studies in Drosophila and prokaryotic tester strains are used as the initial screen for these chemicals and should they be positive, the testing then advanced to mammalian systems of increasing complexity and cost. At either end of this testing spectrum, the technology and genetics have been reasonably well defined, however, other mammalian test systems which are sensitive, reliable, easy to perform, relatively quick and less expensive are required if all medicinal, industrial and environmental chemicals are to be screened. The data obtained thus far suggests that the use of the L5178Y/asn$^-$ mouse leukemia may be very useful in this regard (see below for details.)

The following characteristics illustrate the suitability of the L5178Y/asn$^-$ murine leukemia as an indicator cell for the detection of the chemical mutagens:

a) It is a well established cell line which grows well in suspension culture and in vivo as a single cell ascites tumor with a doubling time of 10-12 hours.

b) Growth in vivo or in vitro is easily interchangeable. When taken from the mouse it will grow in suspension culture or it can be cloned in soft agar to determine cellular viability with high plating efficiency or induced mutation by using the appropriate culture medium.

c) It has a stable, near-diploid chromosomal complement of 42 chromosomes. This would allow the simultaneous comparison of point mutations with cytogenetic aberrations.

d) It has been shown to require an exogenous source of the amino acid L-asparagine (asn) for growth (16). Thus, cloning the cells in an asn-free medium will allow the detection of induced mutation to asn independence. This is the locus studied in the present grant proposal.

e) Since the L5178Y is a neoplastic cell, it metastasizes to all organs of the body and ultimately kills the host. The administration of the chemical in question to the tumor bearing animal with metastatic disease could be of significant value by allowing a study of organ accumulation of the mutagenic chemical by determing the mutant frequency of L5178Y cells in a given organ.

Other mutational systems employing the L5178Y leukemia cell include the thymidine kinase locus (13), drug resistance (14) and HGPRT locus (15). The availability of multiple loci in the same cell line would be of distinct value in the screening of chemicals for their mutagenic potential since some but not necessarily all genes might be expected to be mutated by various chemicals. Further scientific benefit in the use of this cell line is the ability to use it in a host-mediated assay which would allow a consideration of mammalian pharmacodynamic and pharmacokinetic factors such as chemical activation, detoxification, excretion and preferential organ accumulation.

II. Background Data from the Applicant's Laboratory

The induction of asparagine-independent mutants by the action of known chemical mutagens on the asparagine auxotroph of the murine leukemia L5178Y (L5178Y/asn$^-$) was studied. Treatments were carried out both in vitro and in a host-mediated assay whereby the cells were implanted i.p. and the chemical was administered by a distal route. Asparagine-independent cells were identified by soft-agar cloning in asparagine-deficient medium. The average spontaneous mutation rate to asparagine independence was 7.4×10^{-7}/locus/ generation. In the

in vitro system methylmethanesulfonate, melphalan and iodo-deoxyuridine were conclusively mutagenic; 5'-amino-iododeoxyuridine and trifluorothymidine were negative. The mutagenic effects of the alkylating agents ethyl methanesulfonate, N-methyl-N'nitro-N-nitrosoguanidine, di(2-chloroethyl)sulfide (sulfur mustard) and dimethylnitrosamine were studied in vitro and in the host-mediated assay. In the host-mediated assay, all agents were mutagenic and all but dimethylnitrosamine were mutagenic in vitro. Posttreatment incubation in fully supplemented medium (expression time) was required prior to cloning in the selection medium. For all agents there was a dose- and expression time-related increase in mutant frequency. Other factors that affect the mutagenic potency of a chemical include the vehicle and route of administration to the mouse host.

The spontaneous mutant frequency and mutation rate to asn independence of L5178Y asn$^-$ cells grown in vitro are shown in Table 1. The average mutant frequency was 3.2×10^{-6} viable cells with a spontaneous mutation rate of 7.4×10^{-7}/locus/generation. Similar studies of cells grown in the peritoneal cavity of mice revealed a mutant frequency of 6.3×10^{-6} viable cells which compares very favorably with the "in vitro" studies.

Before performing any host-mediated studies, it was important to determine the reproducibility of cell growth in the peritoneal cavity. This is shown in Figure 1. Following the intraperitoneal inoculation of 10^6 cells, there is reproducible, exponential growth if the cells are harvested, diluted, and transplanted to new host mice prior to or just as the cells are entering the plateau phase of growth. A significant increase in doubling time occurs if the cells are transplanted after having been in plateau phase for 2 to 3 days. This information is of importance in deciding when, after having implanted the cells in the mice, one might administer the chemical in question since the growth phase of the cells, i.e., exponential vs plateau, might influence the chemical's effect.

In the assessment of mutant clones, demonstration of phenotypic stability and measurement of the mutant gene product are essential. Randomly selected mutant L5178Y/asn$^+$ colonies were subcultured in asn-free medium and their growth was monitored as is shown in Figure 2. Cells grew quickly when initially suspended in asn-supplemented medium, but there was a slight, initial lag in growth when the cells were suspended in asn-free medium (Fig. 2, point a). However, there was comparable growth with subsequent re-suspension of cells into both asn-supplemented and asn-free medium after initial growth in asn-free medium (Fig. 2, point b). Previous growth of the mutant cells in asn-supplemented medium (Fig. 2, point b) did not affect growth when subsequently subcultured in asn-free medium (Fig. 2, point c). The phenotypic nature of newly derived chemically-induced mutants was confirmed by extracting colonies from the selective cloning medium, and subculturing the cells in asn-free medium; in no instance did a colony so selected fail to grow.

Determination of the asparagine synthetic capability of both sublines were performed through the courtesy of Dr. Robert E. Handschumacher of the Dept. of Pharmacology at Yale University. Using the method of Chou and Handschumacher (17) values of 0.13 mole of asparagine synthesized per 10^6 cells per hour were obtained from the L5178Y/asn$^-$ cells and 2.23 moles of asparagine per 10^6 cells per hour were obtained for the mutant L5178Y/asn$^+$ cells.

Long term observations of the growth of L5178Y/asn$^+$ and L5178Y/asn$^-$ in asn-free or asn-supplemented medium, respectively, revealed comparable doubling times (Fig. 3). In addition, reconstruction experiments did not reveal that either subline eas at a selective advantage or disadvantage with regards to the other. Furthermore, to be certain that the recovery of mutant cells was not the result of selective cytotoxicity, populations of both the auxotrophs and prototrophs were similarly exposed to the chemicals in question and equivalent cytotoxicity was observed.

The mutagenic effects of the alkylating agents EMS (ethylmethanesulfonate), MNNG (N-methyl-N'nitro-N-nitrosoquanidine), SM (sulfur mustard) and DMNA (dimethylnitrosamine) were studied in vitro and in the host-mediated assay (HMA). In order to detect the mutagenic activity of these agents, posttreatment incubation in fully supplemented medium (expression time) was required prior to cloning in the selection (asn-free) medium.

