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ΧΗΜΙΚΑ ΧΡΟΝΙΚΑ

ΝΕΑ ΣΕΙΡΑ

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1975

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THE INFLUENCE OF SOME THIOLS, UNSATURATED ALIPHATIC ACIDS AND BIOGENIC AMINES ON THE INHIBITION OF 3, 4-BENZOPYRENE CANCEROGENESIS

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(Received April 13, 1975)

Summary

The cancerogenic action of 3,4-Benzopyrene (3,4-BP) can be reduced by substances physiologically occuring in organism of animals and human beings, as well as, by several synthetically prepared compounds. Experimental investigations on female mice (NMRI-strains), 4-5 weeks of age, 20-25 g body weight, have revealed that, when 3,4-BP solutions (2.52mg 3,4-BP in 0.5 ml Tricapryline, final concentration) are incubated with some Thiols, unsaturated aliphatic acids or biogenic amines, the tumor development can be partially postponed or completely inhibited; In comparison to the groups treated with only 3,4-BP the cancerogenesis is almost 100%. Some theoretical suggestions have been experimentally investigated in order to explain a probable mechanism for the inhibition of the cancerogenic action of 3,4-BP in higher animals.

Key words: Cancerogenesis, Inhibition, 3,4-Benzopyrene inactivation, Natural and Synthetic anticancerogens, cancerogenic resistant animals.

Introduction

Experimental investigations concerning the cancerogenic action of 3,4-BP on meat producing animals, showed that the pigs and cows which received intramuscularly 145mg and 435mg of 3,4-BP respectively, developed no tumors locally in a period of 12-29 months of observation.[1] Similar results were obtained with beagle dogs and Thailand pigs.[2] On the contrary, in comparative studies with NMRI mice strains, a high rate of tumor development was observed in 95-100% of the laboratory animals, 2-4 months after a single subcutaneous injection of 2.52 mg 3,4-BP. The distinction of various animal species in relation to 3,4-BP-cancerogenesis can be explained from the fact that the initial time required for the development of a cancer cell and the necessary dosis of the corresponding cancerogenic agent for the tumor induction, varies considerably, and probably depends on many other factors besides time and dosis, such as, race, age, sex, exogenic and endogenic influences, etc.

It is also possible, that domestic and meat producing animals could possess an inactivation mechanism able to postpone or even to inhibit totally the cancerogenic action of carcinogens, in this special case of 3,4-BP, by means of one or eventually more substances present in the corresponding "cancerogenic resistant" animal species. Consequently, if this hypothesis is true, the compound or the group of

substances which are able to inactivate 3,4-BP should be absent from the laboratory animals susceptible to cancerogens, or may be present only in small quantities which are not sufficiant enough to inhibit cancerogenesis. To prove this hypothesis experimentally, we studied some reactions between 3,4-BP and natural or synthetic compounds.

The idea was, if the examined compounds react with the K-Region of 3,4-BP then, they could either increase or decrease its cancerogenic activity. In the latter case, we could probably succeed by the mice strains to postpone or to inhibit the 3,4-BP cancerogenesis in the presence of a suitable metabolic product or a synthetically prepared drug.

Another possibility was to block some important reactiv groups of cell components, for example DNA, RNA, etc. with putrescine in order to inhibit their reaction with, 3,4-BP.

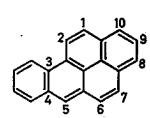
Theoretical considerations

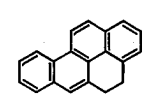
1.- 3,4-Benzopyrene is composed of 5 six membered aromatic rings with 10 conjugated double bonds (Table 1, formula V). Especially the position Δ_6 of the double bond is characterized by a higher π-electron density,[3-6] and it is called K-Region or carcinophore zone. Theoretically, through a selective reduction of the Δ_6 double bond with suitable compounds, a 6,7-Dihydro-3,4-benzopyrene derivative can be formed with 9 conjugated double bonds. As a result of the reduction of the Δ_6 double bond, the π-electron density in the corresponding position may decrease. Thus, the relation of the conjugated double bonds and the π-electron density in the 3,4-BP molecule can be modified and probably also its cancerogenic action.

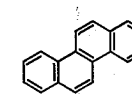
Furthermore, the reduction of the Δ_6 double bond, accompanied with a substitution of the C_6 or/and C_7 position of the 3,4-BP molecule, could also reduce the reactivity between 3,4-BP and several cell components, because the reactive groups of the cancerogen are now either blocked or diminished.[7] Therefore, some cytological alterations due to the action of 3,4-BP, causing the formation of cancer cells, could probably be hindered because of the inactivation of the 3,4-BP molecule.

2.- The chemical structure of 6,7-Dihydro-3,4-benzopyrene (XIII) is an intermediate between 3,4-Benzopyrene (V) which is strong cancerogenic agent, and Chrysene (XIV) which possess almost no cancerogenic activity.[8] Probably, the chemical constitution of 6,7-Dihydro-3,4-benzopyrene could also influence its cancerogenic property which could be theoretically between 3,4-BP (strong cancerogenicity) and Chrysene (weak or no cancerogenicity).

3.- Taken into consideration the above mentioned theoretical suggestions, we tried to find some suitable compounds such as Thiols, unsaturated aliphatic acids, or other substances, which could react selectively with 3,4-BP to reduce the Δ_6 double bond, according to the reactions demonstrated in table I. These reactions are only theoretical models, and they served us for the selection of the corresponding Thiols and acids.







	3,4-Benzopyrene	6,7-Dihydro-3,4-benzopyrene	Chrysene
	(V)	(XIII)	(XIV)
Number of double bonds:	10	9	9

TABLE I. *Inhibition of the cancerogenic action of 3,4-Benzopyrene in presence of some investigated natural and synthetic compounds.* *Brackets = number of mice died due to toxicity, accidents, diseases, cannibalismus, etc. not considered for statistical evaluation. **Brackets = days of observation. ***Inhibition of 3,4-BP cancerogenesis in % calculated for the period of 345-375 days after injection.

Substance	Inject. amount in mg/0.5 ml Tricapryline		Nr of mice*	Nr of mice died within 7 months after 3,4-BP injection		Nr of mice died after 7 months of 3,4-BP inj.		Nr. of mice still alive**		3,4-Benzopyrene cancerogenesis***
	add mg	3,4-BP mg		with tumor	no tumor	with tumor	no tumor	with tumor	no tumor	in %
1) Control (untreated mice)	—	—	28 (2)	—	—	—	—	—	28 (375)	0
2) 3,4-Benzopyrene	—	2.52	30	30	—	—	—	—	—	100
3) 3,4-BP+cis-Aconitic acid	30 + (alc)	2.52	34	3	2	3	1	—	25 (345)	18
4) 3,4-BP+L-Cysteine	<10+ (H_2O)	2.52	30	26	—	4	—	—	—	100
5) 3,4-BP+L-Cysteine-ethylester	20 + (alc)	2.52	32	2	1	—	2	—	27 (345)	6
6) 3,4-BP+Dithiothreitol	10 +	2.52	28 (3)	3	2	—	4	—	19 (345)	11
7) 3,4-BP+Bismuthiol I.	20 + (alc)	2.52	28 (2)	3	1	—	1	—	23 (345)	11
8) 3,4-BP+Mercaptosuccinic acid	20 + (alc)	2.52	31 (1)	7	1	—	—	—	23 (345)	22
9) 3,4-BP+Putrescine	10 +	2.52	28 (2)	—	1	1	2	—	24 (375)	3

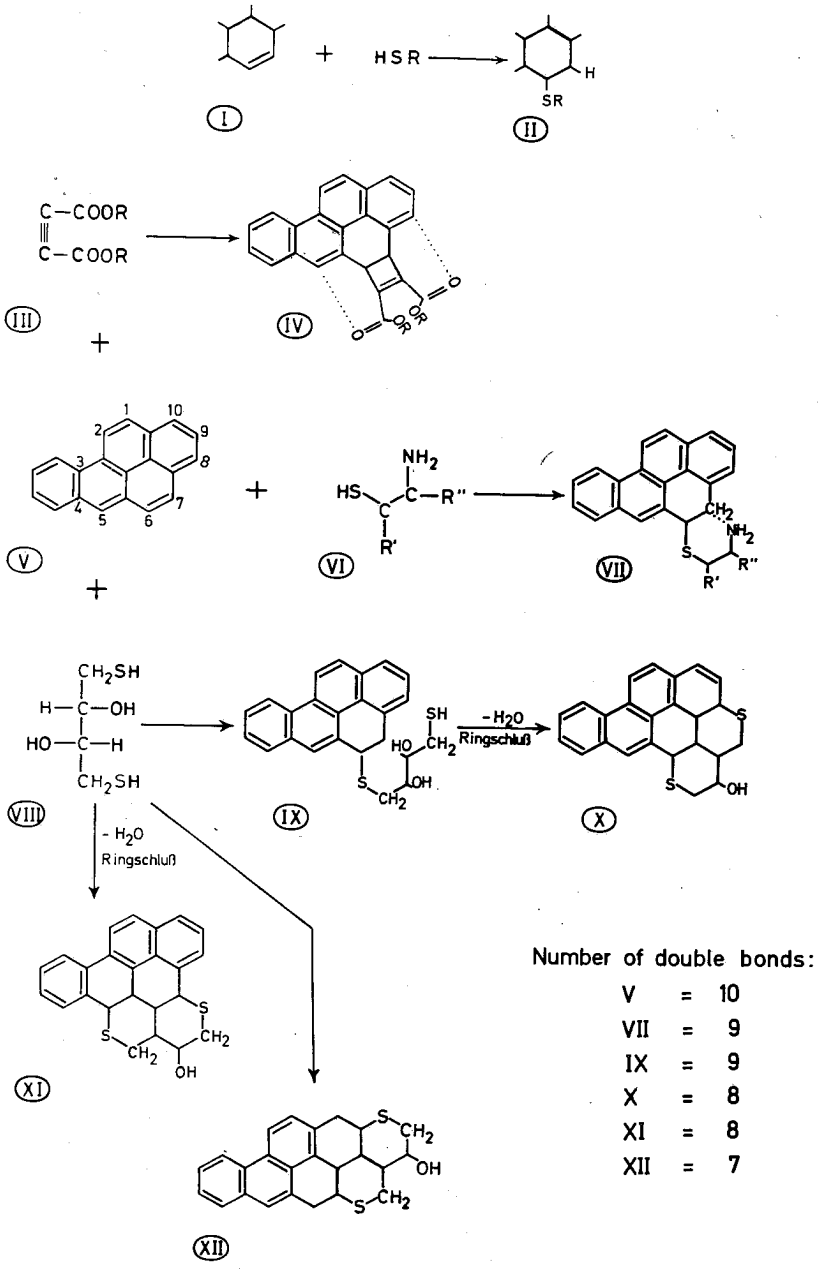


FIG. 1. *Theoretical models concerning possible reactions of Thiols and unsaturated aliphatic acids with the K-Region of 3,4-Benzopyrene.* The selective reduction of the Δ_6 double bond of the 3,4-BP molecule, as well as, the substitution of the positions C_6 and/or C_7 could probably reduce its cancerogenic activity. Notice also the comparison of the 3,4-BP molecule (strong cancerogenic agent) with Chrysene (XIV. lack of cancerogenic activity probably due to the absence of the C_6-C_7 group).

Experimental

Material and Methods

For the experimental investigations two groups of compounds have been used which could react with 3,4-BP reducing selectively the Δ_6 double bond and form addition products.

A) Sulfhydryl compounds physiologically occuring, such as L-Cysteine, Glutathion, Cysteamine, or synthetically prepared, i.e. Bismuthiol I, Mercaptosuccinic acid, 6-Mercaptopurine, L-Cysteine ethyl ester, Dithiothreitol, etc.

B) Unsaturated aliphatic di- and tricarboxylic acids, such as, fumaric, maleic, cis-aconitic acid which are present in different animal species, as well as, some synthetically prepared, i.e. cis-aconitic anhydride, acetylene dicarboxylic acid and its methyl and ethyl esters, etc.

Further, a third group of compounds has been selected which could react with some cell components, thus inhibiting their linkage with 3,4-BP and blocking indirectly the cancerogenesis. The compounds are:

C) Biogenic amines and their decarboxylation products, for example Spermine, Spermidine, Ornithine, Putrescine, Cadaverine, Agmatine, etc.[9]

3,4-Benzopyrene, $C_{20}H_{12}$, Molecular weight 252,32, melting point 176,5° C 99% purity, was purchase from Fa. Schuchardt, München-Germany. Nr. BE 7131. The compounds tested were purchased from different companies i.e. Merck/Darmstadt, Fluka/Buchs, Schuchardt/München, Roth/Karlsruhe, Calbiochem/Lucerne, Kodak/Eastman-USA, Koch-Licht/Colnbrook, etc.

The 3,4-BP solutions were prepared by dissolving the substance in Tricaprylin. The less soluble compounds were first dissolved in a small amount of ethyl alcohol and then Tricaprylin was added to the ethanolic solution. (Occasionally, lipophobe compounds were first dissolved in water and emulsions with tricaprylin were injected). The mixtures of 3,4-BP + the compound tested were injected either directly after mixing them together, or they were first incubated for 48-76h at 38°C, in order to complete the reaction between 3,4-BP and the compound added.

The laboratory animals used, were female mice, NMRI strains, 4-5 weeks of age and 20-25g body weight. Each animal received subcutaneously 0.5ml of the Tricaprylin solution containing 2.52mg 3,4-Benzopyrene + 1 - 30mg of the compound tested. Over 50 different compounds have been already tested if they possess any anti-cancerogenic property. For the preliminary trial, each compound was tested in a group of 10 mice. In order to confirm the most important experimental findings, the results were checked again with a greater number of animals (usually 30 mice) for a statistical evaluation.

Tumors were developed locally, usually 80-100 days after the s.c. injection, and animals died 2-3 months later due to malignant process. Post mortem, the tumors were removed for histological examination (Fasske) as well as, several organs, i.e. Lungs, Liver, Splen, Kidneys, etc.

Animals from different groups died during the experiments because of other reasons not related to the 3,4-BP cancerogenesis (for example, accident, toxicity of the compound tested, pneumonia and other infectious diseases, cannibalismus, etc) were not considered for the interpretation of the results.

