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# ISTITUTO DI RICERCHE FARMACOLOGICHE "MARIO NEGRI,

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## PROGRAMMA DI RICERCA SULLL'EFFETTO PORFIROGENICO DELLA TCDD

## Rapporto Finale Relativo al Programma di Ricerca presentato per l'anno 1981

Milano, Aprile 1982

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Uno degli effetti tossici della TCDD è la sua azione porfirogenica. Questo effetto riveste una particolare importanza in quanto,non solo può essere prodotto negli animali da esperimento, ma è anche stato osservato nell'uomo in seguito a contaminazione con TCDD avvenuta, ad esempio, per esposizione occupazionale (A. Poland, D. Smith, G. Possick, Arch.Environ.Health 22, 316, 1971).
Il tipo di porfiria che viene indotto è classificato come Porfiria Cutanea Tarda (PCT) ed è caratterizzato, nell'uomo, da ipertricosi e da lesioni di tipo dermatologico dovute all'effetto fotosensibilizzante della porfirina e dall'instaurarsi di un caratteristico spettro di escrezione urinaria, dominato da una massiccia preponderanza delle porfirine a sette ed a otto gruppi carbossilici.

Studi sperimentali hanno mostrato che l'alterato pattern di escrezione urinaria delle porfirine è dovuto ad un blocco, principalmente nel fegato, nella sintesi dell'eme, a livello dell'enzima porfirinogeno carbossiliasi (uroporfirinogeno decarbossilasi E.C. 4.1.1.37).

L'inibizione di questo enzima, che decarbossila l'uroporfirinogeno a coproporfirinogeno, il precursore del protoporfirinogeno, che viene poi incorporato nell'eme dell'emoglobina e dei citocromi, provoca l'accumulo dei prodotti di reazione intermedi cioè dei porfirinogeni con un numero di gruppi carbossilici da 7 a 5; con l'aggravarsi del difetto enzimatico l'inibizione si avvicina al 100% con il conseguente accumulo dell'uroporfirinogeno stesso (a 8 gruppi carbossilici).
Il meccanismo per cui si sviluppa il difetto dell'enzima porfirinogeno carbossiliasi è tuttora sconosciuto; il TCDD e gli altri composti porfirogenici potrebbero agire direttamente sull'enzima di per sè o attraverso un metabolita; alternativamente potrebbero provocare un danno specifico a carico degli epatociti tale da modificare di conseguenza questa attività enzimatica. Inoltre la letteratura riporta numerosi esempi di interazione tra il manifestarsi della porfiria, (e quindi l'instaurarsi del blocco enzimatico) ed altri fattori quali alterazioni nel metabolismo del ferro o la somministrazione contemporanea di altri farmaci /A.G. Smith and F. De Matteis, Clin. Haematol. 9(2), 399 (1980)/.

In considerazione di quanto sopra esposto, il programma di ricerca presentato per il 1981 si proponeva uno studio che affrontasse principalmente il problema dell'inibizione dell'enzima porfirinogeno carbossiliasi articolato nei due punti seguenti:

1) Studio dell'enzima porfirinogeno carbossiliasi nell'uomo

Per questo aspetto ci si proponeva di mettere a punto una metodica sufficientemente sensibile da permettere la misura dell'attività enzimatica della porfirinogeno carbossiliasi anche in piccole quantità di tessuto epatico (quale ad esempio è possibile ottenere nel caso di una biopsia) oppure nel sangue (globuli rossi).

2) Studio dell'enzima porfirinogeno carbossiliasi nell'animale da esperimento.

Per questo aspetto ci si proponeva a) di caratterizzare le proprietà cinetiche (Km, Vmax) dell'enzima porfirinogeno carbossiliasi nel tessuto epatico di animali sia controllo sia resi porfirici da un trattamento cronico con TCDD; b) di misurare l'attività dell'enzima porfirinogeno carbossiliasi sia in organi extraepatici quali la milza ed i reni sia nel sangue di animali controllo e resi porfirici con un trattamento cronico con TCDD.

Programma di ricerca svolto nell'anno 1981

1) Messa a punto di un metodo di misura dell'enzima porfirinogeno carbossiliasi ad alta sensibilità.

Come precedentemente esposto nel rapporto semestrale (presentato nel settembre 1981) è stato messo a punto un metodo di misura dell'enzima porfirinogeno carbossiliasi che ha permesso di abbassare di circa 10 volte il limite di sensibilità rispetto al metodo usato precedentemente in Istituto (v. rapporto annuale presentato nel gennaio 1981). Questo metodo utilizza un detector a fluorimetria accoppiato al cromatografo liquido ad alta pressione ed è adatto per la determinazione dell'attività enzimatica sia in tessuti a bassa attività specifica (ad es. il sangue) sia quando sia disponibile solo una piccola quantità di tessuto (come avviene nel caso di una biopsia).
Il metodo e le sue applicazioni sono descritti in dettaglio nell'allegato n. 1 (L. Cantoni, R. Ruggieri, D. Dal Fiume and M. Rizzardini: The determination of uroporphyrinogen decarboxylase in tissues by high performance liquid chromatography coupled to fluorescence detection. In: J.Chrom. 229, 311-318, 1982).

2) Studio dell'enzima porfirinogeno carbossiliasi nell'animale da esperimento

Per questa parte del progetto di ricerca è stato utilizzato un modello sperimentale che consiste nell'indurre porfiria con un trattamento cronico con TCDD nella dose di 25 ug/kg/settimana i.p. x 9 settimane in topi maschi C57Bl/6.

a) Caratterizzazione delle proprietà cinetiche in vitro (Km, Vmax) dell'enzima porfirinogeno carbossiliasi.
Sono state determinate le caratteristiche cinetiche sia dell'enzima presente nel tessuto epatico sia dell'enzima presente nei globuli rossi del sangue in animali controlli e resi porfirici con trattamento cronico con TCDD.

Sono stati scelti questi due tessuti per i seguenti motivi:

i) il fegato è l'organo maggiormente colpito dalla porfiria indotta dal TCDD (vedi anche i risultati di cui si parla al punto b) ed è quindi il più importante per studiarne il meccanismo dell'inibizione enzimatica. Inoltre anche per i pazienti affetti da PCT sempre il fegato è l'organo in cui sono maggiori sia l'accumulo delle porfirine sia il danno istologico.

ii) nell'uomo sembrano esistere almeno 2 forme di PCT, una di tipo ereditario ("familial type") ed una dipendente invece solo da interazioni con composti tossici ("Sporadic type") differenziabili tra loro per la diversa diffusione del difetto a carico della porfirinogeno carbossiliasi, presente nel primo caso sia nel fegato sia nei globuli rossi e nel secondo solo nel fegato. Lo studio dell'enzima del globulo rosso è quindi sembrato importante per verificare se l'interazione con un composto fortemente porfirogenico come il TCDD potesse di per sè essere sufficiente a modificare il comportamento anche dell'enzima presente nei globuli rossi oltrechè quello epatico. Nella tabella No. 1 sono riportati i valori delle Km e Vmax apparenti degli enzimi dei due tessuti nei due gruppi di animali.
I valori di questi parametri cinetici sono calcolati sulla base dell'attività enzimatica misurata in presenza di diverse concentrazioni del substrato uroporfirinogeno III ed espressa come somma totale dei porfirinogeni presenti nella miscela di incubazione (coproporfirinogeno, a 4 gruppi carbossilici; pentacarbossilico porfirinogeno, a 5 gruppi carbossilici; esacarbossilico porfirinogeno, a 6 gruppi carbossilici, eptacarbossilico porfirinogeno, a 7 gruppi carbossilici).