A major utility of the HMA is in the detection of mutagens that require metabolic activation to the active form. A classic example is DMNA, a chemical that is known to require metabolic activation via liver hydroxylases (18,19), thus explaining its lack of cytotoxicity and mutagenicity when L5178Y/asn$^-$ cells were exposed to it in vitro. However, when exposed to DMNA in the HMA, L5178Y/asn$^-$ cells were significantly mutagenized (Figure 4). When the cells were assayed 6 hrs (Day 0) after treatment, no increase in mutants was produced by 2.5 mg/kg and only a slight increase was produced by 5 and 10 mg/kg. However, when the cells were assayed 4 days after treatment, doses of 2.5, 5 and 10 mg/kg produced a significant increase in the mutant frequency.

In contrast, significant attenuation of mutagenic activity of certain chemicals may occur through the HMA. If one were to assume that EMS, MNNG, and SM were distributed in the total body water, then extrapolation of effective doses from in vitro to in vivo exposure should be possible. However, such extrapolation for the above chemicals indicated considerable reduction in mutagenic activity by factors of approximately 4,900- and 10,000-fold for EMS, MNNG, and SM, respectively (Tables 2 to 4). Such attenuation probably results from physicochemical and pharmacokinetic factors such as rapid hydrolysis, local inactivation, distribution, and other factors that would prevent the active mutagen from reaching the target cells within the peritoneal cavity. Some of these

limitations may be circumvented by changes in the vehicle and route of administration. An illustrative example includes the use of the organic solvent polyethylene glycol 200 rather than phosphate-buffered saline (pH 7.4, 5×10^{-2} M) as the vehicle for SM. As shown in Table 4, solutions of SM in polyethylene glycol 200 resulted in a considerable increase in the SM-induced mutant frequency. This is probably due to the slower rate of hydrolysis of SM in organic solution as compared to aqueous solution.

In mutagenicity screening it is of obvious importance to have a control sample which is treated with the vehicle alone to be certain that the chemical in question does indeed increase the mutant frequency. It would also be of value to include a "positive" control, i.e., a dose of known mutagen which would be expected to produce a significant increase in the mutant frequency. This has a dual benefit; it ensures that the genetic locus under consideration is indeed capable of being mutated, and should the chemical in question produce an increase in the mutant frequency, one would have an estimate of the relative potency of the unknown chemical with reference to the standard mutagen. With these thoughts in mind an extensive evaluation of the mutagenic effect of methylmethanesulfonate (MMS) as a function of dose and duration of exposure was undertaken. The data in Figure 5 illustrate a dose-related increase in mutant frequency. Even non-lethal or minimally lethal doses of MMS such as 5 and 10 µg/ml. respectively, produce a significant increase in the mutant frequency. Exposure of the cells to 20 µg/ml for 3 hours kills half of the cells and produces a mutant frequency of 135×10^{-6} viable cells (vs control of 12×10^{-6} viable cells).

For comparative purposes, the effects of a drug, e.g., cytotoxicity, can frequently be expressed in terms of the product of the log dose x duration of exposure. For example, the cytotoxic effect of MMS at a dose of 10 µg/ml for 4 hours (log dose x time = 4) was comparable to the effect of 20 µg/ml for 3 hours (log dose x time = 3.909).

An extensive monograph on the technology of mutagenicity screening (20) suggests a standard format of 48 hours for dealing with the matter of "expression time," i.e., the time required for posttreatment incubation in fully supplemented medium during which the mutation would be phenotypically expressed. The results of our experiments suggest that the matter of expression time must be individually determined for each drug and dose. The closest approximation to the optimal time can be derived by following the posttreatment outgrowth of the cells in fully supplemented medium. The optimal time is probably that time when the growth of the cells approximates that of the control, i.e., a doubling time of 12 hours. When followed on a daily basis, this would allow at least 2 rounds of DNA replication. An evident increase in the mutant frequency at that time would be consistent with the pairing error hypothesis (21). Such an effect may be seen with sulfur mustard (SM). By virtue of its bifunctional alkylating potential, SM is much more cytotoxic than the monofunctional alkylating agent, MMS. As is seen in

Figure 6, SM can exert a profound delay in cell duplication depending on the dose. Thus, four doses of 0.001 µg/ml to 0.01 µg/ml a 2-day expression time would be adequate for the detection of induced matutions. However, for larger, more toxic doses, assay at early time points would not be expected to detect induced mutations, whereas, cloning on days 4 through 8 would detect them.

A photograph of asn$^-$ independent mutants as induced by sulfur mustard are shown in figure 7.

III. SPECIFIC AIMS

A. To determine the mutagenic potential of a wide variety of polycyclic aromatic hydrocarbons derived from different sources, i.e., the coal conversion process (samples supplied by ERDA Research Center, Morganstown, West Va.) - as assayed in the L5178Y/ASN$^-$ murine leukemia host mediated assay

These studies have been carried out in active collaboration with Dr. Lipsky. Preliminary studies utilizing certain crude material fractions derived from the coal conversion process were found to be very insoluble in the usual aqueous vehicles and the concentrations of organic solvents required to dissolve the various fractions superceded the lethal dose of solvent by several orders of magnitude. (For example, in preliminary experiments, the concentration of DMSO which was required to dissolve the material was 100 x the LD_{50} of DMSO for mice. More recently Mazola oil proved to be an excellent solvent here, however its compatability with the bioassay (control) is now being determined. In subsequent experiments, the crude derivatives will again be subjected to separation of the complex mixtures into individual components by means of high efficiency glass capillary column gas chromatography in Dr. Lipsky's laboratory. Part of the gaseous effuent from the column will be continuously diverted from the very sensitive detection system at 5 minute intervals and bubbled through a suitable vehicle which can then be introduced into the bioassay system (see below). If any of these 5 minute fractions (which may contain up to 20-30 individual compounds) prove to be mutagenic, that fraction will be further separated by gas chromatography for further identification of the component(s) responsible for mutagenic activity. In the above experiments DMNA was given to another group of mice as a positive control. In both instances, a DMNA-induced increase in the mutant frequency was observed indicating the responsivity of the cell line under analogous experimental conditions. Obvious studies which need to be performed are of pharmacokinetic nature relating to absorption and distribution of the components of the extract.

Previous problems which had hampered the progress of the host-mediated assay were the occasional appearance of a high spontaneous mutant frequency in the controls and ready flexibility between in vivo and in vitro growth. In experiments conducted over the past 6 months this has been overcome. Clonal isolates from in vitro growth grow readily in suspension and these suspensions when innoculated into mice grow rapidly and when cloned directly from the mice they have a plating efficiency in excess of 60%. In addition, the spontaneous mutant frequency of these new clonal isolates are consistently in the 10^{-6} range.