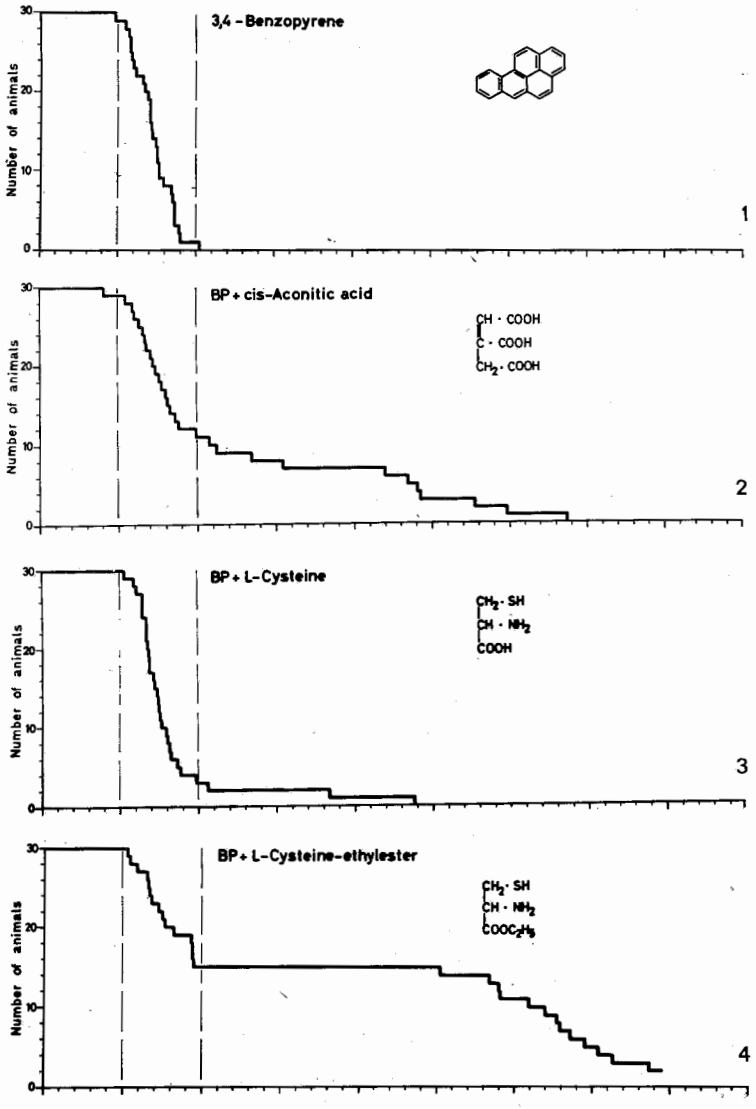


FIG. 2. *Selection of natural and synthetic compounds which when injected together with 3,4-Benzopyrene, were able to postpone as well as, to inhibit cancerogenesis, thus prolonging the life of the mice treated with the cancerogen.*

Days of survival of mice after a single subcutaneous injection of: (1) 2.52mg 3,4-BP, (2) 2.52mg 3,4-BP + <10mg cis-Aconitic acid, (3) 2.52mg 3,4-BP + <10mg L-Cysteine, (4) 2.52mg 3,4-BP + <10mg L-Cysteine-ethylester, (5) 2.52 mg 3,4-BP + 5 mg Dithiothreitol, (6) 2.52mg 3,4-BP + <10mg Mercaptosuccinic acid, (7) 2.52mg 3,4-BP + <10mg Bismuthiol I. and (8) 2.52mg 3,4-BP + 10mg Putrescine. Notice also the increase of the moderate anticancerogenic property of L-Cysteine after esterification. (ordinates: Number of mice pro compound tested).

FIG. 2A. (Continue)

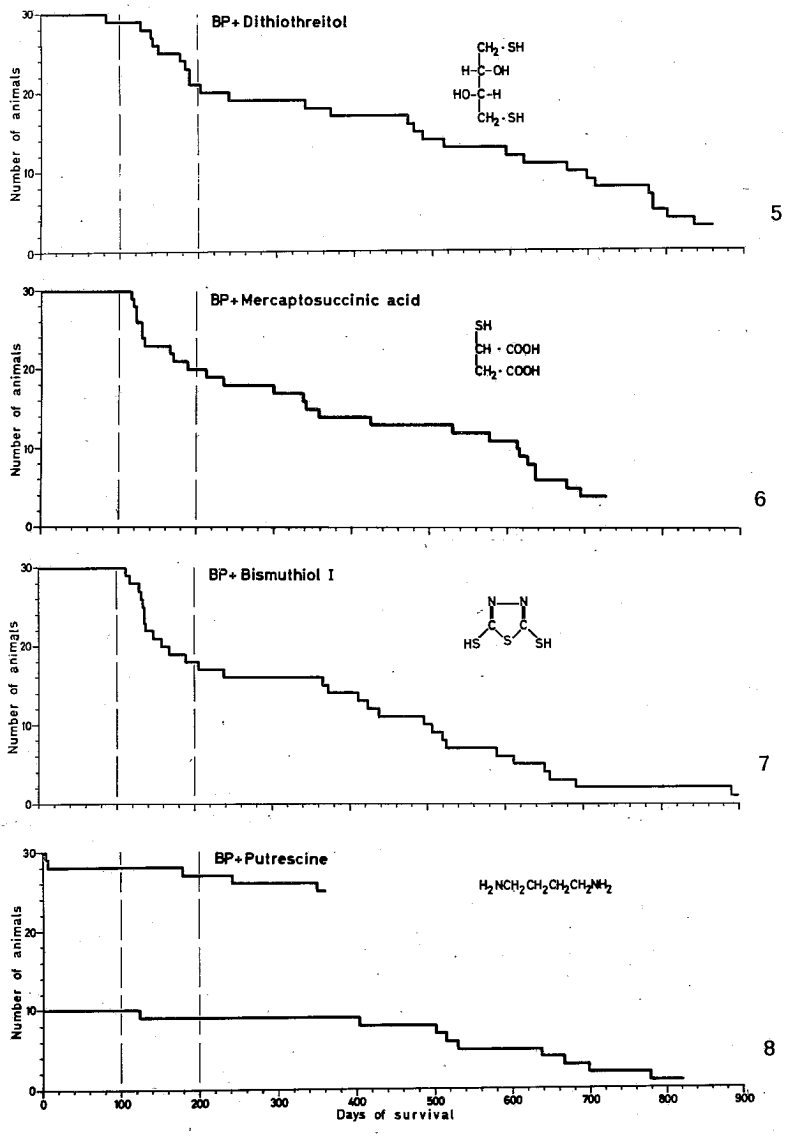
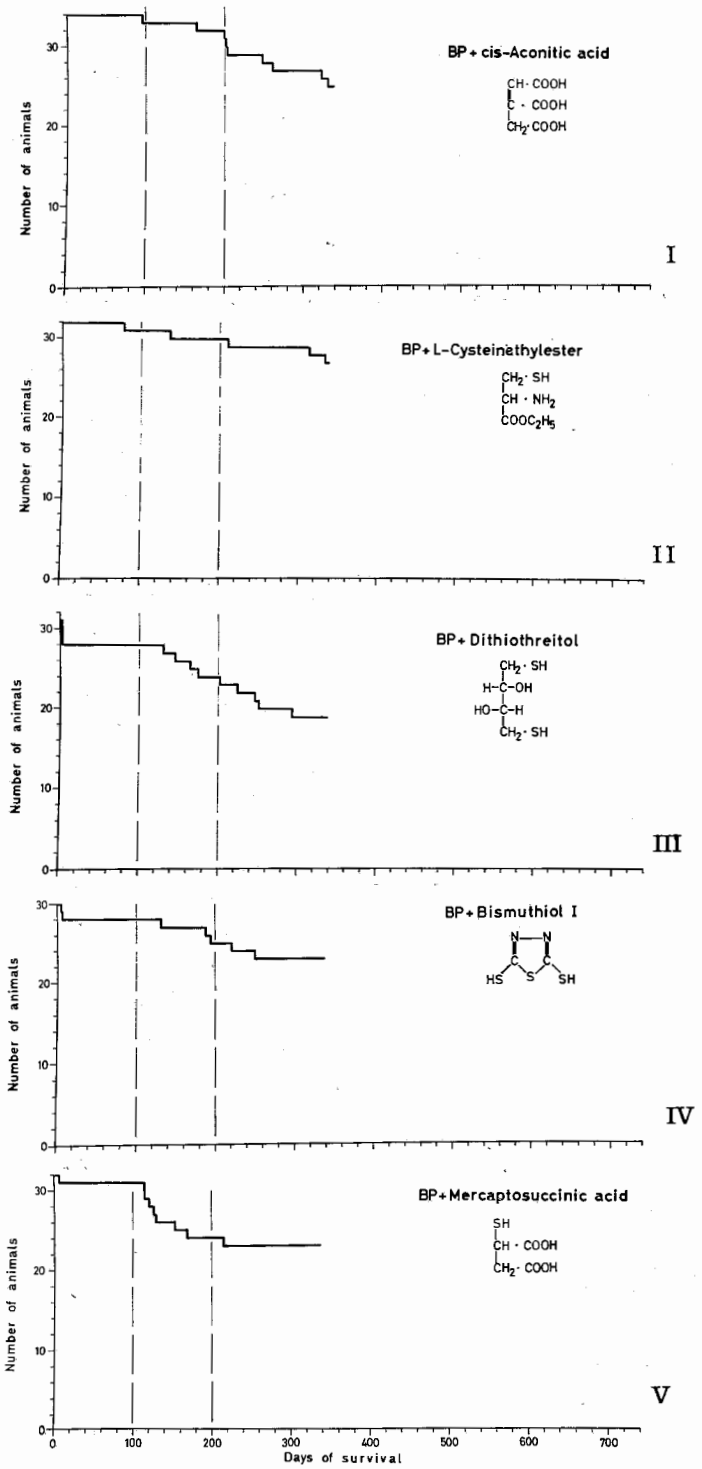


FIG. 2B. *Improvement of the anticancerogenic effect of the selected compounds by increasing their concentration and solubility.* (Addition of few drops of ethyl alcohol or water to the Tricapryline solution). Days of survival of mice after a single subcutaneous injection of: (1) 2.52mg 3,4-BP + 30mg


BP + cis-Aconitic acid
CH · COOH
C · COOH
CH2 · COOH
I
BP + L-Cysteinethylester
CH2 · SH
CH · NH2
COOC2H5
II
BP + Dithiothreitol
CH2 · SH
H–C–OH
HO–C–H
CH2 · SH
III
BP + Bismuthiol I
N N
C C
HS S SH
IV
BP + Mercaptosuccinic acid
SH
CH · COOH
CH2 · COOH
V
Number of animals
0
10
20
30
100
200
300
400
500
600
700
Days of survival

cis-Aconitic acid, (II), 2.52mg 3,4-BP + 20mg L-Cysteine-ethylester, (III) 2.52mg 3,4-BP + 10mg Dithiothreitol, (IV), 2.52mg 3,4-BP + 20mg Bismuthiol I., (V) 2.52mg 3,4-BP + 20mg Mercaptosuccinic acid. Notice that, the high mortality of mice between 100-200 days after the 3,4-BP injection, is considerably decreased in comparison to figure 2, if higher amounts of the anticancerogens are present in the 3,4-BP solution. See also TABLE I & II. (Ordinates: Number of mice pro compound tested).

Results and discussion

The most important results are graphically summarized in fig. 2 and table I.

The strong cancerogenic action of 3,4-Benzopyrene was also confirmed under the above mentioned experimental conditions. A single s.c. injection of 2.52mg 3,4-BP in 0.5ml Tricaprylin caused the development of tumors to all 30 young mice treated. Tumors usually appeared locally few weeks after the injection and within 7 months all animals died because of the malignant process.

But the 3,4-BP cancerogenesis can be reduced by several natural and synthetic compounds, such as unsaturated di- and tricarboxylic acids,[10-12] flavons,[13] quinones[14] peroxides[15] thiols[16-17] biogenic amines,[18] after hydrogenation,[19] as well as, by soil microflora[20] etc.

From the unsaturated aliphatic di-and tricarboxylic acids tested, fumaric and maleic acid were unable to reduce the cancerogenic action of 3,4-BP; but some anhydrides which do not occur physiologically such as maleic and citraconic anhydride are known to reduce the 3,4-BP cancerogenesis.[10] Also cis-aconitic acid possess anticancerogenic properties. The results are summarized in table II. From the 34 mice treated with 2.52mg 3,4-BP + 30mg cis-aconitic acid, only 6 animals developed a tumor; (By three mice the tumor development was postponed). Also by 3 further animals died during the one year of observation, no tumors were found. From this group there are 25 mice still alive without tumors.

The inhibition of the cancerogenic action of 3,4-BP depends upon the cis-aconitic acid concentration. Lower amounts of cis-aconitic acid i.e. 10mg or less added to 3,4-BP solutions were less effective in preventing cancerogenesis (Group C and D).

These results need further attention because of the importance of cis-aconitic acid as a physiological occuring "anticancerogenic compound" for an eventual biological inactivation of the cancerogenic action of 3,4-BP by "cancerogenic resistent" animals. cis-Aconitic acid which is a metabolic product of the "citric acid cycle" could be one of the natural compounds inhibiting cancerogenesis.

Also some Thiols were able to inhibit 3,4-BP cancerogenesis. The anticancerogenic action of some HS-derivatives was first studied experimentally by Crabtree.[16]

In our experiments, L-Cysteine was also unable to reduce the cancerogenic action of 3,4-BP. Most of the mice died between 100-200 days after the s.c. injection of 2.52mg 3,4-BP due to the tumor development; only by 4 mice the tumor induction was postponed and the corresponding animals lived longer (Tab. I).

A very important observation is that when L-Cysteine is *esterified* its moderate anticancerogenic activity can be considerably increased. For example, L-cysteine-ethylester possess a good inhibitory effect against 3,4-BP cancerogenesis. From 32 mice treated with 2.52mg 3,4-BP + 20mg L-cysteine-ethylester, only 2 mice developed a tumor and died because of the malignant process. 3 more animals died within 345 days of observation were free of tumors. 27 mice are 345 days after the s.c. 3,4-BP injection still alive without tumors.