Per quanto riguarda gli animali controllo, l'andamento della cinetica è rappresentato nelle figure 1 e 2 per l'enzima epatico e nelle figure 5 e 6 per l'enzima dei globuli rossi. In queste figure sono rappresentate singolarmente sia le cinetiche di formazione di ognuno dei prodotti intermedi della reazione sia la cinetica della formazione della somma totale dei prodotti di reazione. Gli enzimi dei due tessuti sono differenti sia per quanto riguarda la Vmax apparente, che è circa 8 volte più alta per la carbossiliasi del tessuto epatico sia per quanto riguarda la Km che è circa la metà nell'enzima degli eritrociti rispetto a quello epatico.

Questi dati indicano che l'enzima epatico, anche se ha una affinità minore per il substrato di quello eritrocitario, è però presente in una quantità molto maggiore; di conseguenza l'attività specifica dell'enzima del fegato risulta molto più alta.

Ambedue gli enzimi seguono però una cinetica del tipo di Michaelis Menten (fig. 1,2,5,6 parte b); inoltre se si considerano le singole curve che rappresentano l'andamento dell'accumulo dei prodotti intermedi si nota come si verifichi per gli enzimi di tutti e due i tessuti un effetto di inibizione da substrato per quello che riguarda la formazione di coproporfirina (fig. 1 e 5 parte b).

Il trattamento con TCDD modifica sia la Km che la Vmax dell'enzima epatico (Tabella 1): rispettivamente la Vmax viene ridotta ad un terzo ($p \leqslant 0.02$) e la Km viene dimezzata ($p \leqslant 0.04$) rispetto ai valori dei controlli. Le cinetiche di formazione dei prodotti di reazione sono riportate nelle figure 3 e 4. E' da notare che nella figura 3 non è stata riportata la curva relativa alla formazione di coproporfirina; infatti la formazione di questo prodotto di reazione, anche se misurabile (e perciò considerato per il calcolo sia della Km che della Vmax che tiene conto di tutti i prodotti) era inibita del 97% e quindi difficilmente rappresentabile sullo stesso grafico della attività totale.

La forte riduzione della Vmax indica che la quantità di enzima funzionante presente nel tessuto epatico è molto minore rispetto a quello presente nei controlli; inoltre anche l'affinità della porfirinogeno carbossiliasi per il substrato uroporfirinogeno III è stata modificata dal trattamento con TCDD. Sono però necessari ulteriori esperimenti per chiarire se queste differenze sono dovute ad un effetto del TCDD sulla sintesi dell'enzima (ad es. un blocco) oppure alla formazione in vivo di un inibitore che ne impedisce il funzionamento a sintesi avvenuta.

Il trattamento con TCDD non modifica, al contrario, il comportamento dell'enzima eritrocitario nonostante l'alta dose impiegata e il contemporaneo forte effetto evidenziato sul fegato. Infatti non sono modificati rispetto ai controlli i valori nè della Km nè della Vmax (Tabella 1); per questo motivo le cinetiche dell'enzima eritrocitario degli animali

trattati con TCDD, sovrapponibili a quelle dei controlli, non sono state inserite in questo rapporto. Questo risultato suggerisce che, nel caso di una popolazione contaminata, come a Seveso, non è possibile evidenziare la presenza di un effetto porfirogenico dal solo dosaggio dell'enzima porfirinogeno carbossiliasi eritrocitario.

b) Misura dell'attività dell'enzima porfirinogeno carbossiliasi sia nel fegato sia in organi extraepatici di animali controllo e resi porfirici con TCDD

L'effetto porfirogenico di un trattamento cronico con TCDD è stato valutato nel fegato, nei reni, nella milza, nel cervello e nei globuli rossi misurando sia l'accumulo e il tipo di pattern di porfirine presente in ognuno di questi tessuti sia l'attività dell'enzima porfirinogeno carbossiliasi.
In questo modo si è voluto verificare se il danno indotto dal TCDD fosse limitato oppure no al solo fegato. I dati sono discussi e presentati in dettaglio nell'allegato n. 2. In sintesi, i risultati hanno confermato quanto parzialmente osservato nel lavoro svolto precedentemente sul ratto (Porphyrogenic effect of chronic treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin in female rats. Dose-effect relationship following urinary excretion of porphyrins. L. Cantoni, M. Salmona, M. Rizzardini, Toxicol. Appl.Pharmacol. 57, 156-163, 1981) e cioè che, oltre al fegato, anche nei reni e nella milza c'è accumulo elevato di porfirine, specialmente ad alto numero di gruppi carbossilici, e si verifica un'inibizione dell'enzima uroporfirinogeno carbossiliasi. L'effetto è così distribuito: fegato (93.4% di inibizione calcolata sulla formazione di coproporfirinogeno) > rene (76% di inibizione) e milza (78% di inibizione) (Tabella n. 3 e n. 5 dell'allegato 2). In particolare nella milza si è però osservata anche una forte variabilità nella risposta dell'enzima (v. tabella n. 5 dell'allegato n.2); difatti è stato possibile dividere i dati relativi agli animali trattati con TCDD in due gruppi, in cui rispettivamente si sono verificati una attivazione e una inibizione dell'enzima. Questi due gruppi sono significativamente diversi tra di loro per quanto riguarda l'accumulo di porfirine, che è più alto nel

gruppo inibito. Questa variabilità potrebbe essere legata alla presenza di effetti specifici diversi del TCDD sull'enzima delle popolazioni cellulari che compongono la milza (cellule connettivali, globuli rossi, globuli della serie bianca) e/o alla alterazione da parte del TCDD dei rapporti quantitativi tra le diverse popolazioni.
Per quanto riguarda il fegato ed i reni, il metodo di analisi utilizzato per la determinazione dell'attività enzimatica, che misura contemporaneamente tutti i prodotti presenti alla fine della reazione nella miscela di incubazione, ha permesso di stabilire che, con ogni probabilità, il TCDD colpisce in modo differenziato ogni diverso passaggio di decarbossilazione. Inoltre si è verificato che, per quanto riguarda il meccanismo di inibizione, questo non può essere dovuto ad un effetto diretto sull'enzima delle porfirine accumulate; infatti anche se le porfirine presenti nel tessuto vengono eliminate, attraverso un passaggio su resina, prima dell'incubazione per la misura della attività enzimatica, la porfirinogeno carbossiliasi resta comunque inibita (v. Tabella n. 2 dell'allegato n. 2). Non è stato invece evidenziato nessun effetto sulla porfirinogeno carbossiliasi del cervello e dei globuli rossi (v. Tabella n. 4 dell'allegato n. 2).

Tabella 1 - Determinazione dei valori di apparente Km e Vmax dell'enzima porfirinogeno carbossiliasi nel fegato e negli eritrociti di topi C57Bl/6 controllo e trattati cronicamente con TCDD[a].

| Tessuto | Gruppo | Parametri cinetici | |
|---|---|---|---|
| | | Km apparente (uM) | Vmax (pmol/min/mg P) |
| Fegato | Controllo | 4.994 ± 0.350[b] | 48.314 ± 1.954 |
| Fegato | TCDD | 2.784 ± 0.529** | 17.154 ± 1.195* |
| Eritrociti | Controllo | 1.884 ± 0.112[c] | 5.918 ± 0.107 |
| Eritrociti | TCDD | 2.293 ± 0.103 | 6.619 ± 0.107 |

[a]Gli animali erano trattati per 9 settimane con la dose di 25 ug/kg/settimana i.p.

[b]Ogni valore rappresenta la media ± E.S. di 4 differenti esperimenti, ognuno effettuato su un pool di tessuto proveniente da 5 animali diversi

[c]Ogni valore rappresenta la media ± E.S. di 2 differenti esperimenti, ognuno effettuato su un pool di tessuto proveniente da 5 animali diversi.