B. Methods of Procedure

A schematic for the host-mediated assay is shown in figure 8 and 9. As is shown in figure 8, L5178Y/asn$^-$ cells are innoculated intraperitoneally. Four days later the agent in question is administered by distal route (subcutaneously, or IV) and at varying time intervals the cells can be harvested from the peritoneal cavity and cloned in soft agar (see below) for determination of viability and induced mutant frequency. Absorption and circulation of the agent through the various organs of the mammalian host allows the agent to undergo biotransformation (activation or inactivation) or is excreted or stored.

C. Stock Propagation

1. In vivo

Both the asparagine auxotroph and prototroph of the L5178Y grow well as a single cell suspension in the peritoneal cavity of certain inbred strains of mice, notably the DBA_2, BDF_1, and AKD_2F_1. Male DBA_2 mice (18-20 gm) are purchased from the Jackson Laboratory, Bar Harbor, Maine, and are housed in our animal facilities until they weigh approximately 25 gm. This quarantine period allows the mice to acclimate to their new surroundings and insures us that they are free of diseases which might alter the course of results of our experiments. (Although in regular, repeated purchases from the Jackson Laboratory over the past 10 years, every mouse shipment has been healthy).

Stock lines for study are maintained by the serial dilution and transplantation of 10^6 cells to new host mice at weekly intervals (Figure 1). The mice are sacrificed and the cells are extracted according to the method described above. Appropriate dilutions of the cell suspensions are made in saline and cetrimide solution (to lyse the red blood cells) and then the cells are counted in a Model B Electronic Particle Counter (Coulter Electronics, Hialeah, Florida). The cells are then diluted with 0.9% sodium chloride solution to a concentration of 10^7/ml. One-tenth ml (10^6 cells) of this suspension is then injected into new host mice for either stock propagation or experimental purposes. The cells assume immediate exponential growth which is reproducible if the cells are harvested, diluted and transplanted to new host mice at weekly (7-8 day) intervals.

D. Exposure of L5178Y to the Chemical in vivi Through the Host-Mediated Assay

1. Strain of mice

All studies will be conducted with DBA_2 male mice as described above.

2. Dose

Mutagenic doses for each fraction will be related to its general toxicity to the mouse. Thus the initial evaluation will consist of 4 doses plus the appropriate controls. The doses will be related to the mouse LD_{50}, specifically 1/100 LD_{50}, 1/10 LD_{50}, 1/2 LD_{50}, 1 LD_{50}. The desired dose will be administered in 0.1-0.2 ml volumes using the appropriate diluent as determined by Dr. Lipsky.

If positive mutagenic results are obtained with the smallest (i.e., 1/100 LD_{50}) dose, then further studies will involve smaller doses in an attempt to find the "no effect dose." If negative results are obtained with the largest dose (1 LD_{50}) then the doses will be scaled upward to 2-4 LD_{50}. Due to the host toxicity with these larger doses, the duration of cellular exposure in the host will of necessity be less than 24 hours. In these instances cells will be harvested from the mouse 4-6 hours after treatment and diluted to 1 x 10^4/ml with complete (i.e., asn-supplemented) medium. Thus, *in vitro* incubation will substitute for *in vivo* "expression time."

3. Routes of Administration

The full advantage of host-mediation is provided by administering the chemical by a distal route (i.e., distal to the I.P. site) of innoculation of the indicator cells. However, some chemicals, e.g., sulfur mustard may be inactivated at the site of injection, and thereby provide false negative results. Furthermore, other chemicals, e.g., cycasin, are activated in the gastrointestinal tract; others may be inactivated or not absorbed by the gastrointestinal mucosa. Consequently, in initial studies the chemicals will be administered subcutaneously. If these provide negative results then the studies will be repeated and the chemical will be administered by I.P. injection.

4. Time of Treatment

The chemical will be administered on the 4th day after the intraperitoneal implantation of 10^6 cells. At this time the cells are in mid-logarithmic growth at a concentration of approximately 2-4 x 10^7/ mouse (Figure 1).

5. Removal of L5178Y ascites cells from mice

a. The first study point is 5-6 hours after the administration of a single dose of the chemical. Further determinations will be madeat daily intervals for 4-5 consecutive days to assess the effect of "expression time" on the recovery of mutant cells.

b. Mice are sacrificed by cervical dislocation.

c. The abdominal wall is saturated with 0.5% amphyl solution.

d. The area of incision is sterilized with a red hot #11 scalpel blade.

e. The skin is nicked with the blade and the skin is then peeled back to fully expose the peritoneum.

f. Using a 25 gauge needle, 5 cc of F-S_{10} medium is injected into the peritoneal cavity with moderate force so as to cause turbulence and hence mixing of the ascitic and culture fluids. (F-S_{10}=Fischer's medium without L-asparagine supplemented with 10% horse serum.)

g. As much fluid as possible is withdrawn into the same syringe usually 4.5-5 ml).

h. A new, sterile 25 gauge needle is applied to the syringe to minimize contamination and the contents of the syringe are ejected through the needle onto the side of a 50 ml screw cap centrifuge tube. This procedure plus gentle pipetting and ejection against the side of the tube with a 5 ml pipette 3 times will disrupt any cellular aggregates. The suspension may then be viewed under an inverted microscope to be sure of a homogeneous, single cell suspension.

i. The volume is then brought up to 50 ml with $F^{-}S_{10}$.

j. A 1:20 dilution (0.5 + 9.5 ml) in saline is made, then further diluted 1:10 in cetrimide solution to lyse any contaminating red blood cells. This final dilution is then counted in the Model B Coulter Counter. If significant cell kill has been achieved then the above dilution would have to be altered. Plotting the cells counts as a function of time after treatment provides immediate information on the approximate lethal or cytostatic effect of the chemical on the L5178Y. Extrapolation of the exponential portion of the growth curve back to the ordinate will provide a figure from which the survival fraction of the treated cells can be calculated. This supplies confirmatory evidence for viability determinations using the soft-agar cloning technique.

k. Washing procedure: the cell suspension is now centrifuged at 900 rpm in the international PR-2 centrifuge for 5 minutes; the supernatant is decanted and the pellet is dispersed in the remaining medium (usually 1 ml) by gently tapping the bottle against the palm of one's hand. The volume is then brought to 50 ml with $F^{-}S_{15}$ and the procedure is repeated for a total of 2 washes. After the second wash, the cells are diluted with $F^{-}S_{15}$ to a final concentration of 5×10^{6} cells/ml/ this dilution being pre-determined from the count obtained in "j".

l. Aliquots of this suspension are now taken for the determination of viability and induced mutations.