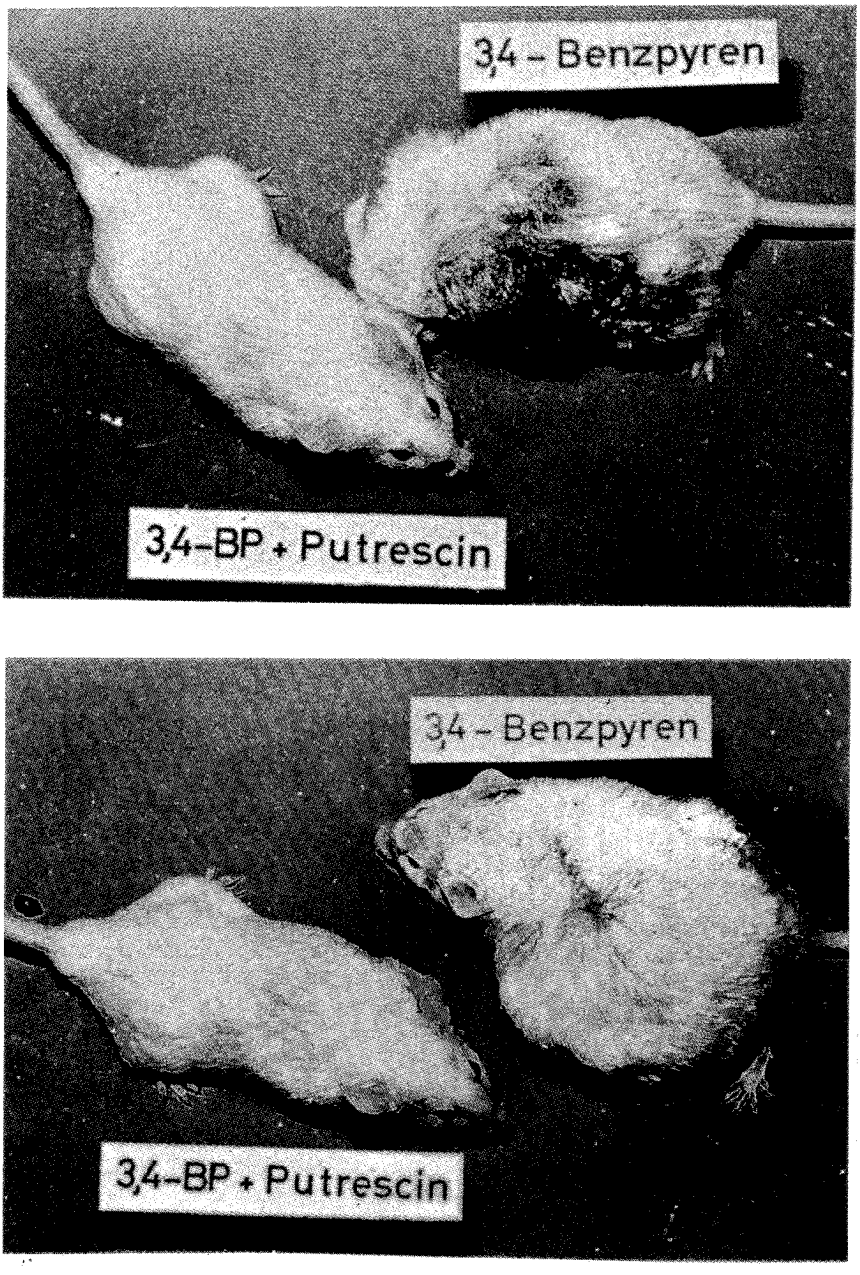


FIG. 3, 4. *Two characteristic photos demonstrating the inhibition of 3,4-Benzopyrene cancerogenesis in presence of Putrescine.*

right: Tumor development 6 months after a single s.c. injection of 2.52 mg 3,4-BP in 0.5ml tricapryline.

left: Complete inhibition of tumor development by adding 10 mg Putrescine to the injected 2.52mg 3,4-BP/0.5 Tricapryline solution.

TABLE II. *The anticancerogenic action of cis-Aconitic acid in relation to its concentration in 3,4-BP solution.* Decrease of 3,4-BP cancerogenesis from 100% to 18%, by increasing the amount of cis-aconitic acid from 0 to 30mg/2.52 mg 3,4-BP. (*Brackets = number of mice died due to toxicity, accidents, diseases, cannibalismus, etc. not considered for statistical evaluation. **Brackets = days of observation. Inhibition of 3,4-BP cancerogenesis in % calculated for the period of 345-375 days after injection.

Substance	Inject. amount in mg/0.5 ml Tricapryline		Nr of mice*	Nr of mice died within 7 months after 3,4-BP inj.		Nr of mice died after 7 months of 3,4-BP inj.		Nr of mice still alive**		3,4-Benzo-pyrene cancerogenesis in %
	add mg	3,4-BP mg		with tumor	no tumor	with tumor	no tumor	with tumor	no tumor	
1) Control (untreated mice)	—	—	28 (2)	—	—	—	—	—	28 (375)	0
2) 3,4-Benzopyrene (A)	—	2.52	30	30	—	—	—	—	—	100
3) 3,4-BP+cis-Aconitic acid (Group D)	<10 (sat)	+ 2.52	35	23	—	4	8	—	—	77
4) 3,4-BP+cis-Aconitic acid (Group C)	10 (alc)	+ 2.52	19 (1)	10	—	2	7	—	—	63
5) 3,4-BP+cis-Aconitic acid (Group B)	30 (alc)	+ 2.52	34	3	2	3	1	—	25 (345)	18

This observation demonstrates that with a suitable substitution some potential anticancerogens can be developed.

Other thiols, such as Dithiothreitol, Bismuthiol I., Mercaptosuccinic acid possess also a strong inhibiting effect against 3,4-BP cancerogenesis. (Figure 2, table I).

The strongest inhibitory effect against 3,4-BP cancerogenesis was achieved with Putrescine (1,4-Diaminobutane). From 30 mice treated with 2.52 mg 3,4-BP + 10 mg Putrescine, 2 mice died few days after the injection, probably due to the toxicity of Putrescine. (Injected amount of Putrescine 400-500 mg/kg). Three more mice died during the one year of observation without tumors. Only by one mouse died 361 days after the s.c. injection, a stomach tumor was found. This is also a very interesting finding because usually experimental tumors are developed locally in the place where the cancerogen is injected, and metastasis is rare. From the Putrescine treated group 24 mice are still alive without tumors.

The inhibition of the tumor development in presence of Putrescine is also demonstrated in two characteristic photos.

Putrescine also needs special attention in relation to the biological inactivation of cancerogenic agents, because it is formed as a metabolic product of bacteria in the digestive tract of different animal species, through the decarboxylation of ornithine.

In conclusion, the above mentioned experimental findings indicated that:

1.- The experimental 3,4-Benzopyrene cancerogenesis in mice can be postponed or completely inhibited by natural and synthetic compounds.

2.- As a result of the postponed tumor induction, as well as, the complete inhibition of tumor development, a prolongation of life of the mice treated with 3,4-Benzopyrene + anticancerogens, is possible.

3.- Some of the compounds tested are occuring in different animal species either physiologically, i.e. cis-aconitic acid, or are degradation products of bacteria present in their digestive tract, i.e. Putrescine. A biological inactivation of the cancerogenic action of 3,4-BP (and eventually other cancerogenic agents) with the above mentioned (and other) compounds in "cancerogenic resistant" animal species, is thus possible.

4.- Natural or synthetic compounds which are able to "inactivate" cancerogens, could be practically applied in different fields of pollution i.e. cigarettes, motors, etc. in order to prevent 3,4-Benzopyrene cancerogenesis.

Acknowledgment

I thank Dr. E. Fasske for the histological examination of the tumors.

Περίληψις

Ἔρευναι ἐπὶ τοῦ μηχανισμοῦ ἀδρανοποιήσεως τῆς καρκινογόνου δράσεως τοῦ 3,4-Βενζοπυρενίου.

Ἐπίδρασις διαφόρων σουλφυδρυλικῶν παραγώγων, ἀκορέστων ἀλιφατικῶν ὀξέων καὶ βιογενῶν ἀμινῶν διὰ τὴν παρεμπόδισιν τῆς 3,4-ΒΠ καρκινογενέσεως.

Εἰς λευκοὺς ποντικούς, ὑποδόριος ἔνεσις 2,52 mg 3,4-Βενζοπυρενίου (3,4-ΒΠ) προκαλεῖ κατὰ μέσον ὅρον μετὰ πάροδον τριμήνου τοπικῶς τὴν

δημιουργίαν ὄγκων εἰς ἀναλογίαν 95-100%. Ἀντιθέτως εἰς χοίρους καὶ ἀγελάδες, μεγαλύτεραι ποσότητες τῆς ἰδίας ἑνώσεως (145 καὶ 435 mg κατὰ ζῶον ἀντιστοίχως) μετὰ πάροδον 12-29 μηνῶν δὲν παρουσίασαν καμμίαν καρκινογόνον ἐνέργειαν.

Διὰ τὴν ἐξήγησιν αὐτῆς τῆς παρατηρήσεως, μεταξὺ τῶν ἄλλων πιθανοτήτων (ἡ δόσις τοῦ 3,4-ΒΠ καὶ ὁ χρόνος παρατηρήσεως δὲν ἦσαν ἐπαρκεῖς) ἐμελετήθη ἐπίσης καὶ ἡ περίπτωσις ἀδρανοποιήσεως τοῦ 3,4-ΒΠ «in vivo» ἀπὸ οὐσίας αἱ ὁποῖαι ἀπαντῶνται εἰς τὸν ὀργανισμὸν μεγαλυτέρων ζώων, καὶ πιθανῶς νὰ μὴν εὑρίσκονται εἰς τὰ μικρότερα ζῶα ἢ νὰ εὑρίσκονται εἰς ἐλαχίστας ποσότητας μὴ ἱκανὰς νὰ ἀδρανοποιήσουν πλήρως τὰς καρκινογόνους οὐσίας καὶ νὰ προστατεύσουν τὰ πειραματόζωα ἀπὸ τὴν καρκινογένεσιν.

Ἕνας πιθανὸς μηχανισμὸς ἀδρανοποιήσεως τῆς καρκινογόνου δράσεως τοῦ 3,4-Βενζοπυρενίου, εἶναι ἡ ἐλάττωσις τῆς πυκνότητος τῶν π-ἠλεκτρονίων κυρίως εἰς τὴν θέσιν Δ_6 τοῦ μορίου αὐτοῦ ὅπου ἀντιστοιχεῖ καὶ ἡ καλουμένη «καρκινογόνος περιφέρεια» μὲ ἑνώσεις αἱ ὁποῖαι δύνανται νὰ ἀναγάγουν ἐκλεκτικῶς τὸν διπλοῦν δεσμόν. Πρὸς τὸν σκοπὸν τοῦτον ἐμελετήθησαν ὡρισμέναι ἀντιδράσεις εἰδικῶς μὲ μερκαπτοπαράγωγα καὶ ἀκόρεστα ἀλιφατικὰ ὀξέα. Ἐκ τῶν πειραμάτων διεπιστώθη ὅτι μία σειρὰ ἑνώσεων αἱ ὁποῖαι εὑρίσκονται φυσιολογικῶς εἰς διαφόρους ὀργανισμούς, ὅπως π.χ. τὸ cis-ακονιτικὸν ὀξύ, εἴτε παρεσκευάσθησαν συνθετικῶς π.χ. Αἰθυλικὸς ἑστὴρ τῆς κυστεΐνης, Διθειοθρεϊτόλη, Μερκαπτοηλεκτρικὸν ὀξύ, Βισμουθειόλη, εἶναι εἰς θέσιν, προστιθέμενα εἰς διαλύματα 3,4-ΒΠ, νὰ ἐξασθενοῦν τὴν ἰσχυρὰν καρκινογόνον δρᾶσιν αὐτοῦ μέχρι τελείας ἀδρανοποιήσεως.

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SYNTHESIS AND MICROBIAL TRANSFORMATION OF 21-CHLOROPREGNA-4,16-DIENE-3,20-DIONE, 21-HYDROXYPREGNA-4,16-DIENE-3,20-DIONE PIVALATE AND 16α, 17α-EPOXY-21-HYDROXYPREGNA-4-ENE-3,20-DIONE PIVALATE

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(Received June 9, 1975)

Summary

The title compounds were synthesized in order to study the possibility of their microbial hydroxylation. Transformation of 21-Chloropregna-4,16-diene-3,20-dione occurred in poor yield. No hydroxylation was observed with 21-Hydroxypregna-4,16-diene-3,20-dione pivalate and 16a, 17a -epoxy-21-hydroxypregna-4-ene-dione pivalate.

Key words: Triamcinolone related compounds,
3β Hydroxy-pregna-5,16-dien-20-one-formate,
Pregna-5,16,20-triene-3β,20-diol-20-acetate, 3-formate,
21-Iodo-pregna-5,6-dien-3β-ol-20-one formate,
21-Hydroxypregna-4,16-diene-3,20-dione pivalate,
16α, 17α-epoxy-pregna-4-en-20-one-3,21-diol 21-pivalate,
16α, 17α-epoxy-21-hydroxypregna-4-ene-3,20-dione pivalate,
21-Chloro-3β-hydroxypregna-5,16-diene-20-one formate,
21-Chloropregna-4,16-diene-3,20-dione

Theoretical

The synthesis of Triamcinolone[1] (9a-fluoro-11b, 16a, 17a, 21-tetrahydroxy-1,4 - pregnadiene-3,20-dione) and related corticoids is of industrial interest, owing to their pharmaceutical value. Microbial transformation plays an important role in the production of corticoids. A possible sequence for the synthesis of Triamcinolone is illustrated in Scheme I. Alternatively microbial hydroxylation of 16a, 17a-epoxy-21-hydroxypregna-4-ene -3,20-dione acetate (9)[2] would afford a product which could be used for the synthesis of the key intermediate 8 of Scheme I. Early microbiological studies[8] have shown that 5 is easily hydroxylated at C_{11} and that at the same time the acetyl group is hydrolysed at C_{21} (5→6). However, it was also found that the transformation of steroid 9 was slow and incomplete.[8]

These observation prompted two lines of investigation. First, improvement in the hydroxylation of 9 by chemical modification at C_{21}. Secondly, the modification of 5 at C_{21} to improve the selectivity of acetylation in the hydroxylated product (avoid hydrolysis at this step since some difficulty having been experienced with step 6 to 7 in Scheme I). Steroids 10 and 11 were synthesized, having a bulky pivalyl group at C_{21} in place of the acetyl group. It was hoped thereby to improve hydroxylation at C_{11} and also avoid the accompanying microbial hydrolysis, thus making selective acetylation unnecessary (step 6 to 7). In addition,