* $p \leq 0.04$ in confronto al valore dei controlli (test t di Student)

** $p \leq 0.02$ " " " " "

LEGGENDA ALLA FIGURA 1

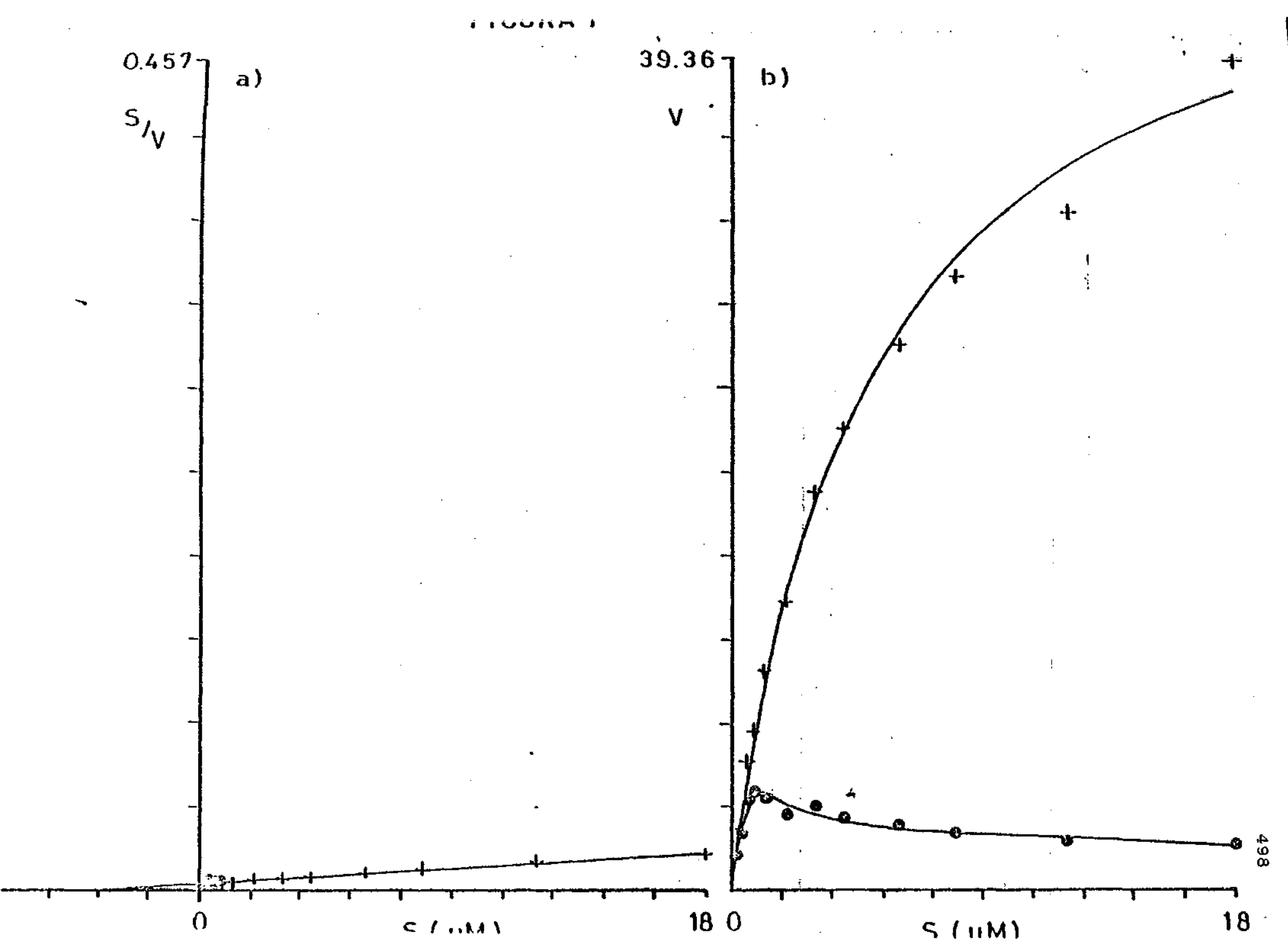
Attività dell'enzima epatico porfirinogeno carbossiliasi di topo C57Bl/6 controllo in presenza di diverse concentrazioni di uroporfirinogeno III.
I punti rappresentano la media di 4 diversi esperimenti ognuno effettuato con un pool di tessuto proveniente da 5 animali.

a) Woolf plot

b) Michaelis-Menten plot

+Attività dell'enzima porfirinogeno carbossiliasi espressa come la somma totale dei prodotti (eptacarbossilico-,+ esacarbossilico-,+ pentacarbossilico-,+ coprocarbossilico porfirinogeno)

*Attività dell'enzima porfirinogeno carbossiliasi espressa come quantità di coproporfirinogeno formato


0.457
a)
S/V
0
18
S (μM)
39.36
b)
V
0
18
S (μM)
498

LEGGENDA ALLA FIGURA 2

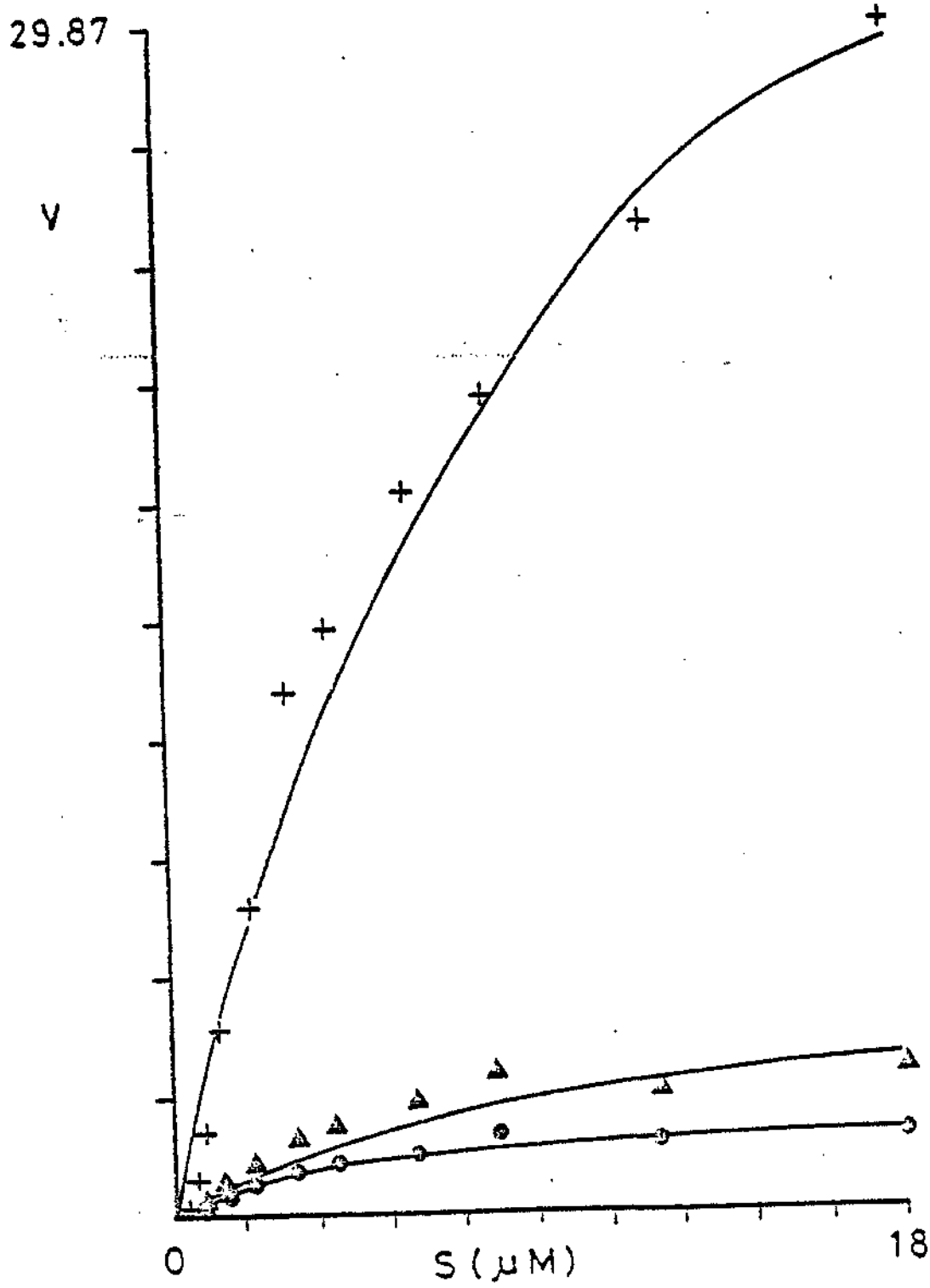
Attività dell'enzima epatico porfirinogeno carbossiliasi di topo C57Bl/6 controllo in presenza di diverse concentrazioni di uroporfirinogeno III.
I punti rappresentano la media di 4 diversi esperimenti ognuno effettuato con un pool di tessuto proveniente da 5 animali.
La cinetica dell'enzima è rappresentata secondo il Michaelis-Menten plot.