6. Viability

Since the induced mutation rate will be calculated from the number of induced mutants per million viable cells, the exact quantitation of cellular viability is essential. This is determined by cloning the cells in soft agar in the presence of 10 g/ml of L-asparagine using a modification of the technique as described by Chu and Fischer (29). Viability as determined by cloning is compared with viability as determined by the outgrowth method.

Soft agar cloning procedure:

a) A 2.2% Noble agar solution is prepared in advance and 5 ml (0.11 gm) is dispensed to 100 ml capacity glass medium bottles. This is then sterilized in an autoclave and the bottles are stored in the refrigerator until needed.

b) F^+S_{15} is pre-heated in a 44° C water bath.

c) Agar is melted in a boiling water bath or with a quick burse in an autoclave. When the agar bottle is cool enough to handle (i.e., warm), 45 ml of pre-warmed (44°C) F^+S_{15} is added to the agar bottle. This is now designated as AF^+S_{15} and is returned to the 44°C water bath.

d) An aliquot of cells from step 5k (in Section C-11-5) is now serially diluted with F^+S_{15} to provide sufficient cell inoculum per flask so as to produce 40-80 macroscopic colonies. E.g., control cells can be plated with an efficiency of 60%. Therefore, 100 cells would be plated. Since the final concentration of cells in step 5k was 5×10^6 ml, this would be diluted in 3 steps:

1) 1:100 (0.1 ml cell suspension + 9.9 ml of F^+S_{15})
2) 1:100 dilution of #1 to provide 500 cells/ml
3) 0.5 ml of dilution of #2 plus 9.5 ml of F^+S_{15}

to provide a final concentration of 25 cells/ml. This cell inoculum is scaled upwards depending on the anticipated cell kill by the chemical. If cell kill from a particular dose cannot be estimated, then 3 or 4 cell inocula covering 3 or 4 orders of magnitude are cloned. Each inoculum is done in duplicate.

e) 6 ml of AF^+S_{15} are added to 30 ml Falcon plastic monolayer flasks in the upright position.

f) 4 ml of the cell suspension from "d" are then added to the flask. Thus one dilution of "d" will be cloned in duplicate.

g) The cell-agar-medium mixture is gently mixed, then placed in a bucket of crushed ice for 5 minutes to allow the agar to gel. The flasks are then placed in the upright position in retainers designed by the applicant and are kept at room temperature for 20 minutes, then placed in a 37°C warm room.

h) Macroscopic colonial growth will be maximum by day 15 at which time the flasks are removed from the incubator. The flasks are now placed horizontally, thereby spreading the soft agar over one side of the flasks and the colonies are counted on a New Brunswick C110 Colony Counter.

i) The plating efficiency (P.E.) is calculated by the following:

$$P.E. = \frac{\text{number of observed colonies}}{\text{number of cells plated}} \times 100$$

The control is normalized to 100% and all treated cultures are expressed as a per cent of this figure, thus enabling the construction of a dose-response curve.

7. Induced Mutation

a. The cell concentration from "5k" above was 5×10^6 ml.

b. Agar is prepared as described for viability cloning except that the agar is diluted with 45 ml of F^-S_{15} (i.e., lacking L-asparagine).

c. The following is then added to a 30 ml Falcon plastic monolayer flask:

9 ml of AF^-S_{15}

5 ml of F^-S_{15}

1 ml of cell suspension from "5k."

This will result in 5×10^6 cells suspended in 15 ml of Agar-medium mixture.

d. The same procedure as in "6g and h" is followed with regards to mixing, agar gelling and colony counting.

e. The mutant frequency is derived from the following:

1. $$\text{Mutant frequency} \times 10^6 \text{ viable cells} = \text{(MF)} = \frac{\text{\# of mutant colonies}/10^6 \text{ plated cells}}{\text{plating efficiency}}$$

E. Significance

The carcinogenic process is undoubtedly a multifactorial one. While it has been estimated that the majority of human cancers are environmentally induced, the unequivocal identification of these substances is far from apparent. Carcinogenicity assays in current use involve whole animal studies that are very time consuming (several years) and costly. While a definitive casual relationship between mutation and the induction of cancer is also not conclusive, it has been shown that most, if not all, known carcinogens are mutagens. Thus, since mutagenicity screening is

far less costly and time consuming, mutagenicity tests would be used in initial screen to identify those chemicals which would be studied more closely in the carcinogenicity tests.

It is recognized that murine physiology and pharmacology may differ from its human counterpart, however, a mammalian host-mediated assay which utilizes a mammalian indicator cell with a stable chromosomal complement should permit a closer analogy to man than the study of non-mammalian or solely in vitro mammalian systems.

F. Facilities available

Bioassays are being carried out in approximately 900 square feet of laboratory space in addition to separate office facilities. Equipment in use includes a Biophysics Cytograph Model 6301 for cell counting, sixing and viability estimated (dye-exclusion technique). Cell culture equipment, constant temperature water baths, colony counter, Hewett-Packard programmable calculator Model 3800, refrigerated centrifuged and microscopes. In addition, all of the facilities of a modern, well-equipped pharmacology department will be available. Some of the major items include a central cell culture facility with a walk-in warm room (37°), CO_2 incubator, roller-cell equipment and inverted microscopes; also available are an electron microscope, amino acid analyzer, high voltage electrophoresis, liquid scintillation counter, Carey Recording Spectrophotometer. Experimental animals are kept in special animal holding rooms under the direction of full time veterinarians.

G. Collaborative arrangement

Active collaboration with Dr. S. R. Lipsky, Professor of Physical Sciences is being carried out in all of these experiments. Dr. Lipsky's laboratory will continue to fractionate the crude material and will also identify the chemical structure of those isolated components which prove to be mutagenic.

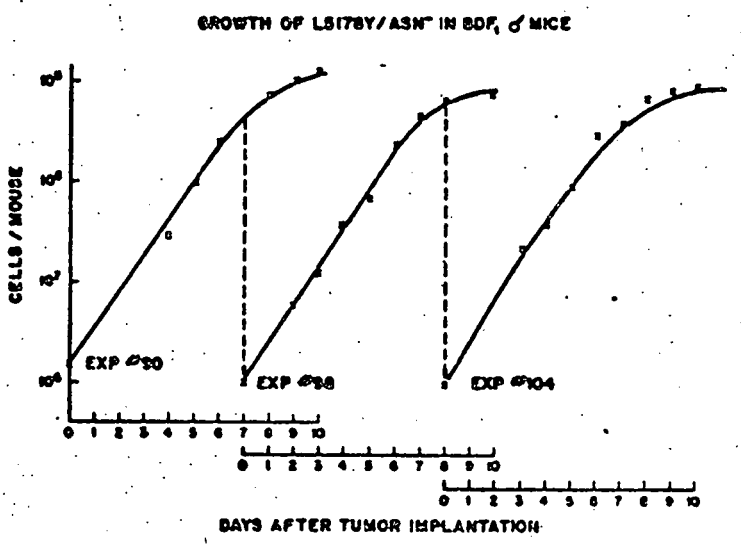


Fig. 1. Cells (10^6) were implanted into the peritoneal cavity of mice and were quantitatively harvested on the indicated days. Each *point* represents the mean cell count from 4 mice. *Arrows*, harvest, dilution, and transplantation from donor mice to new host mice. *BDF*$_1$, BD2F$_1$.