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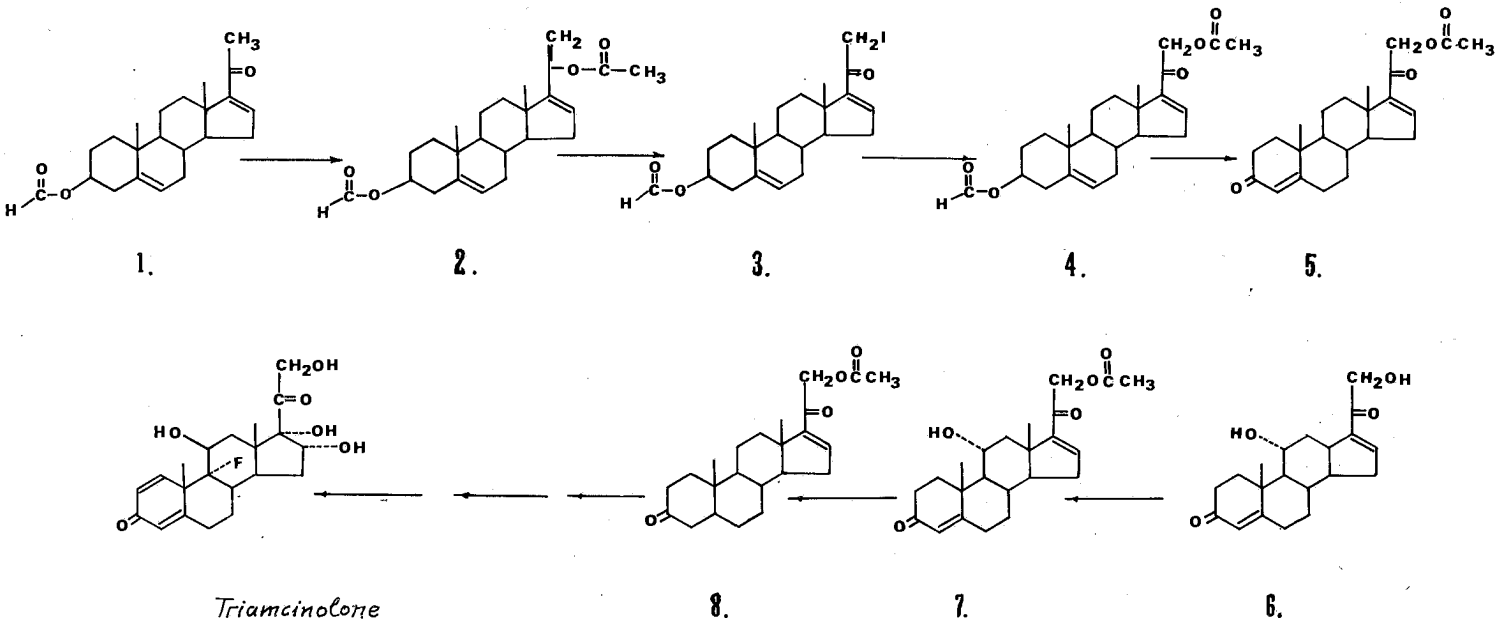
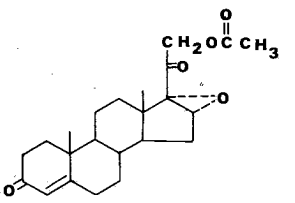


Scheme 1



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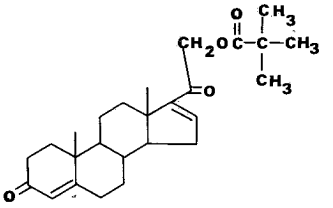

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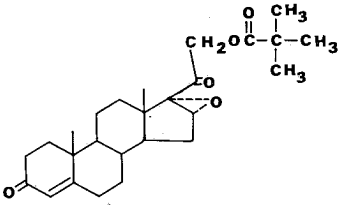

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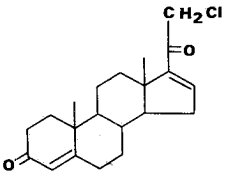

12.

21-chloropregna-4,16-diene-3,20-dione (12) was synthesized which, if microbiologically hydroxylated at C_{11}, should readily undergo later selective manipulations (i.e. acetylation) at C_{21}.

Results of the attempted microbial transformation: Steroids 10 and 11 were unaffected by microbial treatment which hydroxylated steroid 5, however, some transformation occured with 12[8]. More specifically, the condition used for the attempted 11a-hydroxylation were the following: Spores of *Aspergillus Ochraceus* at a concentration of 2×10^8 conidia per ml, substrate 0.5g/liter, phosphate buffer, 1% (pH 7) and incubation temp. 28° C.

Details of the "spore process" may be found in an excellent review paper (technique, advantages, variety of transformation) by Vezina Sehgal and Singh[7] who developped the process for large scale transformation.

Starting materials. 3β-Hydroxy-pregna-5,16-dien-20-one and 16α, 17α-epoxy-5-pregna-3β-ol-20-one acetates were used as starting materials.

Experimental

The procedure used for the synthesis of 10 was the same as that reported[2] for the synthesis of 9. Compound 11 and 12 were synthesized also by basically known procedures.[2,4,5,6]

3β-Hydroxy-pregna-5,16-dien-20-one- formate (1)

3β-Hydroxy-pregna-5,16-dien-20-one (1) (500 g) was heated with 4.5 l of acetoformic anhydride at 70° C. with stirring for 30 minutes. T.L.C. (20% of ethyl acetate in benzene) indicated that the formylation was complete. The reaction mixture was cooled, poured into 14 l of water and stirred for an additional 30 minutes. The precipitate was filtered, washed to neutrality with water and dried at 50° C. under vacuum yielding 540 g (98%) of material pure on T.L.C. m.p. 170-174° C. N.M.R. and I.R. were in agreement with the proposed structure. Recrystallization of a small amount from acetone gave an analytical sample m.p. 173-174.5° C.

Anal. calcd for $C_{22}H_{30}O_3$: C,77,15; H, 8.83, Found: C, 77.20; H, 8.82.

Pregna-5, 16, 20 -triene-3β, 20-diol 20 acetate, 3-formate (2)

A solution of pregna-5,16-dien-3-ol-20-one formate (96 g), *p*-toluene sulfonic acid (15.3 g; *p* -toluene sulfonic acid monohydrate, dried by dissolving it in benzene which was removed by distillation) and isopropenyl acetate (2.04 l -dried and redistilled) were placed in a 5 l 3-necked flask fitted with a 10-inch fractionating column. The solvent was slowly distilled for 12 hours with the volume maintained above 1.5 l by addition on fresh isopropenyl acetate as required. Afterwards a solution of 10 g of $NaHCO_3$ in 1 liter of H_2O was added slowly with stirring. When the evolution of CO_2 stopped, 1.25 l of water was added whereupon the steroid was precipitated. The precipitate was filtered, washed with water and dried at 50° C. in vacuum. Benzene (500 ml) was added to the two-phase filtrate. The organic phase was separated, washed with water (3 × 500 ml), dried and concentrated in vacuum at 40° C. The residue was slurried with methanol and filtered. The filtrate obtained was concentrated again, slurried, treated with methanol and filtered. The residue was washed with cold methanol yielding pure product m.p. 141-142° C (89g, 89%).

21-Iodo-pregna-5,6-dien-3β-ol-20-one formate (3).

Pregna-5,16, 20-triene-3β, 20-diol-20-acetate 3-formate (2 g) was dissolved in dioxane to which 1,3-diiodo-5,5-dimethylhydantoin (1.6 g) was added. The mixture was stirred under dry nitrogen and heated to 70° - 80° for one hour after which time the reaction was complete. Then it was cooled, treated with potassium iodide solution, followed by thiosulfate solution and water (equal volume to that of dioxane used in the reaction). The precipitate was collected by filtration, washed with water and dried in vacum at 40-50° C. yielding 2.35 g (97%) of the title product. Analytical sample m.p. 138°-9° C. (dec) was prepared by recrystallization from aceton. Spectral data were in agreement with the proposed structure.

Anal. Calcd. for $C_{22}H_{29}O_3I$:C, 56.45; H, 6.45% Found: C, 56.24; H, 6.19%.

21-Hydroxypregna-4, 16-diene-3,20-dione pivalate (10)

Δ5, 16-Pregnadiene-21-iodo-3-ol-20-one 3-formate (3 g) was dissolved in acetone and added dropwise to a mixture of sodium bicarbonate (4.6 g) and pivalic acid (4.7 g) in acetone (200 ml) which was preheated for a while (until the evolution of CO_2 ceased) at 40° C. After the addition was completed the mixture was refluxed and when the reaction was completed about (30 min) it was poored into water, filtered washed and dried, yielding 2.6 g of Δ5,16-pregnadiene-3,21-diol-20-one 3-formate-21-pivalate, m.p. 166-68° C. The latter product was dissolved in a mixture of toluene (150 ml) and cyclohexanone (23 ml). After some solvent was removed through a Dean-Stark water trap aluminum isopropoxide (2.15 g). dissolved in toluene was added. The mixture was refluxed for three hours, cooled and while still warm (50-70° C) an aqueous solution of 10% NaCl was added (50 ml) and filtered.

After separation of phases the organic phase was steam distilled. The residue of the steam distillation was extracted (with benzene and ether) and concentrated to an oil. Treatment of the oil with either yielded 1.6g of 21-hydroxypregna-4,16-diene-3,20-dione pivalate m.p. 155-156° C, as white crystals. The crystalline material was collected by filtration and the filtrate concentrated to an oil which chromatographed on neutral alumina (woelm, activity III, using benzene-hexane (1:1) as solvent) yielding an additional 500 mg of same product, m.p. 155-156° C. Total yield 2,1g (79.5%). Spectral data were in agreement with the proposed structure.

Anal. Cald for $C_{26}H_{36}O_4$:C, 75,68; H, 8.79% Found: C, 75.70; H, 8.69%.

16α, 17α-epoxy-pregna-4-en-20-one-3,21-diol 21-pivalate

A solution of 20g 16,17-oxido-5-pregnen-3β-ol-20-one acetate in 400 ml of acetic acid-carbon tetrachloride (1:1) chilled to 18° C., was treated with a solution of 8.6g of bromine in 60 ml of carbon tetrachloride. Upon complete decolorization. there was added 30 ml of 32% solution of hydrogen bromide in acetic acid. The solution let to stand for 10 min at room temperature then a second molar equivalent of bromine, 8.6g in 60 ml of CCl_4, was added portionwise at room temperature with stirring during a period of one hour. The reaction mixture was allowed to stand for an additional 20 minutes. Then evaporated in vacuo with minimum heating. The remaining was poured in water, filtered and the separated solid washed with water and dried overnight at 50° C. The solid 36 g was dissolved in 150 ml of benzene - 350 ml methanol mixture and then 10.4 g. of hydrogen bromide in 30 ml MeOH were added. The solution was allowed to stand at room temprature overnight. The reaction mixture was diluted with water and extracted with ether. The ethereal extracts were combined, washed with water, dried, concentrated to an oil and diluted with benzene. After the addition of 73 g of sodium iodide in 350 ml of absolute ethanol the mixture was allowed to stand at room temperature for 24 hours. It was then diluted well with water and extracted with ether. The extract was washed with 3% sodium thiosulfate solution, then water and dried. Ether was evaporated and the residue was dissolved in acetone and added dropwise to a mixture of 25 g $NaHCO_3$ and 30.6 g of pivalic acid, wich was preheated at 40° C. for a while. After the addition was completed the mixture was refluxed and when the reaction was completed (about 30 minutes) it was poured into 1.5 liter of water, filtered, washed and dried yielding 17.6 g of the title product (84% overall). The analytical sample was crystallized from ether and then from methanol - methylene chloride as follows: steroid dissolved on minimum amount of CH_2Cl_2 and then MeOH added. Methylene chloride was removed by boiling producing incipient cloudiness. At that point left for crystallization. Crystals so obtained treated with hot hexane and filtered, m.p. 204.5° - 206.5°.

Anal. Calcd for $C_{26}H_{38}O_5$: C, 72.57; H, 8.90
Found: C, 72.33; H, 9.00

16α, 17α-epoxy-21-hydroxypregna-4-ene-3,20-dione pivalate (11).

4-pregnen-16,17-oxido-20-one-3,21-diol-21-pivalate (2.5 g) was dissolved in a mixture of toluene (120 ml) and cyclohexanone (26 ml). After some solvent was removed through a Dean-Stark water trap 1.3 g of aluminum isopropoxide dissolved in toluene was added. Then the mixture was refluxed for two hours, cooled and while still warm (50-70° C.) aqueous solution of 10% NaCl was added (30 ml) and filtered. After separation of phases the organic phase was steam distilled. The residue of steam distillation extracted with benzene, dried over Na_2SO_4 and concentrated under vacuum to an oil. Treatment of the oil with ether yielded 2.5 g of crude product. This product was crystallized from ether and washed with hexane giving compound 11 (1.5 g) T.L.C. one spot (ethylacetate-benzene. 2:8). Further crystallization from benzene-hexane gave material with the following characteristics; m.p. 199.5-200.5° C, T.L.C. one spot, spectral data in agreement with the proposed structure for compound 11.

Anal. Calcd. for $C_{26}H_{36}O_5$:C, 72.54; H, 8.90 Found: C, 72,33; H, 9.00.

21-chloro-3β-hydroxypregna-5,16-dien-20-one formate

Pregna-5,16,20-triene-3β, 20-diol 20 acetate 3-formate (15 g) was dissolved in dioxane (300 ml) to which 1,3-dichloro-5,5 dimethyl hydantion (15 g) was added. The mixture was stirred under dry nitrogen and heated to 70° for 30 minutes,

by which time the reaction was complete. To the cooled reaction mixture water (150 ml) was added whereupon the steroid was precipitated. The precipitate was collected by filtration, washed with water, and then with small amount of methanol, and air dried on the funnel. Further drying in vacuo at 40° C. yielded 8.3 g (56%) of the title product m.p. 187-89° C. which was used without further purification for the next step of the synthesis.

21-chloropregna-4,16-diene-3,20-dione (12)

21-Chloro-3β-hydroxypregna-5,16-diena-20-one 3-formate was dissolved in a mixture of toluene (500 ml) and cyclohexanone (72 ml). After some solvent was removed through a Dean-Stark water trap 7.2 g of aluminum isopropoxide dissolved in toluene was added. Then the mixture was refluxed for two hours, cooled and while still warm (50°-70° C.), an aqueous solution of sodium chloride was added (160 ml) and filtered. After separation of phases the organic phase was steam distilled. The residue of the steam distillation extracted with methylene chloride dried over Na_2SO_4 and concentrated to solid residue. The residue recrystallized from acetone hexane to give compound 12, m.p. 164°-66° C. $(a)_D$ + 142.9° (55g. 72.4%).

I.R.-U.V. and N.M.R. confirmed its structure.

Anal. Calcd for $C_{21}H_{29}O_2Cl$: C, 72.71; H, 7.87; Cl, 10.02%
Found: C, 72.57; H, 8.03; Cl, 9.80%

Περίληψις

Σύνθεσις τῆς 21-χλωροπρέγνα-4,16-διεν-3,20-διόνης καὶ τῶν πιβαλικῶν ἐστέρων τῆς 21-ὑδροξυπρέγνα-4,16-διεν-3,20-διόνης καὶ τῆς 16α, 17α-εποξυ-21-ὑδροξυπρέγνα-4-εν-3,20-διόνης.