+ Attività dell'enzima porifirinogeno carbossiliasi espressa come formazione di porfirinogeno eptacarbossilico.

▲ Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno esacarbossilico.

• Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno pentacarbossilico.

FIGURA 2



LEGGENDA ALLA FIGURA 3

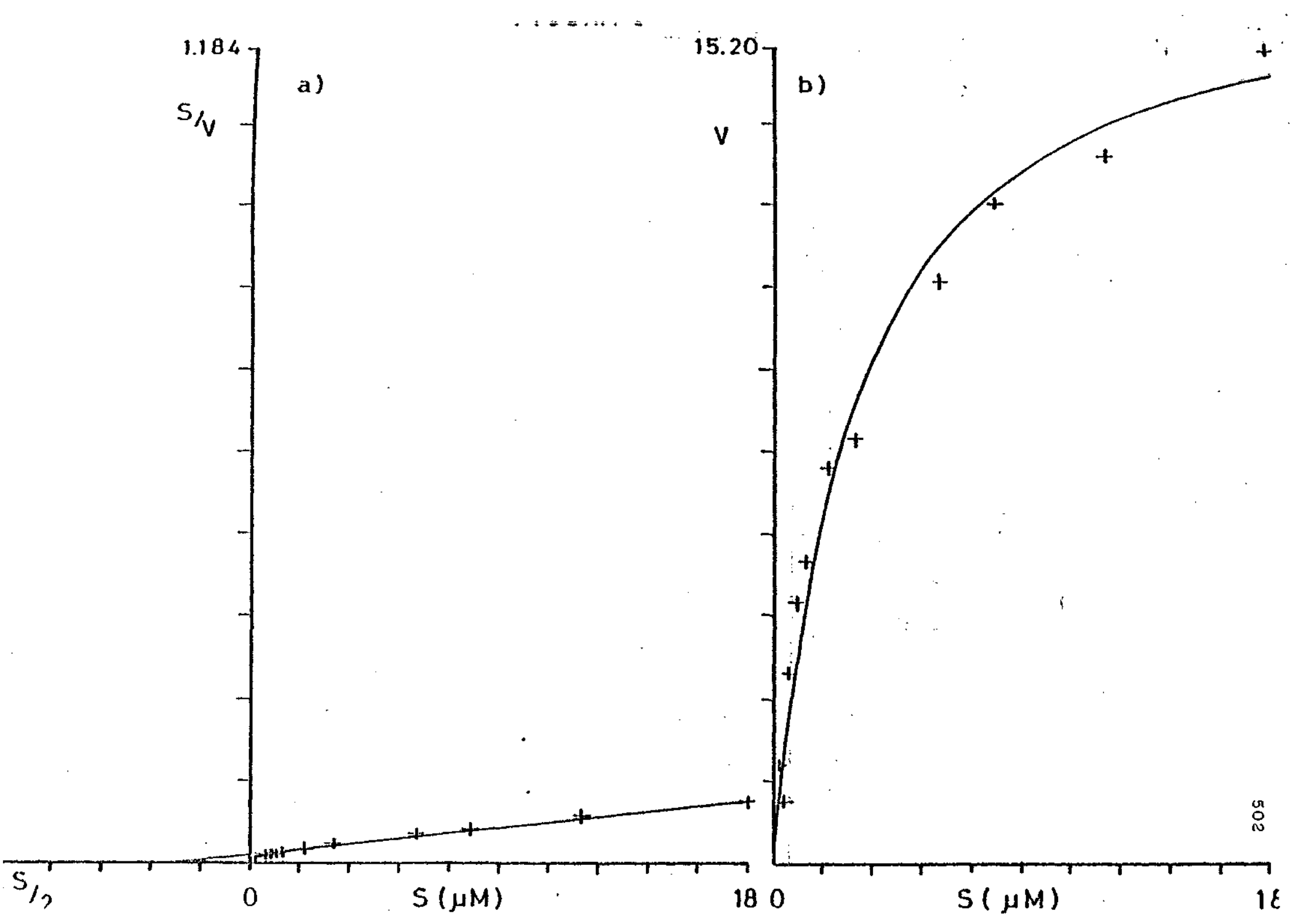
Attività dell'enzima epatico porfirinogeno carbossiliasi di topo C57Bl/6 trattato cronicamente con TCDD in presenza di diverse concentrazioni di uroporfirinogeno III.

I punti rappresentano la media di 4 diversi esperimenti ognuno effettuato con un pool di tessuto proveniente da 5 animali.

a) Woolf plot

b) Michaelis - Menten plot

L'attività dell'enzima è espressa come la somma totale dei prodotti (epta-carbossilico-,+ esacarbossilico-,+ pentacarbossilico-,+ coprocarbossilico porfirinogeno).