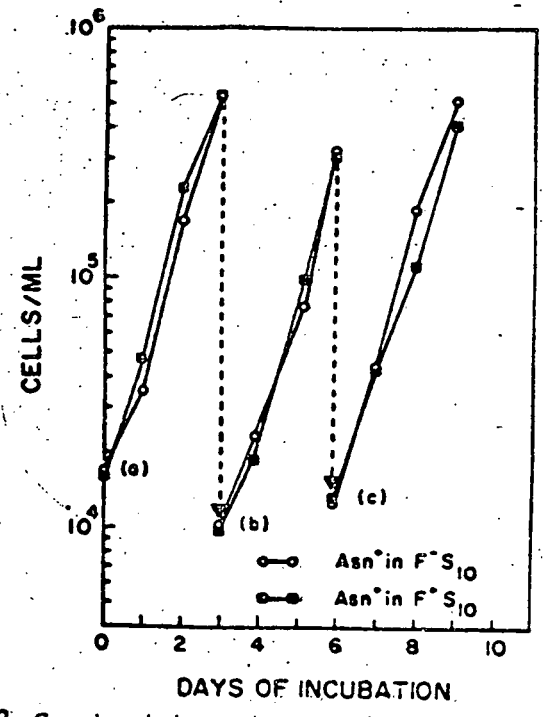


Fig. 2. Growth and phenotypic nature of cells from newly derived L5178Y/Asn$^+$ clones. At *Point a*, aliquots from the same colonies were suspended in asparagine-deficient (F^-S_{10}) and asparagine-supplemented (F^+S_{10}) medium and observed from Days 0 to 3; at *Point b*, cells that had grown in F^-S_{10} were then diluted into F^+S_{10} and F^-S_{10} and observed from Days 3 to 6; at *Point c*, cells from F^+S_{10} were then diluted into F^+S_{10} and F^-S_{10} and observed from Days 6 to 9.

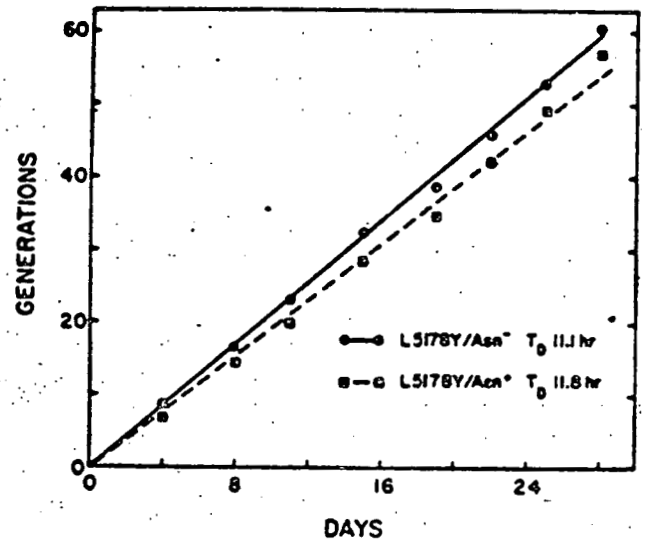


Fig. 3 . Comparison of the growth of L5178Y/Asn⁻ in an asparagine-supplemented medium and L5178Y/Asn⁺ in asparagine-deficient medium. T_D, doubling time.

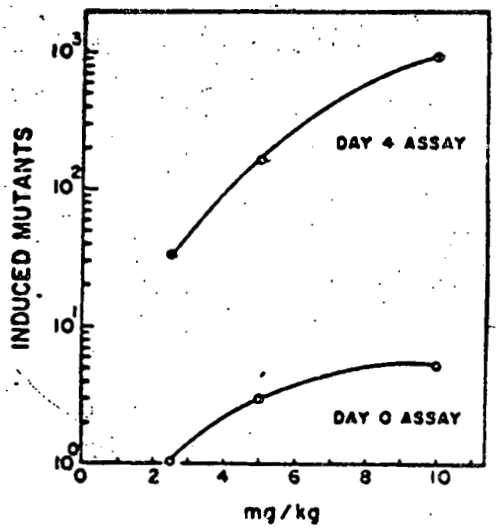
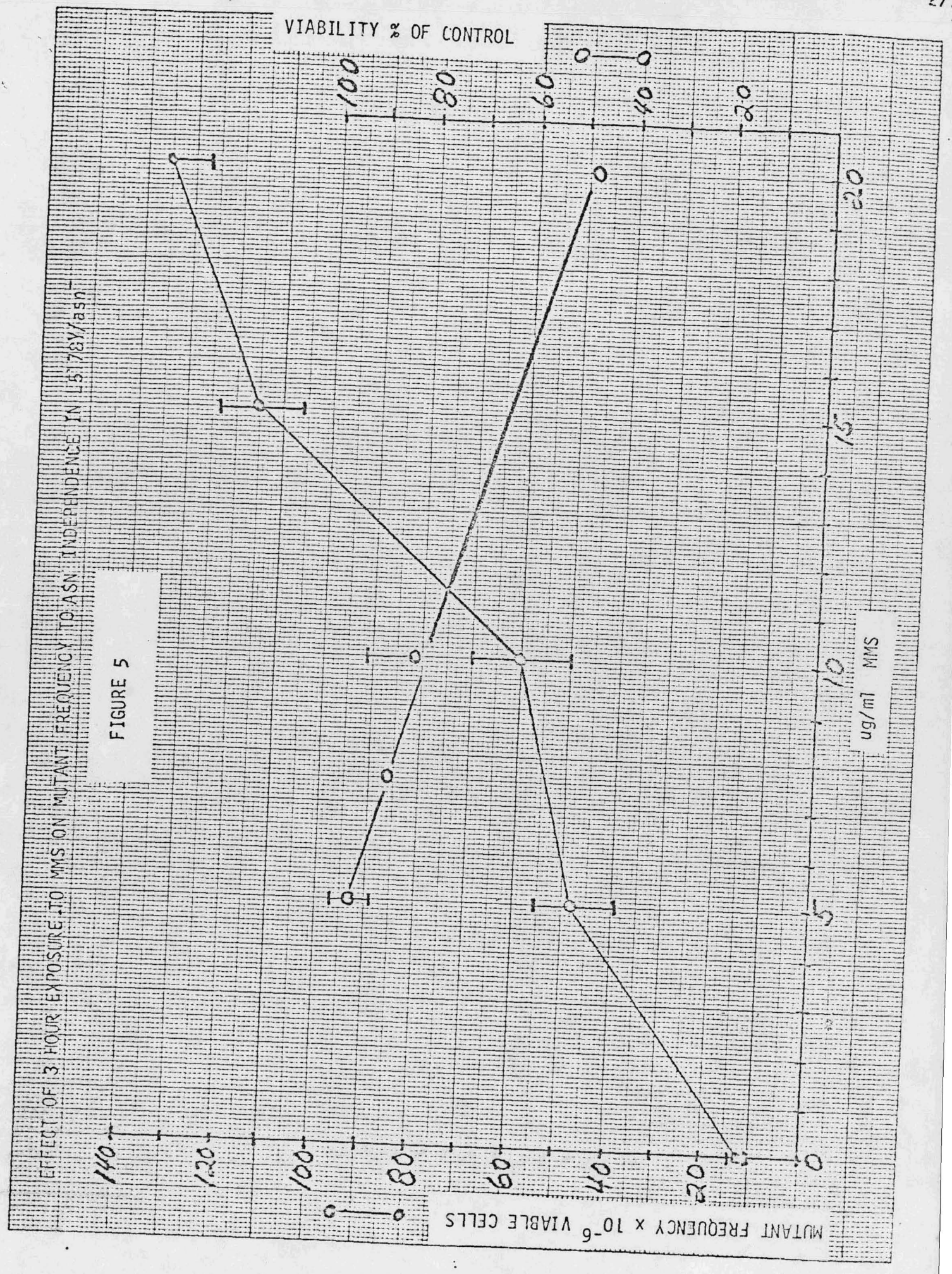