Ἡ σύνθεσις τοῦ κορτικοειδοῦς Triamcinolone, καὶ συναφῶν ἐνώσεων, παρουσιάζουν μεγάλο βιομηχανικὸν ἐνδιαφέρον λόγῳ τῆς φαρμακευτικῆς των ἀξίας. Κατὰ τὴν ἀναζήτησιν μεθόδου καὶ συνθηκῶν βελτιωμένης βιομηχανικῆς συνθέσεως τῆς Triamcinolone συνετέθησαν αἱ ὑπὸ τὸν τίτλον ἑνώσεις διὰ νὰ μελετηθοῦν ὡς ὑποστρώματα διὰ μικροβιακὴν ὑδροξυλίωσιν. Ὡς γνωστόν, ἡ μικροβιακὴ ὑδροξυλίωσις εἰς τὴν θέσιν 11α εἶναι οὐσιώδης διὰ τὴν καθόλου σύνθεσιν τῶν κορτικοειδῶν. Ἡ συντεθεῖσα 21-χλωροπρεγνα-4,16-διεν-3,20-διον ἔδωσε πτωχὰ ἀποτελέσματα ὑδροξυλιώσεως, ἐνῶ τὰ ἄλλα δύο στεροειδῆ οὐδεμίαν ὑπέστησαν μετατροπήν. Ἡ σύνθεσις τῶν ὑπὸ τὸν τίτλον ἑνώσεων ἐν τούτοις διελεύκανε ὡρισμένα ἐρωτηματικὰ ὡς πρὸς τὰς προϋποθέσεις δομῆς τοῦ ὑποστρώματος εἰς τὴν θέσιν C_{21} διὰ τὴν μικροβιακὴν ὑδροξυλίωσιν εἰς $C_{11\alpha}$. Αἱ παρατιθέμεναι μέθοδοι συνθέσεως εἶναι αἱ εὑρεθεῖσαι ὡς βέλτισται, ὄχι μόνον διὰ τὴν σύνθεσιν τῶν ὑπὸ τὸν τίτλον ἑνώσεων, ἀλλὰ καὶ διὰ μίαν βέλτιστον πορείαν συνθέσεως πρὸς τὴν Triamcinolone ὡς αὕτη δίδεται εἰς τὸ Σχ. 1.

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DETERMINATION OF TRUE KINETIC PARAMETERS OF A FIRST-ORDER SURFACE REACTION
An experiment on Heterogeneous Catalysis

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Summary

The method is described of obtaining *true* kinetic parameters in heterogeneous catalysis by using a simple, slightly modified, gas-chromatograph. Typical results are presented for the dehydrobromination of 1-bromo-2-methylpropane on alumina modified with 10% sodium bromide.
Key words: Kinetic parameters, surface reactions.

Introduction

It is well known that complicated experimental arrangements are usually required to carry out kinetic experiments on reactions of gases catalyzed by solid surfaces. Moreover, the kinetic parameters so determined are usually apparent, and not true, ones. The "stopped-flow technique", developed by Phillips and his co-workers,[1] is a comparatively new method which requires a very simple experimental set-up, namely, a slightly modified gas-chromatograph, and leads to the determination of *true* kinetic parameters. This technique, used by us,[2,3] proved to be an accurate and easy method for studying heterogeneous catalytic reactions, and can be carried out by students having the usual laboratory experience.

The average time required for the final kinetic experiment is 1 to 2 hrs. Using this technique the student, not only is acquainted with the concepts and the laws of chemical kinetics, but also is helped to distinguish between homogeneous and heterogeneous kinetics.

Theory

Suppose that a substance A, capable of undergoing a simple first-order reaction

$$A \xrightarrow{k} B + C, \qquad (1)$$

is injected into a gas-chromatographic column. If the column is filled with a solid, which acts both as a catalyst for the reaction and a separating phase of product B from the reactant A, the chromatographic trace on the recorder will look somewhat like Fig. 1. In this Figure it is assumed that the product B has a smaller retention time than the reactant A, and C is not detected (e.g. H_2O with a flame ionization detector).

The rate of the reaction, expressed as rate of formation of B, is

$$\frac{d\,(B)}{dt} = kf\,[(A)_o - (B)] \qquad (2)$$

where $(A)_o$ is the amount of A injected, (B) the amount of B produced for contact time t, k the rate constant for the reaction, and f the fraction of reactant molecules on the surface of the catalyst.

On integration, Eq. (2) gives the amount of B as a function of contact time t:

$$(B) = (A)_o\,(1-e^{-kft}) \qquad (3)$$

Eq. (3) is easily changed into a linear form to calculate k, provided (B), $(A)_o$ and t can be determined experimentally. The amount of B is relatively easy to measure, since at any point of the crhomatographic trace, say 0, (B) is proportional to the area under the curve of B (shown shaded in Fig. 1). The amount of A injected is of the order of μl for a liquid and this cannot be measured accurately. More difficult, however, is the determination of the contact time t corresponding to a certain point of the trace, say 0, because of the finite retention time of B on the column, and the assymetry of the peak of B owing to its continuous production from A.

These deficulties can be overcome by stopping the flow of the carrier gas through the column for a definite time Δt. On restoring the flow of the carrier gas, the amount Δ(B) of B produced during this time interval appears as an extra symmetrical peak "sitting" on the tail of the main peak of B, as shown in Fig. 2. If Δt is small compared with the half-life of the reaction, the quotient Δ(B)/Δt can be taken, as a first approximation, equal to the rate of formation d(B)/dt of the product B. The relation between the latter and the contact time can be found by differentiating* Eq. (3) with respect to t:

$$\frac{\Delta\,(B)}{\Delta t} \simeq \frac{d\,(B)}{dt} = (A)_o kfe^{-kft} \qquad (4)$$

Now, Δ(B) is proportional to the area under the curve of the extra "stop-peak" produced, Δt is a measurable quantity and t can be taken equal to the time corresponding to the middle of the interval Δt. Thus, by repeatedly stopping and restoring the restoring the flow of the carrier gas, the *rate* of the reaction can be determined as a function of time. This contrasts with most methods in chemical kinetics, where a *concentration,* rather than a rate, is measured as a function of time.

Inspection of Eq.(4) shows that a plot of the logarithm of the rate Δ(B)/Δt against t should give a straight line from the slope of which kf can be found. Having determined f, as described later, the true rate constant k of the surface reaction can be calculated. For constant stopped-flow intervals Δt, log[Δ(B)] can be plotted against t, since Eq.(4) gives

$$\log[\Delta(B)] = \log[(A)_o kf\Delta t] - \frac{kf}{2.303} \cdot t \qquad (5)$$

It is worth noting that the retention time of the stop-peaks, measured from the moment of each restoring of the carrier gas, does not remain constant, but decreases

*This derivation is different from the original one of Phillips et al.[1]

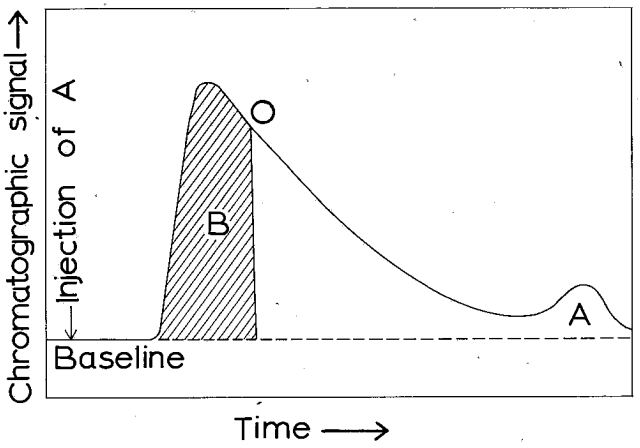

FIG. 1. *Gas chromatographic trace when a substance A undergoes a first-order reaction giving B, on a column which acts both as a catalytic reactor and as a chromatographic column for separating B from A.*

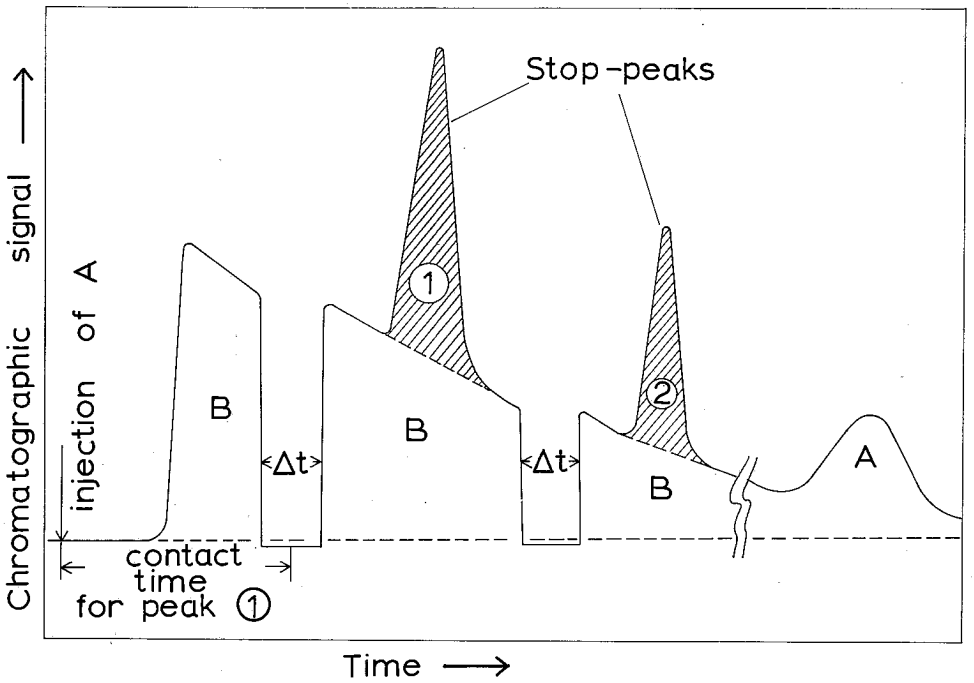

FIG. 2. *Gas chromatographic trace showing the stopping of the carrier gas through the column for time Δt, when after restoring it the extra "stop-peaks" 1 and 2 appear.* The shaded area is proportional to Δ(B).

with increasing number of stops, since the reactant A moves along the column, leaving less and less column lenght for chromatographing the product B.

The measurement of the retention time of the reactant permits the calculation of f, since it is equal to $[(V^{\circ}_R/V^{\circ}_M) - 1] / (V^{\circ}_R/V^{\circ}_M)$, V°_R being the corrected retention volume of the reactant and V°_M the dead volume of the column. The latter can be found by injecting a non-absorbed gas onto the column, such as air (using a thermal conductivity detector) or methane (when a flame ionization detector is employed).

In some cases the reacting substance is not eluted from the column, either because its retention volume is too large or because the rate of its decomposition is high. In both cases, the retention time of the reactant, necessary to calculate f, can be found indirectly by plotting the retention time of the stop-peaks versus the corresponding time from the injection moment to the middle of the relevant stop-flow interval Δt (after subtracting all previous stop intervals). A straight line results whose x intercept gives the retention time of the reactant A, since this corresponds to zero retention time of the product, i.e. to the time when A would be found at the exit of the column. The correctness of this method has been confirmed in many cases.[4]

Determination of true rate constants at various temperatures leads to the calculation of the true activation energy, E_a, and frequency factor, A, for the surface reaction, by the use of the well known Arrhenius equation:

$$\log k = \log A - \frac{E_a}{2.303R} \cdot \frac{1}{T} \qquad (6)$$

Experimental

Preparation of the catalyst: Aluminum oxide (active, acidic, Brockmann grade 1, for chromatographic analysis, BDH chemicals Ltd) was sieved, the 100-120 mesh fraction being retained. This (45 g) was added to an aqueous solution (35 ml) of sodium bromide (5 g). The resulting mixture was left in a beaker for a 24 hr period, following which the excess of water was evaporated on a steambath. The remaining material was spread on a filter paper and left in the air for 24 hr. It was then sieved and the 100-120 mesh fraction was used to fill a stainless steel gas-chromatographic column 60 cm long with an O.D. of 6.4 mm (¼″).

Apparatus and procedure: The above column was fitted into a conventional gas-chromatograph equipped with a single flame ionization detector (Pye-Unicam, Series 104). The whole epxerimental set-up is diagrammatically shown in Fig. 3.

The conditioning of the column was performed *in situ* by heating it for two successive 30 min periods at 100° and 200°C in a stream of carrier gas *(ca.* 15 ml/min). During the conditioning period the end of the column was disconnected from the detector to avoid contamination of the latter. The working temperature was then adjusted to 85.0°C, and four injections (0.5 μl each, every 15 min) were made of the reacting substance (1-bromo-2-methylpropane obtained either from Fluka AG or Merck AG) to establish constant catalytic activity. The kinetic experiments were performed after 8 hr standing at the working temperature.

The end of the column was reconnected to the detector, a small amount of methane was injected to determine the dead volume of the column, following which an injection (0.2 μl of the reacting substance was made to measure the rate of the dehydrohalogenation reaction.

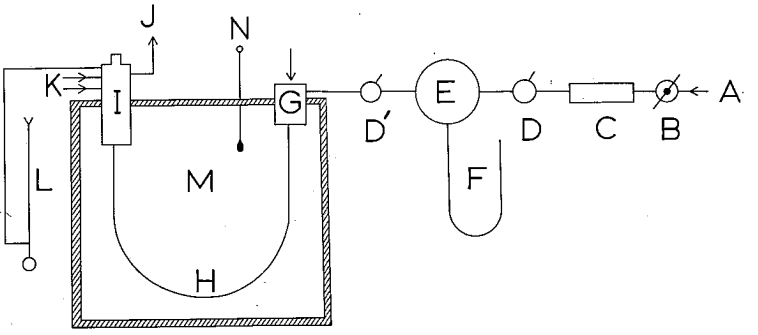

FIG. 3. *Schematic arrangement for kinetic studies.*

A: carrier gas (nitrogen, 99.98%) for the catalytic column, B: control valve, C: gas drying tube (molecular sieve 4A), D, D′: shut-off valves for closing and opening the carrier gas through the column, E: 500 ml volume reservoir to prevent pressure variations during closing and opening the gas flow, F: open manometer to detect pressure variations and measure the pressure drop along the catalytic column, G: injector to the column heated at oven temperature, H: catalytic column, I: flame ionization detector, K: hydrogen and air (dried) to the detector, J: signal to amplifier and recorder, L: buble flowmeter, M: gas-chromatograph oven (temperature stability better than ±0.1°C), N: calibrated mercury thermometer.

$$\underset{\text{(A)}}{CH_3{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}{-}CH_2{-}Br} \xrightarrow{k} \underset{\text{(B)}}{CH_3{-}\underset{\displaystyle CH_3}{\underset{|}{C}} = CH_2} + HBr \qquad (7)$$

The time of injection was recorded and after about 9 min commenced the elution of product 2-methylpropene (B), as shown in Fig. 2. The by-product hydrogen bromide was retained by the alumina catalyst, but even if it were not retained, it would not be detected by the flame ionization detector.