1.184
a)
S/V
S/2
0
S (μM)
18
15.20
b)
V
0
S (μM)
18
205

LEGGENDA ALLA FIGURA 4

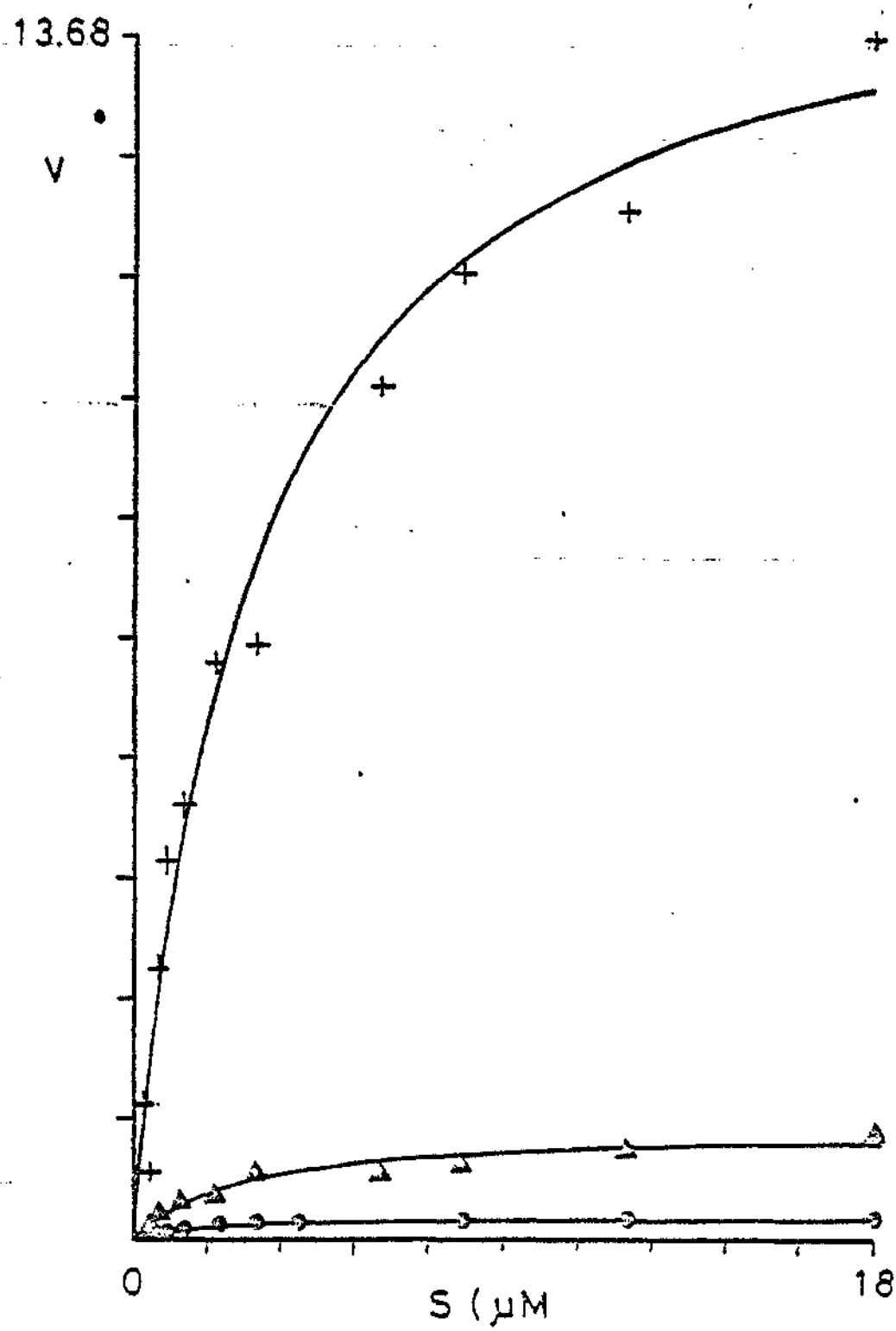
Attività dell'enzima epatico di topo C57Bl/6 trattato cronicamente con TCDD porfirinogeno carbossiliasi in presenza di diverse concentrazioni di uroporfirinogeno III.
I punti rappresentano la media di 4 diversi esperimenti ognuno effettuato con un pool di tessuti proveniente da 5 animali.
La cinetica dell'enzima è rappresentata secondo il Michaelis-Menten plot.

+ L'attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno eptacarbossilico.

▲ Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno esacarbossilico.

• Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno pentacarbossilico.

FIGURA 4



LEGGENDA ALLA FIGURA 5

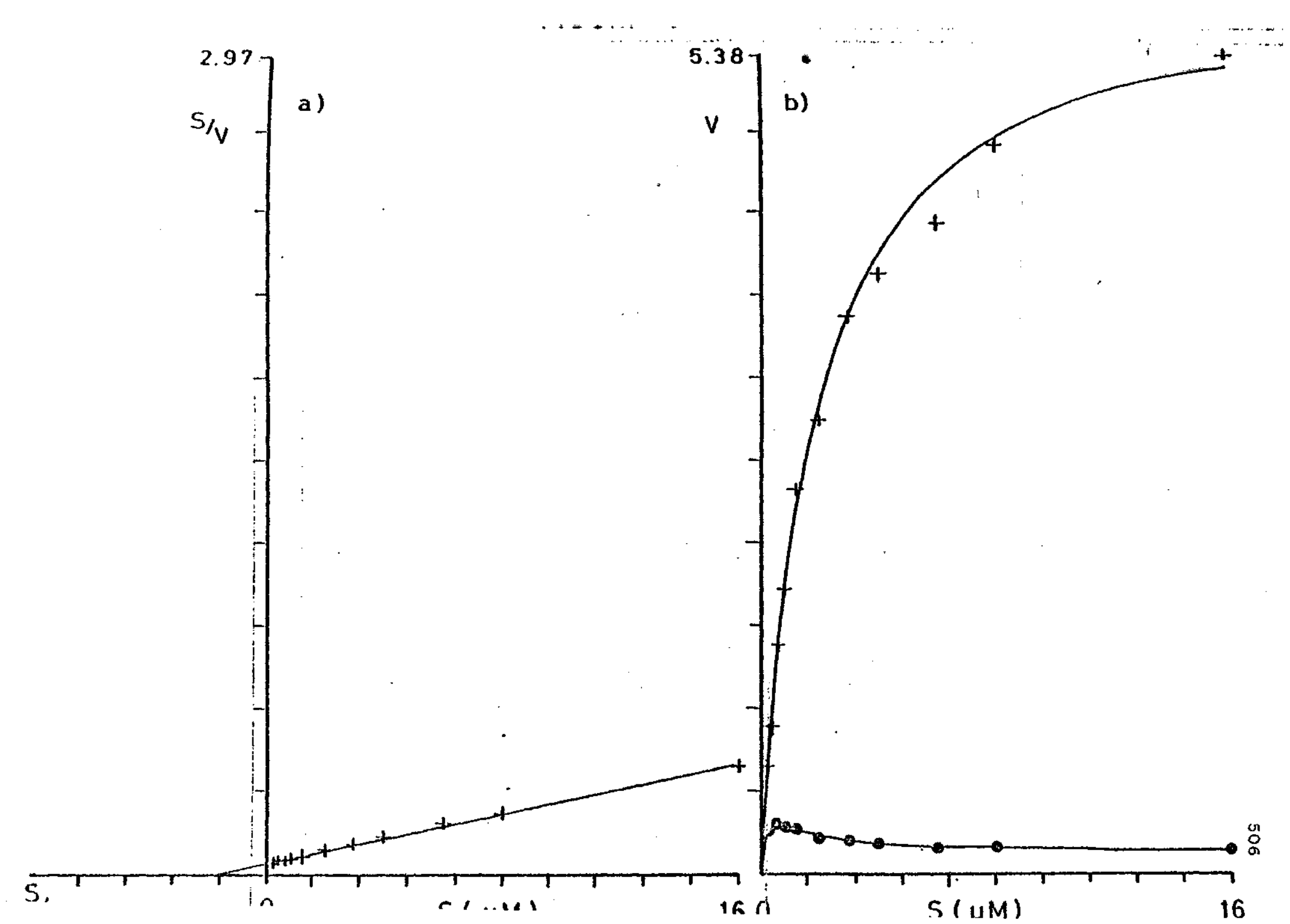
Attività dell'enzima eritrocitario porfirinogeno carbossiliasi di topo C57Bl/6 controllo in presenza di diverse concentrazioni di uroporfirinogeno III. I punti rappresentano la media di 2 diversi esperimenti ognuno effettuato con un pool di tessuto proveniente da 5 animali.

a) Woolf plot

b) Michaelis-Menten plot

+Attività dell'enzima porfirinogeno carbossiliasi espressa come la somma totale dei prodotti (eptacarbossilico-, esacarbossilico-, pentacarbossilico-, coprocarbossilico porfirinogeno).

•Attività dell'enzima porfirinogeno carbossiliasi espressa come quantità di coproporfirinogeno formato.