Fig. 4 DMNA-induced mutants to asparagine independence. Four days after the i.p. inoculation of 10^6 L5178Y/Asn⁻ cells, mice were treated with a single s.c. dose of DMNA. The cells were then harvested, washed in F-S_{10}, and cloned for viability and mutant frequency 6 hr later (Day 0 assay) and 4 days later.


VIABILITY % OF CONTROL
100
80
60
40
20
0
MUTANT FREQUENCY x 10^{-6} VIABLE CELLS
140
120
100
80
60
40
20
0
0
5
10
15
20
ug/ml MMS
EFFECT OF 3 HOUR EXPOSURE TO MMS ON MUTANT FREQUENCY TO ASN INDEPENDENCE IN L5178Y/asn
FIGURE 5
EUGENE DIETZGEN CO.
MADE IN U. S. A.
NO. 340-M DIETZGEN GRAPH PAPER
MILLIMETER

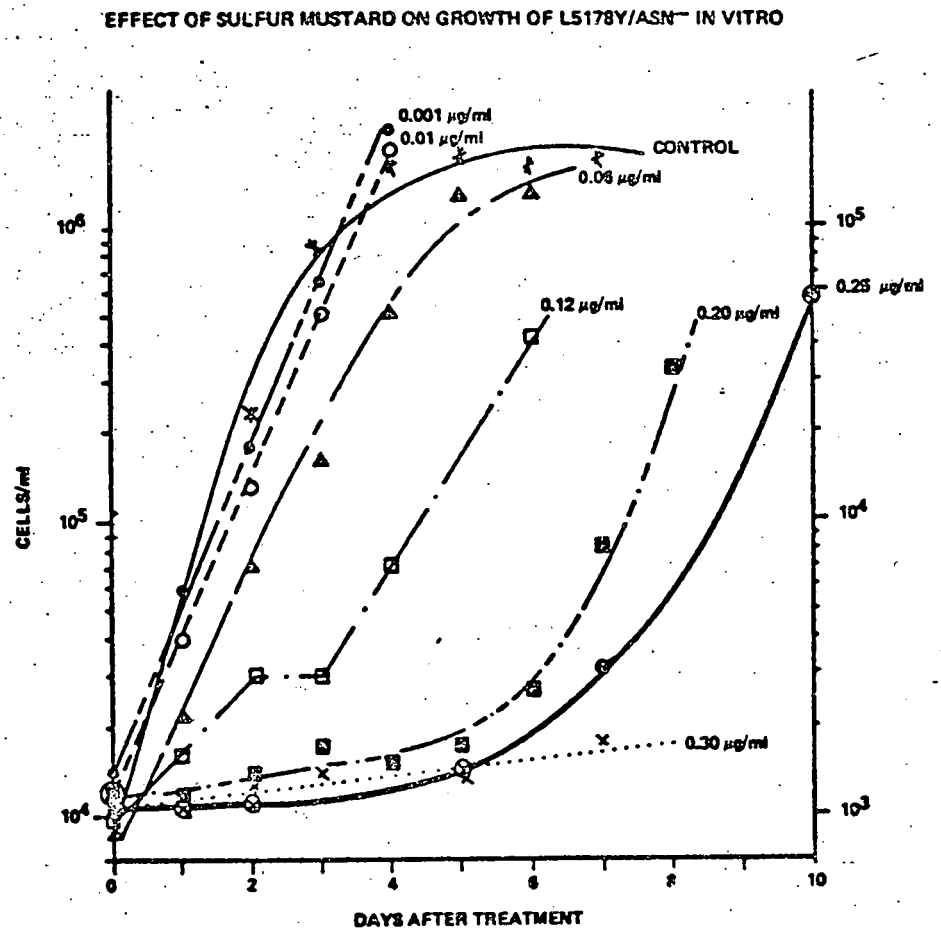
LEGENDS TO FIGURE 5

Figure 5

L5178Y/asn$^-$ cells were exposed to increasing doses of MMS for 3 hours. Cells were then washed free of drug and cloned immediately in fully supplemented medium to determine the degree of cell kill. Another aliquot of cells was re-suspended in fully supplemented medium and incubated for 72 hours at which time they were cloned again to determine viability and mutant frequency is that produced after 72 hours expression time. Data points are mean $\pm$ SEM.

FIGURE 6

L5178Y/asn$^-$ cells were exposed to the stated doses of sulfur mustard for 30 minutes, then washed with drug-free medium. Cells were then resuspended in fully supplemented medium and daily counts were taken.