A series of 1 min stops of the carrier gas through the catalytic column was made by simultaneously closing and opening both valves D and D′ (see Fig. 3). The first stop started 24 min after the injection, while each following stop was made as soon as the elution of the previous stop-peak was complete. No reacting substance (A) appeared at this temperature owing to its great retention time.

The procedure was repeated at three other temperatures, after a few preliminary injections and a 5 hr period standing at each temperature.

Identification of the product-peaks was achieved by injecting small amounts of the four isomeric butenes onto the same column.

Typical Results

The chromatograms obtained as described show only one stop-peak initially. However, after a certain time three other smaller peaks appear after each stop of the carrier gas flow. The first of these is not a butene, but probably a product from an impurity. The second small peak is 2-methylpropene formed through isomerization of the reactant to 2-bromo-2-methylpropane, whilst the third peak (appearing after the main product) consists of a mixture of butenes. All these peaks are of insignificant size and need not concern us here.

The main product-peak was 2-methylpropene and by plotting the logarithm of the area $\Delta(B)$ of each stop-peak versus contact time t according to Eq.(5) a straight line is expected. Since the stop-peaks were all symmetrical and had the same width at their half-height, the logarithm of their height, h, rather than their area, was plotted versus t. Such a plot at 85.0°C is shown in Fig. 4. The remarkable linearity of this and similar plots, obtained at three other temperatures, shows that the experimental data are consistent with a simple first-order reaction.

The true rate constants of the surface reaction, calculated from the slopes of these plots (and the corresponding values of f) by standard least-squares procedures, are compiled in the Table.

TABLE: *Rate constanct of the dehydrohalogenation reaction of 1-bromo-2-methylpropane on 10% (w/w) $NaBr/Al_2O_3$ at four temperatures*

Temperature (°C)	$f \times 10^2$	$k \times 10^2$ (min^{-1})
79.4	99.86	1.26 ± 0.01
85.0	99.80	2.32 ± 0.02
89.9	99.76	3.98 ± 0.05
112.5	98.18	27.4 ± 0.1

The relevant Arrhenius plot is shown in Fig. 5. The calculated true activation energy and frequency factor are $E_a = 25.0 \pm 0.7$ kcal/mole, $\log A = 13.6 \pm 0.4$ (A in min^{-1}).

The only values of true kinetic parameters, found in the literature for the same reaction on analogous surfaces, are given below.

Catalyst	E_a (kcal/mole)	log A (A in min^{-1})	Reference
9.1% KBr/Al_2O_3	19.3	9.8	2
10% $NaCl/Al_2O_3$	29	14.5	3

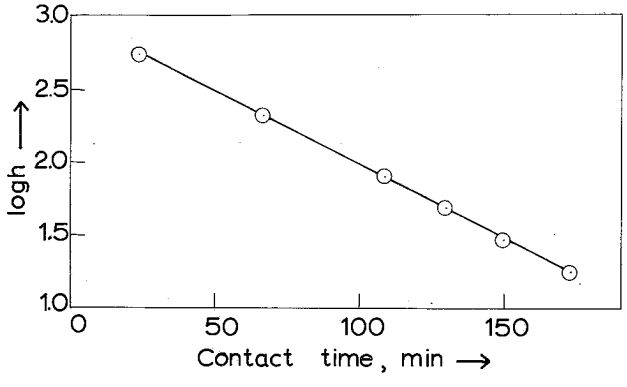

FIG. 4. *Kinetic run for dehydrohalogenation of 1-bromo-2-methylpropane on 10% (w/w) $NaBr/Al_2O_3$ at 85.0°C.*

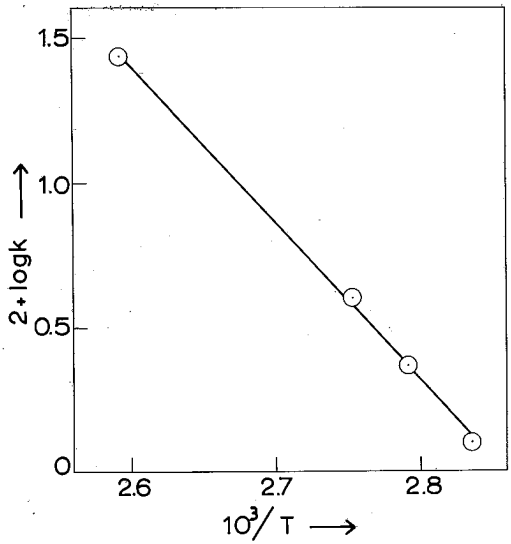

FIG. 5. *Arrhenius plot for dehydrohalogenation of 1-bromo-2-methylpropane on 10% (w/w) $NaBr/Al_2O_3$.*

Περίληψις

Προσδιορισμὸς ἀληθῶν κινητικῶν παραμέτρων ἐπιφανειακῶν ἀντιδράσεων πρώτης τάξεως.

Περιγράφεται ἡ μέθοδος, διὰ τῆς ὁποίας λαμβάνονται *ἀληθεῖς* κινητικαὶ παράμετροι ἑτερογενῶς καταλυομένων ἀντιδράσεων, τῇ χρήσει ἁπλοῦ, ἐλαφρῶς τροποποιηθέντος, ἀεριοχρωματογράφου. Παρουσιάζονται τυπικὰ ἀποτελέσματα διὰ τὴν ἀντίδρασιν ἀφυδραλογονώσεως τοῦ 1-βρωμο-2-μεθυλοπροπανίου ἐπὶ ὀξειδίου τοῦ ἀργιλίου, τροποποιηθέντος διὰ βρωμιούχου νατρίου.

References and Notes

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Chimika Chronika, New Series, 5, 145-151 (1976)

DEPTH PROFILING OF METAL - SILICON INTERFACES BY ION ETCHING AND AUGER ELECTRON SPECTROSCOPY.

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Summary

Gold films have been evaporated under ultra high vacuum (u.h.v) conditions on u.h.v freshly cleaved silicon (111) surfaces. Depth profiling of these metal-silicon interfaces has been carried out using simultaneously the technique of argon ion beam etching and the technique of Auger electron spectroscopy. Calibration of the sensitivity of Auger spectroscopy has been achieved by depth profiling studies of gold contacts to silicon which has been cleaved and deliberately contaminated with a monolayer of oxygen. No atoms other than those of gold and silicon have been detected at the interface between the gold film and the u.h.v freshly cleaved silicon surface. This result is the most direct evidence that metal contacts to u.h.v freshly cleaved silicon are indeed intimate.

Key words: Thin-films, Surfaces, Adsorption, Contacts, Schottky-barriers, Contamination, Devices.

Introduction.

Results on metal contacts to ultra high vacuum (u.h.v) freshly cleaved silicon surfaces published recently by Thanailakis[1,2] appear to confirm the Heine[3] theory about the effect of metal wavefunction tails on the potential barrier existing at a metal — semiconductor interface. Those results have also shown that about a monolayer of adsorbed residual gas atoms on cleaved silicon surfaces can lead to metal — silicon contacts with substantially different properties. It is, therefore, very important that the degree of contamination at a metal — semiconductor interface is known as accurately as possible.

Measurements of the work function of u.h.v. cleaved silicon surfaces as a function of their exposure time to the residual gases in a u.h.v. chamber provide a measure of their contamination before the metal evaporation (Thanailakis[1]). Surface analysis of the cleaved silicon specimens using Auger electron spectroscopy (Chang[4]) gives information about the type and degree of surface contamination down to a very small fraction of a monolayer again before the metal evaporation. Depth profiling studies, however, using simultaneously the technique of argon ion beam etching and the technique of Auger electron spectroscopy, as described by Palmberg,[5] enable information about the nature and amount of contamination at metal — semiconductor interfaces to be obtained after the contacts have been made.

In this paper are presented, for the first time as far as we know, experimental results on the depth profiling of u.h.v. prepared contacts of gold films to freshly cleaved silicon specimens, and also to silicon specimens which have been cleaved and deliberately contaminated with oxygen to about a monolayer coverage.

Experimental.

Silicon single crystal specimens were cleaved along the (111) plane in a u.h.v. system at a base pressure of about 5×10^{-11} Torr. The cleaved surfaces with dimensions 5 mm × 3.5 mm exhibited the step-like topography discussed by Henzler.[6] This step-like topography has an adverse effect on the resolution of the depth profiling method as discussed later in this paper. Immediately after cleavage gold films were evaporated to cover part or the whole area of the freshly cleaved silicon surfaces. The base pressure during gold evaporation was in the range of 10^{-10} Torr. The specimens were then taken outside the u.h.v. chamber and stored in air for several weeks before they were transferred in to another u.h.v chamber fitted with the depth profiling apparatus.

Depth profiling was achieved using simultaneously the technique of argon ion beam etching, in which material is continuously removed from the specimen surface by a focused ion beam, and the technique of Auger electron spectroscopy, in which the Auger spectrum of the freshly revealed surface of the specimen is repeatedly plotted on an x-t recorder. The experimental arrangement for the depth profiling studies is shown in Fig. 1. The energy of the argon ion beam was 1 KeV and the peak current density in the ion beam was 6.2 $\mu A/cm^2$. The FWHM of the ion beam was 6.0 mm and the angle of incidence was 73°. The Auger electron analysed area, as defined by the incident electron beam, was an ellipse of minor axis 0.7 mm and major axis 2.0 mm. A Faraday cup assembly was used to ensure that the area of the specimen being Auger electron analysed coincided with the centre, and therefore the most flat-bottomed part, of the etch pit. The ion impingement rate on the region of the specimen being analysed was 1.1×10^{13} Ar^+/cm^2 - sec.

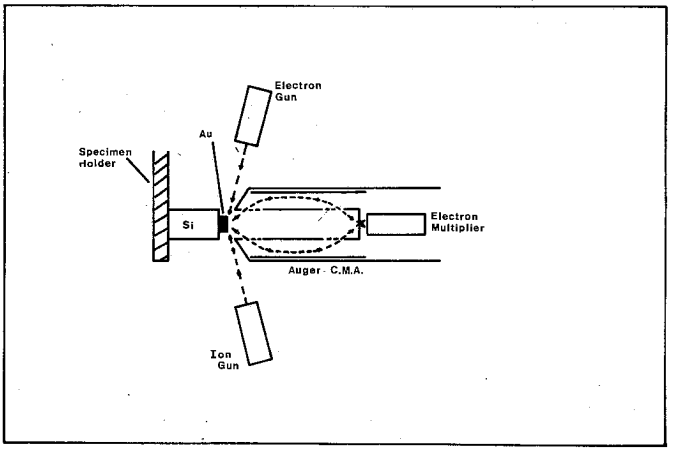

FIG. 1. *Experimental arrangement for simultaneous ion beam etching and Auger electron spectroscopy.*

The u.h.v system with the gold-silicon contact mounted on its holder inside the chamber was initially pumped down to about 3×10^{-9} Torr using an ion pump, but it was not baked out to avoid any possible metallurgical changes at the gold-silicon interface. Then the ion pump was switched off and a steady argon pressure of 5×10^{-5} Torr was maintained in the chamber by means of a liquid nitrogen - trapped mercury diffusion pump.

The energy of the incident electron beam (for the Auger electron spectroscopy, see Fig. 1) was 1.5 KeV and the current density of this beam was about 300 $\mu A/cm^2$. A cylindrical mirror analyser fitted with an electron multiplier (Fig. 1) was used to energy analyse and detect the Auger electrons emitted from the surface atoms of the Auger electron analysed area of the specimen.

Results and Discussion

The Auger electron spectrum of the free surface of gold films, before the ion etching commenced, consisted of carbon, sulphur, gold and oxygen peaks. The carbon, sulphur and oxygen peaks were due to atoms adsorbed on the gold films during their exposure to air before the specimens were placed in the u.h.v chamber. The adsorbed layer was of the order of a monolayer and it was sputtered off within a minute or so after the argon ion gun was switched on, as this was indicated by the disappearance of the corresponding Auger peaks. At the same time the heights of the gold Auger peaks increased to their saturation values and remained constant until the gold-silicon interface was reached at a particular location of the Auger electron analysed area, when peaks corresponding to silicon atoms were also observed.

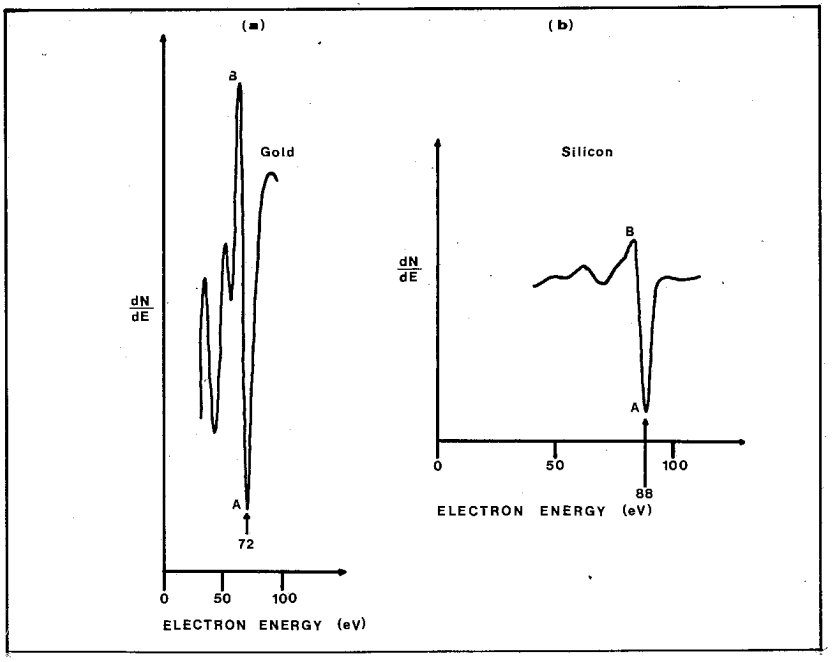

FIG. 2. *a) Part of the Auger electron spectrum of gold. b) Part of the Auger electron spectrum of silicon.* (The dN/dE scale for silicon is compressed by five times with respect to that for gold).