2.97
a)
S/V
5.38
b)
V
S (µM)
16
506

LEGGENDA ALLA FIGURA 6

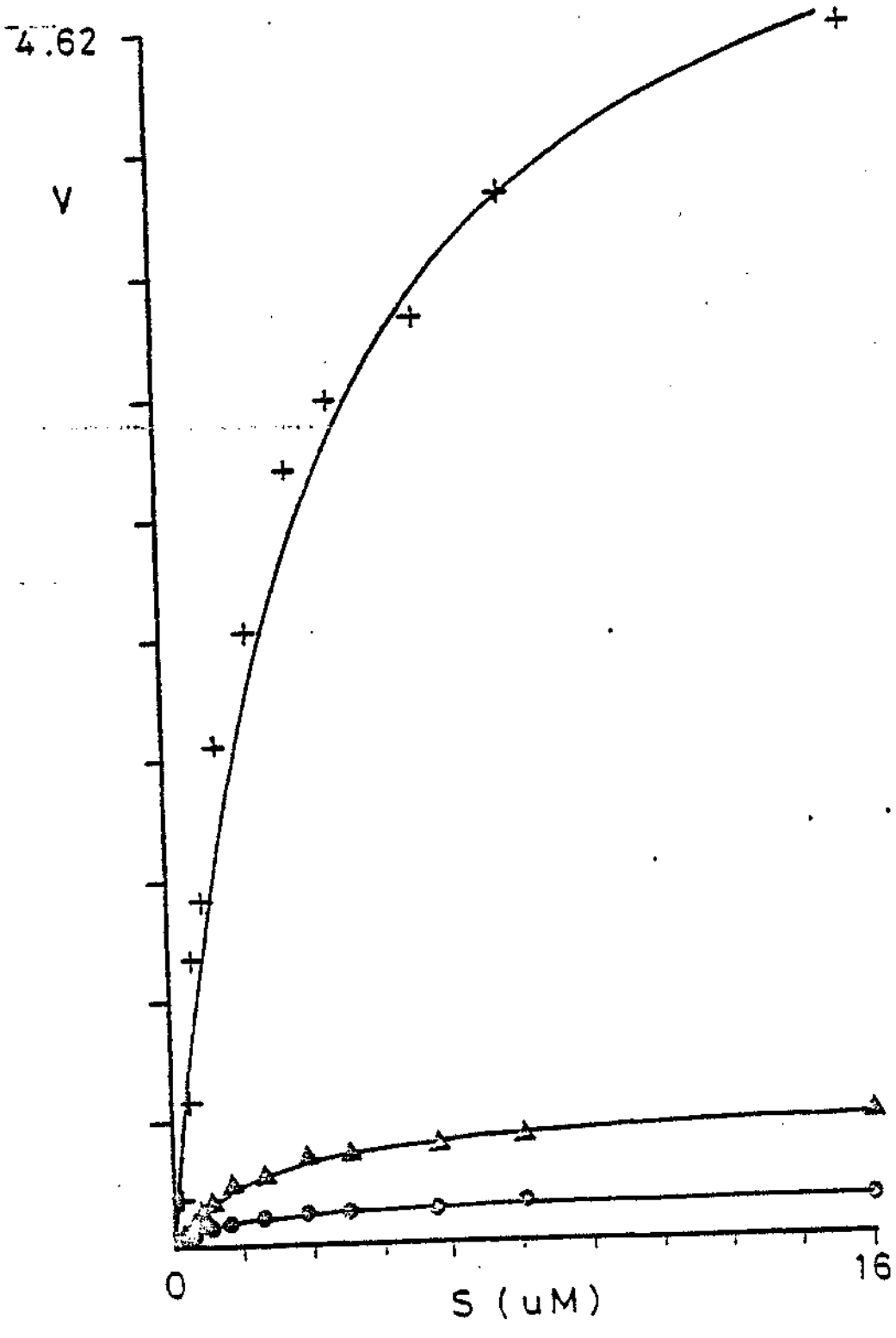
Attività dell'enzima eritrocitario porfirinogeno carbossiliasi di topo C57Bl/6 controllo, in presenza di diverse concentrazioni di uroporfirinogeno III.
I punti rappresentano la media di 2 diversi esperimenti ognuno effettuato con un pool di tessuto proveniente da 5 animali.
La cinetica dell'enzima è rappresentata secondo il Michaelis-Menten plot.

+ Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno eptacarbossilico.

▲ Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno esacarbossilico.

• Attività dell'enzima porfirinogeno carbossiliasi espressa come formazione di porfirinogeno pentacarbossilico.

FIGURA 6



ALLEGATO No. 1

# THE DETERMINATION OF UROPORPHYRINOGEN DECARBOXYLASE IN TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENCE DETECTION.

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SUMMARY

High performance liquid chromatography coupled to fluorescence detection was utilized for the separation and quantitation of porphyrins as methyl esters. The sensitivity of the method permitted quantitation down to 0.2 nanograms of porphyrins per sample and was developed for biochemical investigation of porphyrias. One of possible application is the study of the enzyme uroporphyrinogen decarboxylase. No significant difference was found between two methods of methylation and extraction of the samples prior to chromatography.

## INTRODUCTION

Porphyrins are tetrapyrrolic compounds synthesized in a variety of biological tissues. They differ in the type and number of carboxylic side chains.

Disturbances of the porphyrin metabolism attributed to drugs, environmental contaminants and genetic errors (1,2,3) have been described. In many of these situations it is necessary to measure the porphyrin content of biological fluids and tissues.

Recently high performance liquid chromatography (HPLC) analysis of porphyrin mixtures, both methylated (4,5,6) and as free acids (7), has been developed with some advantages in comparison with the widely used TLC separation of porphyrin methyl esters as regards accuracy and time required. In these methods, detection is usually by a UV detector, so sensitivity remains a major problem because in many experimental models employing tissue culture (8) or human biopsies (9), the porphyrins present are in the nanogram range. In these situations radioactive methods were often used (9).

This paper reports an analytical technique utilizing HPLC coupled to a fluorometric detector, permitting the quantitation of porphyrins in sub-nanogram amounts. The method was developed for the biochemical investigation of porphyrias. At present we have applied it to the determination of the activity of the enzyme uroporphyrinogen-decarboxylase (URO-D) which is markedly affected in the liver of patients with porphyria cutanea tarda and of animals intoxicated with porphyrogenic compounds (9,2).

## EXPERIMENTAL

### Chemicals

Mesoporphyrin IX dimethyl ester, protoporphyrin IX dimethyl ester coproporphyrin III tetramethyl ester, pentacarboxylporphyrin I pentamethyl ester, hexacarboxyl porphyrin I hexamethyl ester, heptacarboxyl porphyrin I heptamethyl ester and uroporphyrin III octamethyl ester were purchased from Porphyrin Products (Logan, Utah, U.S.A.).

ethyl acetate and n-heptane (reagent grade) were obtained from Farmitalia Carlo Erba (Milan, Italy). Solvents were filtered under vacuum through a polycarbonate membrane (0.4 µm) (Nucleopore Corp., Pleasanton, Canada) before use.
Chloroform (Aristar grade) was purchased from BDH Ltd. (Poole, England) and was always washed with distilled water before use.

Apparatus and conditions

The high pressure liquid chromatograph was a Model Series 3 Microprocessor controlled pump module (Perkin Elmer Corp., Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 injector supplied with 175 microliter sample loop injector (Rheodyne, Berkeley, Ca., U.S.A.). The column (0.26 ID x 25 cm) was packed with Silica A 10 µm (Perkin Elmer Corp., Norwalk, CT, U.S.A.).
A model 3000 fluorescence spectrometer (Perkin Elmer, Norwalk, CT, U.S.A.) was used with the excitation wavelength set at 404 nm (slit width 10 nm) and the emission wavelength at 623 nm (slit width 10 nm).

The attenuation of the fluorescence spectrometer was varied appropriately for each sample, depending on the expected porphyrin concentration.
The UV detector was a Model LC 56 B (Perkin Elmer Corp., Norwalk, Ct., USA) with the detection wavelength set at 400 nm.
Separation of the porphyrins was obtained by multilinear gradient elution. The initial conditions were 30% ethyl acetate vs. n-heptane for 6 minutes after injection; then the percentage of ethyl acetate was raised to 45% within 1 minute. Over the next 10 minutes the ethyl acetate percentage was raised linearly up to 80% and this condition was held for 6 minutes. Elution was completed with a 3-minute purge period at 80% of ethyl acetate. Between two analyses the column was reconditioned for 10 minutes with the starting solvent mixture. Flow rate was kept constant at 0.8 ml/min.