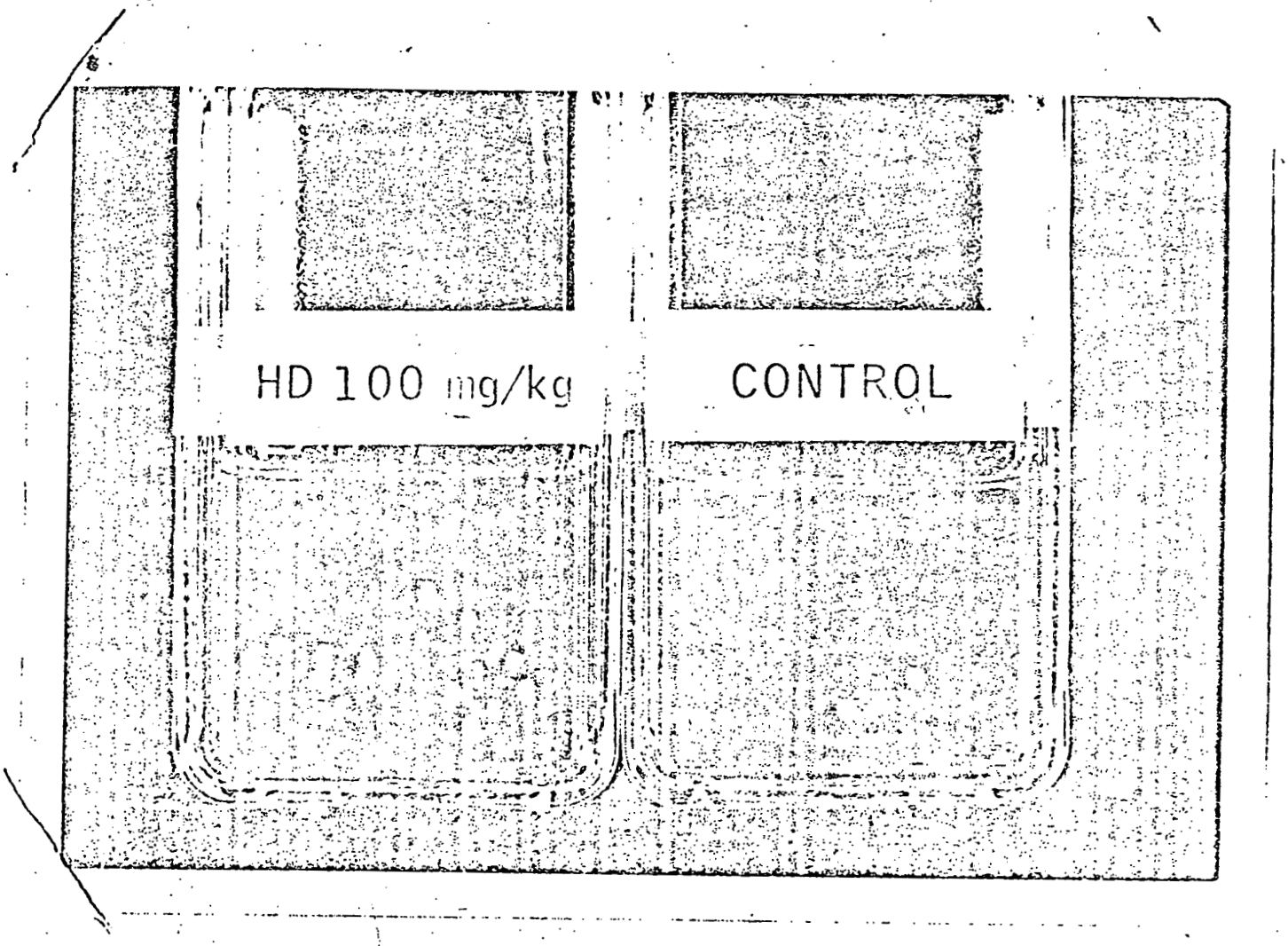
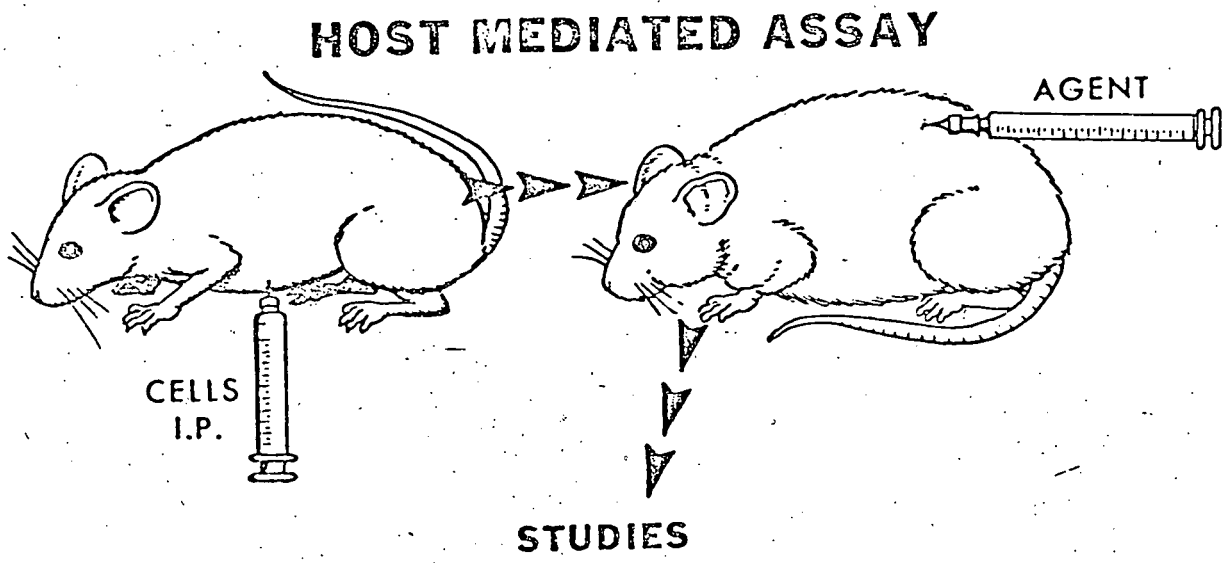


Figure 7

Mice bearing the L5178Y/asn$^-$ were treated with phosphate buffered saline (control) or 100 mg/kg of sulfur mustard subcutaneously. Four days later the cells were harvested and 5 x 10^6 cells were cloned in soft agar. Macroscopic colonies are visible to the naked eye and can be quantitated within 10-14 days.