For each element of the Periodic Table there are several Auger transitions, and hence several peaks at different energies are observed in the derivative Auger electron spectrum (Chang[4]). In Fig. 2 (a) and (b) are shown only parts of the Auger electron spectra for gold and silicon respectively. They include the main 72 - eV gold Auger peak and the 88 -eV silicon Auger peak which have been chosen for the measurements of this paper. On the assumption that the etching rate is constant and that the peak to peak heights in the derivative Auger electron spectrum (i.e. the distance from A to B in Fig. 2) are proportional to atomic concentrations in the surface layer, a plot of Auger peak to peak heights against time is equivalent to atomic concentration against depth.

After the gold-silicon interface at a particular point of the Auger electron analysed area was reached, the gold Auger peak was found to decrease gradually to zero and the silicon Auger peak to increase gradually to its saturation value corresponding to the case where all traces of gold had been sputtered off. The Auger electron analyser was operated at its maximum sensitivity and care was taken to scan through a sufficiently wide range of energy to cover Auger transitions of all elements in the Periodic Table. No atoms other than those of gold and silicon have been detected at the interfaces between gold films and u.h.v freshly cleaved silicon surfaces.

Typical results on the depth distribution of gold and silicon atoms at the gold — u.h.v freshly cleaved silicon interfaces are shown in Figure 3. The depth scale is about 5 Å/min. The width of overlap region in Figure 3 (defined as the distance between the two points along the depth axis which correspond to 10% and 90% of the saturation value of the gold Auger peak to peak signal respectively) corresponds to about 20% of the total thickness of the gold film. This compares with the corresponding value of 10% which has been obtained from similar studies of gold contacts to chemically etched silicon surfaces. This relatively poor resolution for

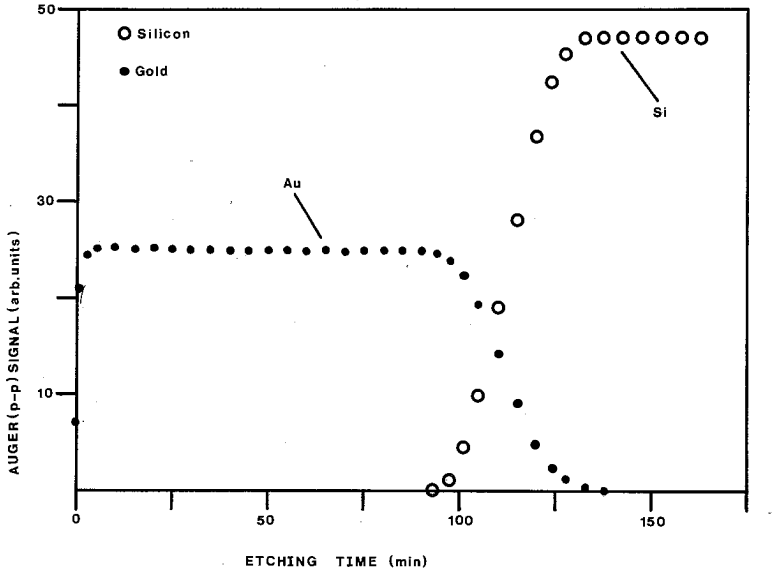

FIG. 3. *Depth distribution of gold and silicon atoms at the interface between gold and u.h.v freshly cleaved silicon.* The depth scale is about 5 Å/min.

cleaved silicon surfaces is due to the fact that these surfaces are rough on a microscopic scale, as it has been reported by Henzler.[6]

To establish the lower limit of contamination which could be detected in the above experiments, depth profiling studies were carried out on gold contacts to oxygen contaminated u.h.v cleaved silicon surfaces prepared as follows:
Silicon single crystal specimens were cleaved, and then they were deliberately contaminated to about a monolayer coverage by letting high purity oxygen gas into the u.h.v chamber in a controlled fashion. The adsorption of oxygen on to cleaved silicon was monitored by measuring the adsorption-induced changes of the silicon work function. The work function against exposure of cleaved silicon to oxygen gas was calibrated against an experimental plot of silicon exposure to oxygen versus surface coverage (expressed in units of atomic layers) obtained by Auger electron analysis of the silicon surface (not the gold-silicon interface).

Depth profiling studies of gold contacts to cleaved silicon surfaces which had been contaminated with a monolayer of oxygen showed the existence of oxygen atoms at the gold-silicon interface. No atoms other than those of gold, oxygen and silicon were detected at the metal-semiconductor interface. Typical results on the depth distribution of gold, oxygen and silicon atoms at the gold-oxygen contaminated cleaved silicon interfaces are shown in Figure 4. It is obvious from this plot that contamination at the gold-cleaved silicon interface to a degree of 1/10 of a monolayer or less can be detected using the technique of simultaneous ion beam etching and Auger electron spectroscopy. Comparison of Figures 3 and 4 shows that there is no significant contamination at the interface of u.h.v prepared metal contacts to freshly cleaved silicon surfaces. Therefore, contacts prepared in this way are trully intimate, and the modern theories of metal-semiconductor contacts proposed by Heine[3] and Inkson [7,8] can be safely applied.

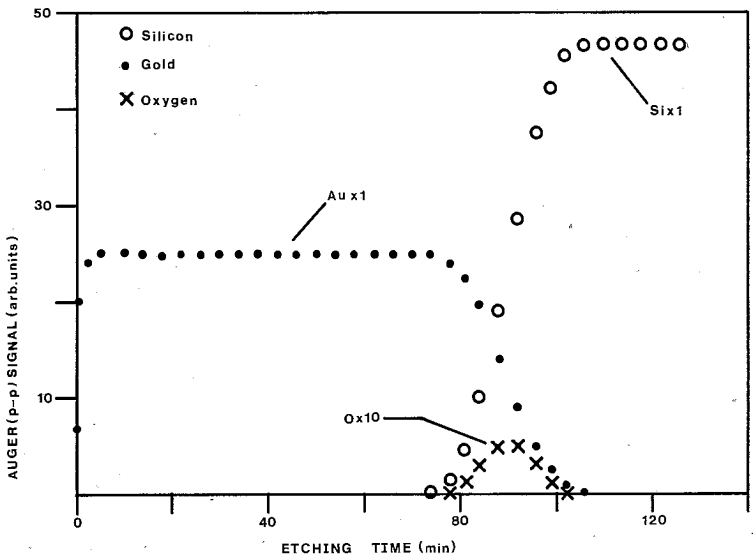

FIG. 4. *Depth distribution of gold, oxygen and silicon atoms at the interface between gold and u.h.v cleaved silicon surface, which prior to metal evaporation was contaminated with a monolayer of oxygen.* The depth scale is about 5 Å/min.

It should be pointed out that the cleaved silicon substrates were in all cases kept at room temperature during the oxygen adsorption and the metal evaporation, so that significant interdiffusion of gold and silicon cannot occur. Therefore, the overlap region in Figures 3 and 4 is due mainly to limitations on the in-depth resolution of the technique imposed by the unevenness of the cleaved silicon substrate combined with the relatively large cross-sectional area of the grazing incidence electron beam. To improve the in-depth resolution of the technique in the present case of cleaved silicon surfaces it is necessary to use an electron beam of cross-sectional diameter of the order of a micron or less at normal incidence.

Conclusions

The technique of simultaneous argon ion beam etching and Auger electron spectroscopy can be used to detect contamination at the interface between metals and cleaved silicon surfaces to a degree of about 1/10 of a monolayer. Depth profiling studies of gold contacts to u.h.v freshly cleaved silicon surfaces using this technique revealed the existence of only gold and silicon atoms at the interface. This result is the most direct evidence that metal contacts to u.h.v freshly cleaved silicon are indeed intimate, and therefore results obtained from studies of such contacts can safely be used to compare with the predictions of the most recent theories of intimate metal-semiconductor junctions.

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Περίληψις

Προσδιορισμὸς τῆς κατανομῆς τῶν διαφόρων ἀτόμων εἰς τὴν διαχωριστικὴν ἐπιφάνειαν μετάλλου-πυριτίου, διὰ τοῦ βομβαρδισμοῦ τῆς ἐπιφανείας ὑπὸ δέσμης ἰόντων καὶ τῆς ἠλεκτρονικῆς φασματοσκοπίας AUGER.

Ὑμένια χρυσοῦ ἀπετέθησαν δι' ἐξαχνώσεως ὑπὸ συνθήκας ἐξαιρετικῶς ὑψηλοῦ κενοῦ ἐπὶ ἐπιφανειῶν (111) πυριτίου, αἱ ὁποῖαι ἐσχηματίσθησαν διὰ προσφάτου σχισμοῦ ὑπὸ τὰς ἀνωτέρω συνθήκας κενοῦ. Ἡ μελέτη τῆς κατανομῆς τῶν διαφόρων ἀτόμων κατὰ μῆκος τῆς διευθύνσεως καθέτως πρὸς τὴν διαχωριστικὴν ἐπιφάνειαν τῶν οὕτω σχηματισθεισῶν ἐπαφῶν χρυσοῦ-πυριτίου ἐγένετο τῇ χρήσει τῆς μεθόδου τῆς συνεχοῦς ἀπομακρύνσεως ἐπιφανειακῶν ἀτόμων, ἡ ὁποία ἐπιτυγχάνεται διὰ βομβαρδισμοῦ τῆς ἐλευθέρας ἐπιφανείας ὑπὸ δέσμης ἰόντων τοῦ ἀδρανοῦς ἀερίου ἀργοῦ, καί, ταυτοχρόνως, τῇ χρήσει τῆς ἠλεκτρονικῆς φασματοσκοπίας Auger, ἡ ὁποία προσδιορίζει τὸ εἶδος καὶ τὸν ἀριθμὸν τῶν διαφόρων ἀτόμων ἐπὶ τῆς ἑκάστοτε ἐλευθέρας ἐπιφανείας. Ἡ βαθμολογία τῆς εὐαισθησίας τῆς φασματοσκοπίας Auger ἐπετεύχθη δι' ἐκτελέσεως παρομοίων πειραμάτων ἐπὶ σχισμογενῶν ἐπιφανειῶν πυριτίου, αἱ ὁποῖαι πρὸ τῆς ἐπ' αὐτῶν ἀποθέσεως τῶν ὑμενίων χρυσοῦ εἶχον σκοπίμως καλυφθῆ ὑπὸ ἑνὸς ἀτομικοῦ στρώματος ὀξυγόνου. Τὸ φάσμα Auger τῆς διαχωριστικῆς αὐτῆς ἐπιφανείας περιελάμβανε καὶ ἠλεκτρόνια ἐνεργείας χαρακτηριστικῆς τῶν ἀτόμων ὀξυγόνου, ἐπιτρέπον τοιουτοτρόπως τὴν

βαθμολογίαν τῆς μεθόδου. Τὸ βασικὸν συμπέρασμα τῆς ἐργασίας αὐτῆς εἶναι ὅτι ὁ βαθμὸς δηλητηριάσεως τῆς διαχωριστικῆς ἐπιφανείας τῶν, ὑπὸ συνθήκας ἐξαιρετικῶς ὑψηλοῦ κενοῦ, σχηματισθεισῶν ἐπαφῶν λεπτῶν ὑμενίων χρυσοῦ μετὰ προσφάτως σχισθέντων δειγμάτων πυριτίου, εἶναι κατὰ πολὺ χαμηλότερος τοῦ 1/10 τοῦ ἑνὸς ἀτομικοῦ στρώματος. Τὸ ἀποτέλεσμα τοῦτο ἀποτελεῖ τὴν πλέον ἄμεσον ἀπόδειξιν ὅτι αἱ ἐπαφαὶ αὗται εἶναι ὄντως συναφεῖς.

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COMPOSITION AND DISTRIBUTION OF LIPIDS IN THE TISSUES OF TRITURUS CRISTATUS*

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Summary

Total lipids from brain, liver, skin and muscle tissue of *T. cristatus* were separately isolated and fractionated by silicic acid chromatography into four lipid classes (neutral lipids, glycolipids, sulfolipids, phospholipids). The phospholipid fractions were further fractionated into six individual phospholipid fractions.

Each of the nine fractions so obtained was submitted to systematic analysis by quantitative determinations of characteristic residues and groups of the lipid molecules, as well as by thin layer chromatography. All the lipid classes examined were found to contain considerable amounts of glycerylether analogs and — with the exception of muscle phospholipids — significant amounts of plasmalogens.

A general figure of the relative turnover rates of lipids in the studied tissues of *T. cristatus* was also obtained by specific radioactivity measurements of individual lipid fractions 24 hours after [1^{-14} C] palmitate injection.

Introduction

In spite of the wide use of amphibians as experimental animals in studies on regeneration and other investigations, the lipids have been largely neglected among the molecular constituents of their tissues.

There is a brief note by Hess[1] on the distribution of unsaturated fatty acids in the regenerating tail of *Xenopus laevis* tadpoles and a series of mainly histochemical observations by Schmidt[2-9] on the neutral fats, fatty acids, phospholipids, cholesterol and lipoproteins in regenerating amphibian tissues.

Reported in this paper is the composition and distribution of neutral lipids, glycolipids and phospholipids in the main tissues of the normal adult salamander *Triturus cristatus,* namely of its liver, brain, skin and muscle.

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Experimental Procedures

Analytical Methods.

Phosphorus, acyl esters, plasmalogens, glycerol, inositol, choline, ethanolamine and serine were assayed according to the methods described by Dittmer and Wells.[10] Hexose was determined by the direct phenol-sulphuric acid method previously described.[12] Cross-determination of glyceryl - ether and plasmalogen content of phospholipid fractions were also carried out by phosphorus assays on hydrolysates prepared by the selective hydrolysis procedures effectively exploited by Dawson[13] and Dawson *et al.*[14] Quantitative determinations of individual phospholipids in phospholipid mixtures or in total lipid extracts were carried out in some instances by phosphorus assays after T.L.C. separation by a simple and accurate procedure devised in this laboratory.[15] According to this procedure, digestion with perchloric acid is carried out on the silica gel portions of the plates corresponding to individual phospholipid spots and after development of the phosphomolybdenum-blue color, the color is extracted with ethyl acetate and its optical density is measured in 820 mμ.

Extraction and Fractionation of Lipids.

The experimental animals *(T. cristatus)* were of Italian origin, kept alive by feeding them thrice a week with ox heart.

Their skin, liver, muscle tissue and brain were dissected in this order after the animals were anaesthesized. Immediately they were mixed with chloroform-methanol, 1:1(v/v) and homogenized in an Omni-Mixer homogenizer (Ivan Sorvall, Inc.) for 3-5 min at 8-10,000 rpm, while kept in ice bath. Extraction of total lipids was continued according to the method of Bligh and Dyer.[16]

The total lipid extracts so obtained were submitted to silicic acid column chromatographic fractionation of lipids in classes, by elution as follows: (a) Eight bed volumes of chloroform plus two bed volumes of chloroform-methanol, 19:1 (v/v) (Neutral Lipid Fraction), (b) eight volumes of chloroform -acetone, 1:1 (v/v) (Glycolipid Fraction), (c) eight bed volumes of acetone (Sulfolipid Fraction) and (d) eight bed volumes of methanol (Phospholipid Fraction).