Sample preparation from tissues

Porphyrinogens formed as reaction products of the *in vitro* urodecarboxylase activity were oxidized to porphyrins, adsorbed on Zerolit FF (ip) resin (BDH Ltd, Poole, England), methylated for 48 h with methanol-sulphuric acid and extracted into chloroform as described by Smith et al. (10). Alternatively the methylation was performed with boron trifluoride (14%, Merck-Schuchardt) as described by Smith and Francis (11) with slight

modifications: to 2 ml of $BF_3$, 4 ml of chloroform were added, the tubes were mixed on a Vortex for 40 sec and centrifugated at 6500 rpm for 10 min. This procedure was repeated twice with a mixture of chloroform-methanol (6 ml in the ratio 2:1 and 2 ml in the ratio 1:1). The pooled extracts were combined and washed twice with distilled water. After the second washing the chloroform layer was taken, mixed with ethanol (10 ml) and evaporated under a stream of $N_2$ at 37°C. Precautions were taken during the whole procedure to avoid contact with direct light, and samples were kept in the dark at -20°C until injected. Immediately before the HPLC analysis, samples were dissolved with a chloroform solution of mesoporphyrin dimethyl ester (0.5 nmoles/ml). This compound, not usually present in biological tissues, was used as internal standard on account of its similarities with the other porphyrins.

Calibration curves

Porphyrin methyl ester standard mixtures at known concentrations for the calibration curve were prepared from individual porphyrin methyl ester solutions in chloroform. The concentrations of these original solutions were determined spectrophotometrically by measuring the adsorption at the Soret band and using the extinction coefficients reported by Falk (12) for meso-, proto-, copro- and uroporphyrin and by Doss (13) for pentacarboxylic-, hexacarboxylic- and heptacarboxylic porphyrin. The standard mixtures were prepared ready to dissolve shortly before use with 2.5 ml of a chloroform solution of mesoporphyrin dimethyl ester (0.5 nmoles/ml). When dissolved, they were stable for at least one week if stored in the dark at -20°C.

## RESULTS

The multilinear gradient elution previously described permitted the separation of two dicarboxylic porphyrins (mesoporphyrin IX, and protoporphyrin IX) and of copro-, pentacarboxylic-, hexacarboxylic-, heptacarboxylic and uroporphyrin. All these porphyrins were in the form of the methyl ester and a typical example of the separation, obtained with a mixture of pure compounds, is shown in Fig. 1. No interfering peak(s) were found in extracts from several tissues (liver, spleen, kidneys and red blood cells); occasionally, additional peaks were observed, quite likely due to the formation of trace amounts of the zinc complex of the porphyrins.

Absolute quantification of the amounts of porphyrins present in samples was performed by comparison with a calibration curve obtained by plotting the peak area ratio of each porphyrin to mesoporphyrin versus the concentration of the porphyrin standard solutions. In our conditions, utilizing the fluorometric detector, linearity was present for all the porphyrins considered within a range of concentrations from 20 to 2000 nanograms per ml. Fig. 2 shows the part of the curves up to 1000 nanograms. The relative correlation coefficients (which also take into account the 2000 ng/ml concentration point) were all greater than 0.999.

The calibration curves sloped differently depending on the specific fluorescence of each porphyrin. The best sensitivity was obtained for coproporphyrin tetramethyl ester. On some occasions, the HPLC system was coupled to the UV detector and the two methods of detection were compared. Fig. 3 presents the profiles obtained using a UV or a fluorometric detector injecting the same sample containing the reaction products of a preparation of human red blood cell uroporphyrinogen decarboxylase. In this example, the chromatogram was obtained at the highest sensitivity of the UV detector (0.02 AUFS) (Panel A). With the fluorometric detector (Panel B), sensitivity could be further enhanced from 5 to 10 times depending upon the porphyrin without changes in the signal-to-noise-ratio.

In our method of HPLC separation, porphyrins need to be present in the methylated form; Tables I and II report the results of recovery experiments to study the effectiveness of two methylation and relative extraction procedures, one with methanol- sulphuric acid and one with boron trifluoride. Known amounts of porphyrins (copro-, pentacarboxylic-, hexacarboxylic-, heptacarboxylic- and uroporphyrin) were adsorbed as free acids on Zerolit FF resin and then methylated and extracted as previously described.

In both procedures, no interfering peaks were visible in the chromatograms and recovery was not influenced by changes in the amount of porphyrin

adsorbed on the resin. The efficiency of methylation and extraction, relative to each porphyrin, increased in the following order: uro> copro> penta> hexa > hepta; the percentage of recovery was always higher than 57%.

As an example of the applicability of the method, Table III reports the results of determination of the activity of the enzyme URO-D in animal and human tissues. The decarboxylated products (copro-, penta-, hexa-, and heptacarboxylic porphyrin) were obtained with 1 hour of incubation of the enzymatic preparation at 37°C under $N_2$, were oxidized to porphyrins with light and then methylated with methanol-sulphuric acid. The amount of uroporphyrin present at the end of incubation represents the residual substrate.

With all the tissues considered, it was possible to measure quantitatively all the reaction products simultaneously.

## DISCUSSION

This paper describes a method for measuring porphyrins present in tissues or formed in in vitro incubations down to a concentration of 0.2 nanograms per sample. This sensitivity was achieved by the use of fluorescence detection which is more sensitive than the more commonly used UV detection (4,5,6). The amount of sample in the optimal range for analysis with fluorescence detection is about one tenth that with UV detection. Another major advantage of the use of a fluorometric detector is its specificity which makes it easy to obtain a clean baseline in the chromatogram, eliminating many interfering substances present in biological samples which adsorb light but do not fluoresce in the same range of porphyrins. This could be important in the sample preparation. Furthermore the gradient elution program described here efficiently separates all the porphyrins without the need for a second derivative system coupled to the detector as described by other authors (14).

HPLC methods of separation of free porphyrins are still in the development stage and have been applied mainly to analysis of urines (7,15). As a consequence, methylation of the porphyrins is still a necessary step in sample preparation especially when the amounts present are fairly low.
In this paper we have shown that two commonly used procedures of methylation do not seem to differ significantly in efficiency.
A somewhat higher percentage of recovery and better reproducibility were obtained with the methanol-sulphuric acid method, but the boron trifluoride method has the advantage of considerably shortening analysis time.
Finally we have shown that the use of HPLC coupled to fluorescence detection provides a technique suitable for the biochemical investigation of porphyrias. In this respect, one possible application is the measurement of the activity of the enzyme URO-D. By our method all the reaction products can be determined simultaneously with advantages in specificity in comparison to radioactive assays, and in sensitivity and accuracy in comparison to TLC separation coupled to spectrophotometric quantitation previously described by other authors (9,10,11). This should facilitate the characterization of the various steps of this enzymatic process and of its response to stimuli of different origin.

ACKNOWLEDGEMENTS

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Table I - Recovery of porphyrins after methylation with methanol-sulphuric acid

| Amount of each porphyrin added to the incubation mixture (nmoles) | RECOVERY (%)* | | | | |
|---|---|---|---|---|---|
| | Copro | Penta | Hexa | Hepta | Uro |
| 0.083 | 90±5 | 83±5 | 74±5 | 65±1 | 106±1 |
| 0.160 | 90±2 | 81±1 | 73±3 | 61±3 | 102±8 |
| 0.800 | 93±1 | 84±1 | 77±1 | 67±0 | 103±1 |
| 1.600 | 98±2 | 90±1 | 84±5 | 68±1 | 107±2 |

*Values represent the mean ± S.E. of two determinations.