1. CELLULAR VIABILITY (IN VIVO; IN VITRO)
2. SOMATIC MUTATION

FIGURE 8

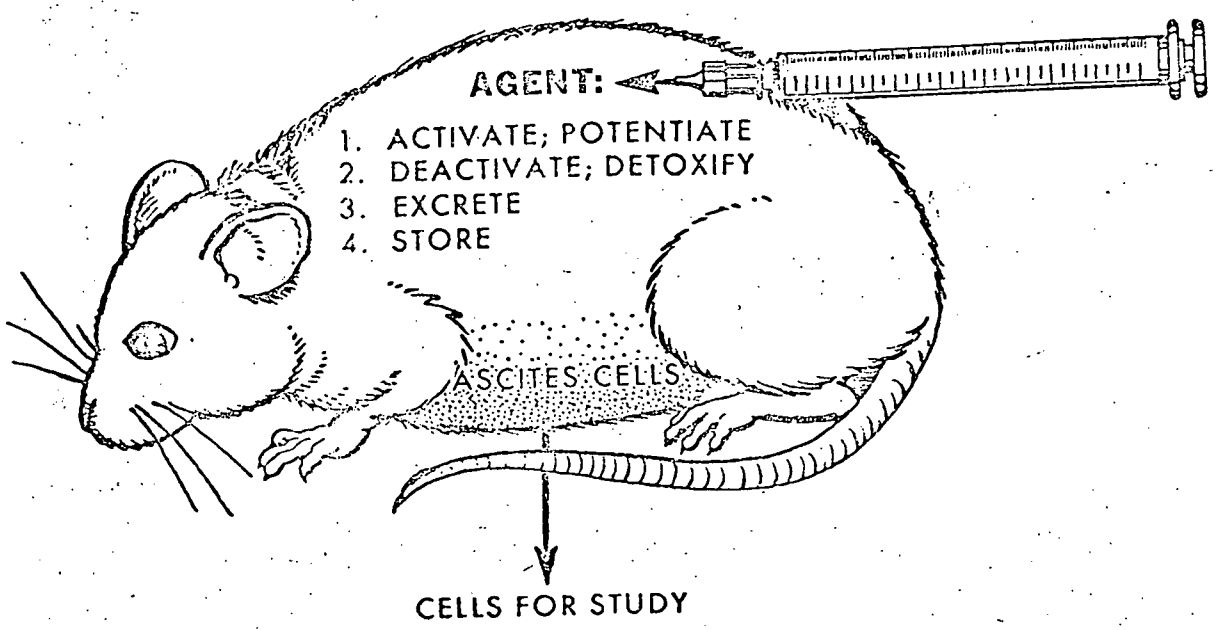

HOST MEDIATED ASSAY
AGENT:
1. ACTIVATE; POTENTIATE
2. DEACTIVATE; DETOXIFY
3. EXCRETE
4. STORE
ASCITES CELLS
CELLS FOR STUDY

FIGURE 9

Table 1

Results of fluctuation tests to determine the number of L5178Y/Asn⁺ cells in cultures of L5178Y/Asn⁻ cells

Ten replicate platings were performed in each of 10 experiments. Total population assayed was 500 × 10^6 cells.

No. of experiments	10
Mean no. of mutants/5 × 10^6 cells	15.3
Variance	246.1
Mutant frequency/10^6 survivors	3.2
Mutation rate × 10^{-7} [a]	7.4

[a] Mutations/locus/generation according to $r = aN_t \ln(CaN_t)$ of Luria and Delbrück

Table 2

Effect of treatment of L5178Y/Asn⁻ cells with EMS in vitro and in the HMA

After 2 hr of exposure to EMS *in vitro* the cells were washed free of drug with F^-S_{10} and then cloned in F^+S_{10} and F^-S_{10} for the determination of viability and mutant frequency, respectively, at various times after incubation in complete medium (expression time). In the HMA, mice were treated with a single s.c. dose of EMS 4 days after i.p. inoculation of 10^6 cells. Cells were harvested 72 hr later, washed, and cloned as above.

Exposure	Dose	Expression time (hr)	Viability (% of control)	Mutation frequency (× 10^{-6} viable cells)
In vitro	0	0	100	3.2
		24	100	2.3
		48	100	2.4
		72	100	2.3
In vitro	5×10^{-6} M	0	58	2.0
		24	55	6.5
		48	53	16.6
		72	85	15.8
In vitro	10^{-5} M	24	20	13.1
		48	31	21.0
		72	48	26.2
In vivo	0.9% NaCl solution control	72	100	4.2
In vivo	10 mg/kg, s.c.	72	90	1.9
In vivo	25 mg/kg, s.c.	72	100	0.8
In vivo	50 mg/kg, s.c.	72	100	6.9
In vivo	250 mg/kg, s.c.	72	63	26.1

Table 3

Effect of treatment of L5178Y/Asn⁻ cells with MNNG in vitro and in the HMA

After 2 hr of exposure to MNNG *in vitro*, the cells were washed free of drug with F^-S_{10} and then cloned in F^+S_{10} and F^-S_{10} for the determination of viability and mutant frequency, respectively, at various times after incubation in complete medium (expression time). In the HMA, mice were treated with a single s.c. dose of MNNG 4 days after the i.p. inoculation of 10^6 cells. Cells were harvested 72 hr later, washed, and cloned as above.

Exposure	Dose	Expression time (hr)	Viability (% of control)	Mutation frequency ($\times 10^{-6}$ viable cells)
In vitro	0	0	100	3.2
		24	100	2.3
		48	100	2.4
		72	100	2.3
In vitro	2.5×10^{-7} M	0	85	0.59
		24	60	10.2
		48	48	24.6
		72	65	29.0
In vitro	5×10^{-7} M	0	35	3.7
		24	24	15.6
		48	26	52.4
		72	45	55.7
In vivo	0.9% NaCl solution control	72	100	10.0
In vivo	25 mg/kg, i.m.	72	60	118.0

Table 4

Effect of treatment of L5178Y/Asn⁻ cells with SM in vitro and in the HMA

SM was dissolved in 0.05 M PBS*, pH 7.2, or polyethylene glycol 200. After 30 min of exposure *in vitro*, the cells were washed free of drug with F^-S_{10} and then cloned in F^+S_{10} and F^-S_{10} for the determinations of viability and mutant frequency, respectively, at various times after incubation in complete medium (expression time). In the HMA, mice were treated with a single s.c. dose of SM, 4 days after the i.p. inoculation of 10^6 cells. Cells were harvested 96 hr later, washed, and cloned as above.

Exposure	Dose	Expression time (hr)	Viability (% of control)	Mutation frequency ($\times 10^{-6}$ viable cells)
In vitro	0	0	100	4.4
		96	100	1.2
	0.001 mg/l in PBS	0	100	4.4
		96	98	6.2
	0.005 mg/l in PBS	0	90	4.4
		96	90	12.6
	0.01 mg/l in PBS	0	95	5.8
		96	85	10.3
In vivo	PBS control	96	100	2.6
	1 mg/kg in PBS, s.c.	96	81	4.8
	10 mg/kg in PBS, s.c.	96	75	3.9
	100 mg/kg in PBS, s.c.	96	66	24.0
	Polyethylene glycol 200 control	96	100	5.0
	50 mg/kg in polyethylene glycol, s.c.	96	53	160.0
	100 mg/kg in polyethylene glycol, i.m.	96	45	59.8

* PBS, phosphate-buffered saline.

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