Phospholipid fractions were further resolved into six fractions by rechromatography on silicic acid columns, eluted respectively with: (1) Chloroform, (2) Chloroform-methanol, 19:1 (v/v), (3) Chloroform-methanol, 4:1 (v/v), (4) Ethyl acetate-methanol, 3:2 (v/v), (5) Choroform-methanol, 1:1 (v/v) and (6) Chloroform-methanol, 1:9 (v/v).

Thin layer chromatographic analysis was performed on silica gel G (0.25 mm thick), developed with the following solvent systems: (a) Petroleum ether-diethylether - acetic acid, 90:10:1 and 70:30:1 (v/v/v) for neutral lipids, (b) Chloroform-methanol-water, 90:10:1 (v/v/v) for glycolipids and sulfolipids and (c) Chloroform-methanol-water, 95:35:4 (v/v/v) for phospholipids. Localization of spots was effected by exposure to iodine vapors, by charring at 100° after spraying with 50% sulfuric acid) and by using the molydbenum-blue[17] and ninhydrin spray reagents for detection of phospholipids and aminolipids respectively.

Results and Discussion

The total phospholipid content of the studied tissues of *T. cristatus* was found as expected, quite different from one another. Namely, the total lipid-phosphorus values in mg/gr of wet tissues were found 1.13 for liver, 0.5 for brain, 0.06 for

muscle and 0.23 for the skin.

Thin chromatographic separation of the above total lipid extracts revealed a rather non-uniform distribution of lipids in the four tissues studied (Fig. 1). To this conclusion lead also the results of the class-separation of total lipids by silicic acid column chromatography and the corresponding analytical data depicted in Table 1. A figure for the relative neutral lipid, glycolipid, sulfolipid and phospholipid content of each tissue is given in the last column of Table 1.

As shown in Table I, all the lipid classes of the four tissues studied contain considerable amounts of glyceryl-ether analogs. Significant is also the plasmalogen content of the phospholipid fractions with the exception of muscle phospholipids.

The main constituents of the neutral lipid fraction of all tissues studied are triglycerides and cholesterol esters, as indicated by thin layer chromatographic analysis. Only minor amounts of free cholesterol occur decreasing in the order: skin, liver, muscle, brain.

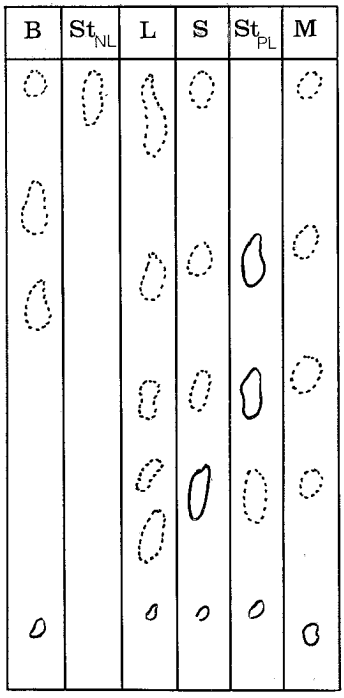

FIG. 1 *A drawing of the thin - layer chromatographic separation of total lipid extracts from brain (B), liver (L), skin (S) and muscle tissue (M) of T. CRISTATUS.* St_{NL} = Neutral - lipid standard, composed of triolein, oleic acid, cetyl alcohol, cholesterol and selachyl alcohol. St_{PL} = Phospholipid standard, composed of phosphatidylethanolamine, phosphatidylserine plus phosphatidylinositol, phospatidylcholine and sphingomyelin (from up to down).
Solvent system: Chloroform-methanol-water, 95:35:4 (v/v/v).

Thin layer chromatographic analysis of the glycolipid and sulfolipid fractions revealed the presence of at least two glycolipids and two sulfolipids in mixture with residual mono-and diglycerides. Further investigation on these fractions was not carried out owing to the low quantities isolated.

The phospholipid fractions were investigated in more detail, starting with a silicic acid column chromatographic fractionation into six fractions. Each was then submitted to thin layer chromatographic analysis and to a series of analytical determinations of the main building stones of lipid molecules (Table 2).

By combining all these data, an overall composition for each of the phospholipid fractions of the four tissues of *T. cristatus* investigated is summarized in Table 3.

As shown in Table 2, variable amounts of glyceryl ether and plasmalogen analogs occur in most individual phospholipid fractions. Another point of interest, not indicated in Table 3, is that about 1/3 of the phosphatidylethanolamine fraction from liver was very tentatively characterized as either N-methyl- or N-dimethyl-phosphatidylethanolamine, on the basis of the following data:

(a) The thin layer chromatographic mobility of the component in chloroform-methanol-water, 95:35:4 (v/v/v) is slightly higher than that of phosphatidyl serine.

(b) It appears as a normal, well-defined spot without any tailing effect, which indicates a non-acidic nature.

(c) With ninhydrin spray it reacts very slowly, yielding a faint spot.

In order to obtain a general figure concerning the relative turnover rates of the lipids in the tissues of *T. cristatus*; (1^{-14} C)-palmitic acid in saline solution was administered intraperitoneally into two animals and the tissues were separated and extracted by the same procedure, described above, 24 hours after the

TABLE I: *Analytical Data of the Lipid Classes of the Tissues of Triturus Cristatus.*

Lipid Class	Lipid phosphorus (μmoles)	Acyl esters (μEq)	Glyceryl ethers (μmoles)	Hexose (μmoles)	Plasma-logens (μmoles)	Esters (% of total per tissue)
LIVER						
Neutral Lipids	—	42	9.3	—	—	25.2
Glycolipids	—	40	10.1	10.9	—	24.0
Sulfolipids	—	3.8	8.7	2.5	—	2.3
Phospholipids	37	81	11.1	—	4.9	48.5
BRAIN						
Neutral lipids	—	14.5	1.1	—	—	30.5
Glycolipids	—	15.7	1.3	1.2	—	33.1
Sulfolipids	—	4.1	1.6	1.0	—	8.6
Phospholipids	3.7	7.9	1.2	—	2.2	27.8
MUSCLE						
Neutral Lipids	—	27	5.6	—	—	19.2
Glycolipids	—	31	6.2	7.1	—	22.0
Sulfolipids	—	8.1	7.2	4.6	—	5.8
Phospholipids	39	68	6.5	—	0.7	53.0
SKIN						
Neutral Lipids	—	51	4.0	—	—	33.7
Glycolipids	—	32	3.8	1.8	—	21.0
Sulfolipids	—	8	5.1	1.1	—	5.3
Phospholipids	42	72	16.0	—	6.0	40.0

TABLE II: *Analytical Data of Individual Phospholipid Fractions Obtained by Re-chromatography on Silicic Acid Columns (see text)*

Assay		Fraction Number 1	2	3	4	5	6
LIVER							
Lipid phosphorus	(μmoles)	0.3	19.3	1.6	5.0	4.8	1.0
Lipid phosphorus	(%)	0.9	60.4	5.0	15.6	15.0	3.1
Plasmalogens	(μmoles)	0.8	1.2	1.1	0.7	0.6	0.5
Esters	(μEq)	19.7	37.7	2.0	10.2	9.7	0.6
Glyceryl ethers[a]	(μmoles)	2.8	3.2	1.0	2.4	0.5	1.2
Glycerol	(μmoles)	9.5	16.8	2.8	5.6	3.8	—
Inositol	(μmoles)	—	—	0.3	1.6	—	—
Choline	(μmoles)	—	—	—	2.6	4.4	0.95
BRAIN							
Lipid phosphorus	(μmoles)	0.13	0.71	0.09	1.9	0.71	0.15
Lipid phosporus	(%)	3.2	17.8	2.2	47.4	17.7	3.7
Esters	(μEq)	0.8	1.4	0.2	3.9	1.5	0.1
Inositol	(μmoles)	—	—	0.06	0.19	—	—
Choline	(μmoles)	—	0.31	0.28	0.20	0.42	—
MUSCLE							
Lipid phosphorus	(μmoles)	0.65	7.50	5.75	23.10	1.65	0.35
Lipid phosphorus	(%)	1.7	19.3	14.7	59.2	4.2	0.9
Plasmalogens	(μmoles)	0.10	0.10	0.15	0.30	—	—
Esters	(μEq)	4.3	14.8	8.7	40.1	0.5	—
Glyceryl ethers[a]	(μmoles)	0.90	0.70	0.30	2.70	1.60	0.35
Glycerol	(μmoles)	1.6	6.9	5.6	21.0	—	—
Inositol	(μmoles)	—	—	0.9	1.1	—	—
Choline	(μmoles)	—	—	0.1	19.1	1.7	—
SKIN							
Lipid phosphorus	(μmoles)	1.90	3.10	5.15	14.50	12.95	4.40
Lipid phosphorus	(%)	4.5	7.3	12.3	31.6	30.8	10.5
Plasmalogens	(μmoles)	0.60	1.50	1.50	1.45	1.60	1.50
Esters	(μEq)	7.8	6.7	9.7	25.3	22.7	—
Glyceryl ethers[a]	(μmoles)	1.2	2.1	1.2	4.5	3.1	4.6
Glycerol	(μmoles)	3.7	1.4	4.5	12.7	11.1	0.1
Inositol	(μmoles)	—	—	0.6	2.6	—	—
Choline	(μmoles)	—	—	—	11.8	31.1	4.3

[a] Or sphingosine, in sphingolipid fractions.

TABLE III: *Overall Composition of the Phospholipid Fractions of the Tissues of Triturus cristatus.*

Phospholipid	Content (per cent of total phospholipid) Liver	Brain	Muscle	Skin
Cardiolipin	0.9	3.2	1.7	4.5
Phosphatidyl ethanolamine	60.4[a]	17.8	28.9	18.2
Phosphatidyl serine	6.9	1.0		
Phosphatidyl inositol	5.9	6.8	5.2	7.6
Phosphatidyl choline	21.9	51.4	58.4	59.2
Sphingomyelin	3.1	19.8	4.8	10.5

ε[a] Plus N-methyl- or N, N-dimethyl-phosphatidyl ethanolamine (see text)

administration of the radioactive palmitate. The lipids were fractionated also as described above and submitted to radioactivity measurement and to acyl ester determinations. The results are depicted in Table 4. As shown in the Table:

(1) The labeled palmitate is incorporated into the lipids of all the tissues studied. However, liver and muscle lipids possess higher specific activities, indicating higher metabolic rates of lipid metabolism in these tissues, although the technique used for injection (intraperitoneally) may have altered the situation for muscle tissue.

(2) Among phospholipids, in all tissues the phosphatidyl choline fraction has higher specific radioactivity, the highest value obtained in the phosphatidyl choline fraction from liver. Also in liver, the phosphatidyl ethanolamine fraction has a high specific radioactivity value in comparison with the corresponding fractions from other tissues.

(3) The specific radioactivities of neutral lipids, glycolipids and sulfolipids are higher in muscle, brain and skin, indicating a relatively higher turnover rate of these lipid classes in tissues other than liver.

TABLE IV: *Specific Radioactivities (cpm/mEq ester) of individual Lipids in tissues of T. Cristatus*

	Liver	*Muscle*	*Skin*	*Brain*
Neutral Lipids	560	1160	42	165
Glycolipidis	7	550	81	176
Sulfolipids	24		205	20
Total Phospholipids	635	520	163	91
Cardiolipin	240	—	—	—
Phosphatidyl ethanolamine	425[a]	135	73	—
Phosphatidyl serine	47	—	—	—
Phosphatidyl inositol	43	—	—	—
Phosphatidyl choline	2080	770	230	—
Sphingomyelin	470	230	153	—

[a] Plus N-methyl- and N-dimethyl-phosphatidyl ethanolamine (see text)

Περίληψη

Σύσταση καὶ κατανομὴ λιποειδῶν στοὺς ἱστοὺς τοῦ Trifurus Cristatus

Τὰ συνολικὰ λιποειδῆ τοῦ ἐγκεφάλου, τοῦ ἥπατος, τοῦ δέρματος καὶ τοῦ μυϊκοῦ ἱστοῦ τοῦ T. cristatus ἀπομονώθηκαν χωριστά, μὲ ἐκχύλιση κατὰ τὴν μέθοδο Bligh-Dyer.[16] Τὸ κάθε ἐκχύλισμα διαχωρίστηκε κατὰ τάξεις μὲ στήλη χρωματογραφίας πυριτικοῦ ὀξέος καὶ ἀπομονώθηκαν ἔτσι τέσσερα κλάσματα: Οὐδετέρων λιποειδῶν, Γλυκολιποειδῶν, Σουλφολιποειδῶν καὶ Φωσφολιποειδῶν.

Ἀκολούθησε συμπληρωματικὸς διαχωρισμὸς (μὲ στήλη πυριτικοῦ ὀξέος) τῶν κλασμάτων φωσφολιποειδῶν σὲ ἕξη ἐπὶ μέρους κλάσματα. Τὸ καθένα ἀπὸ τὰ ἀρχικὰ καὶ τελικὰ ἀνωτέρω κλάσματα ὑποβλήθηκε σὲ μιὰ πλήρη σειρὰ ἀναλύσεων (προσδιορισμοὶ φωσφόρου, ἐστέρων, γλυκεριναιθέρων, πλασμαλογόνων, αἰθανολαμίνης, σερίνης, ἰνοσίτου, χολίνης) καθὼς καὶ σὲ χρωματογραφικὴ ἀνάλυση μὲ λεπτὴ στιβάδα.

Τὰ ἀναλυτικὰ ἀποτελέσματα τῶν προσδιορισμῶν αὐτῶν δίδονται στοὺς πίνακες I, II ἐνῷ στὸν πίνακα III δίδεται ἡ προκύπτουσα σύσταση τῶν λιποειδῶν τῶν ἱστῶν ποὺ μελετήθηκαν.

Τέλος, στὸν πίνακα IV, δίδονται τὰ ἀποτελέσματα γιὰ μιὰ συγκριτικὴ ἐκτίμηση τῆς μεταβολικῆς δραστηριότητας (ὡς πρὸς τὸν μεταβολισμὸ τῶν λιποειδῶν) τῶν ἱστῶν τοῦ T. Cristatus, ποὺ ἔγιναν μὲ μετρήσεις τῆς εἰδικῆς ραδιενεργείας τῶν λιποειδικῶν κλασμάτων, 24 ὧρες, μετὰ τὴν ἐνδοπεριτονιακὴ χορήγηση [^{14}C—1] παλμιτικοῦ ὀξέος σὲ δύο πειραματόζωα.

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