Table II- Recovery of porphyrins after methylation with boron trifluoride

| Amount of each porphyrin added to the incubation mixture (nmoles) | Recovery (%)* | | | | |
|---|---|---|---|---|---|
| | Copro | Penta | Hexa | Hepta | Uro |
| 0.083 | 83±3 | 73±1 | 67±3 | 58±6 | 89±6 |
| 0.160 | 88±3 | 77±3 | 67±6 | 57±6 | 90±6 |
| 0.800 | 88±3 | 81±3 | 73±3 | 61±5 | 98±3 |
| 1.600 | 86±6 | 75±10 | 69±6 | 58±5 | 95±8 |

*Values represent the mean ± S.E. of two determinations

TABLE III -

| Tissue | Uroporphyrinogen added (nmoles) | Porphyrins recovered (nmoles)* | | | | | Recovery % | Uro-D activity (pmoles/1'/mg P) |
|---|---|---|---|---|---|---|---|---|
| | | Copro | Penta | Hexa | Hepta | Uro | | |
| Rat liver | 8 | 1.223±0.14 | 0.190±0.01 | 0.459±0.04 | 1.820±0.14 | 3.933±0.16 | 95 | 13.209 ± 1.16 |
| Rat kidneys | 8 | 0.455±0.04 | 0.116±0.00 | 0.314±0.01 | 1.336±0.03 | 4.778±0.20 | 87 | 12.724 ± 0.43 |
| Rat spleen | 8 | 0.311±0.02 | 0.103±0.00 | 0.296±0.01 | 1.394±0.04 | 5.910±0.02 | 100 | 9.162 ± 0.40 |
| Human liver | 8 | 0.051±0.00 | 0.033±0.00 | 0.100±0.00 | 0.671±0.07 | 5.997±0.59 | 86 | 10.490 ± 0.47 |
| Human blood | 8 | 0.304±0.02 | 0.088±0.00 | 0.270±0.01 | 1.964±0.17 | 3.074±0.31 | 71 | 3.820 ± 0.04 |

*Values represent the mean ± S.E. of two samples. Each sample was done in duplicate. The enzymatic preparations had a protein concentration of 2.5 - 4.6 mg/ml except blood (11.4 mg/ml).

LEGEND TO FIGURES

Fig. 1 - HPLC separation of standard mixtures of porphyrin methyl ester.
1: mesoporphyrin dimethyl ester; 2: protoporphyrin dimethyl ester; 3: coproporphyrin tetramethyl ester; 4: pentacarboxyl-porphyrin pentamethyl ester; 5: hexacarboxylporphyrin hexamehtyl ester; 6: heptacarboxylporphyrin heptamethyl ester; 7: uroporphyrin octamethyl ester.
Conditions of elution as described in the text.

Fig. 2 - Calibration curves for standard porphyrin esters.

Fig. 3 - Comparison of the profiles obtained with UV (A) and fluorometric (B) detectors injecting a sample containing the reaction products of a preparation of human red blood cell uroporphyrinogen decarboxylase.
1: mesoporphyrin dimethyl ester; 2 : coproporphyrin tetramethyl-ester; 3: pentacarboxyporphyrin pentamethyl ester; 4 : hexacarboxyl-porphyrin hexamethyl ester; 5 : heptacarboxyl porphyrin heptamethyl-ester; 6 : uroporphyrin octamethyl ester.
Conditions of elution as in Fig. 1.

FIG. 1

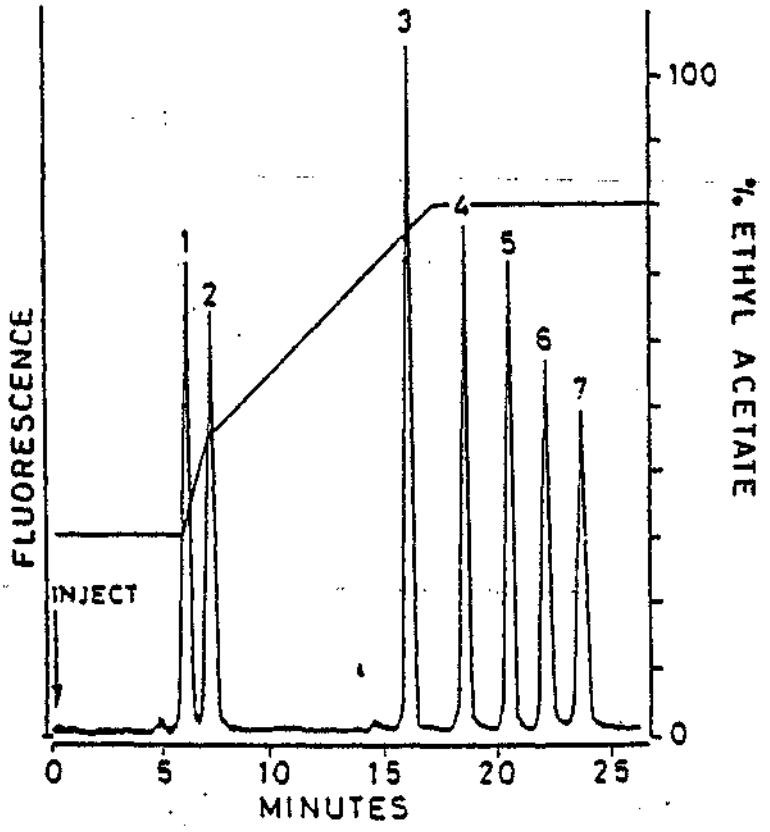

FLUORESCENCE
INJECT
1
2
3
4
5
6
7
100
0
% ETHYL ACETATE
0
5
10
15
20
25
MINUTES

FIG. 2

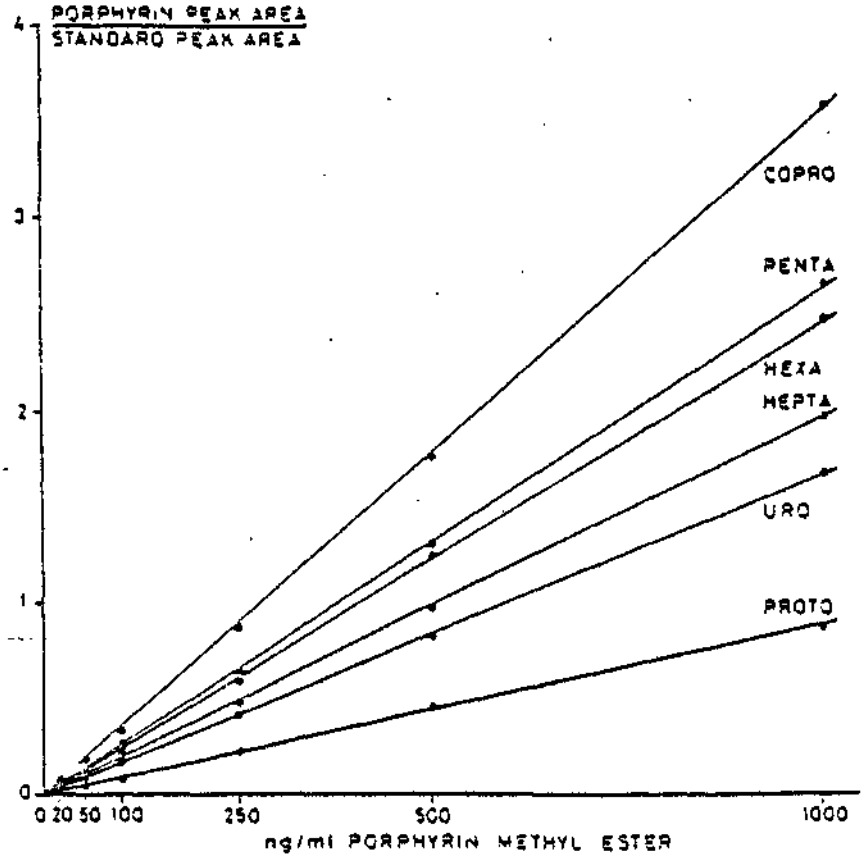
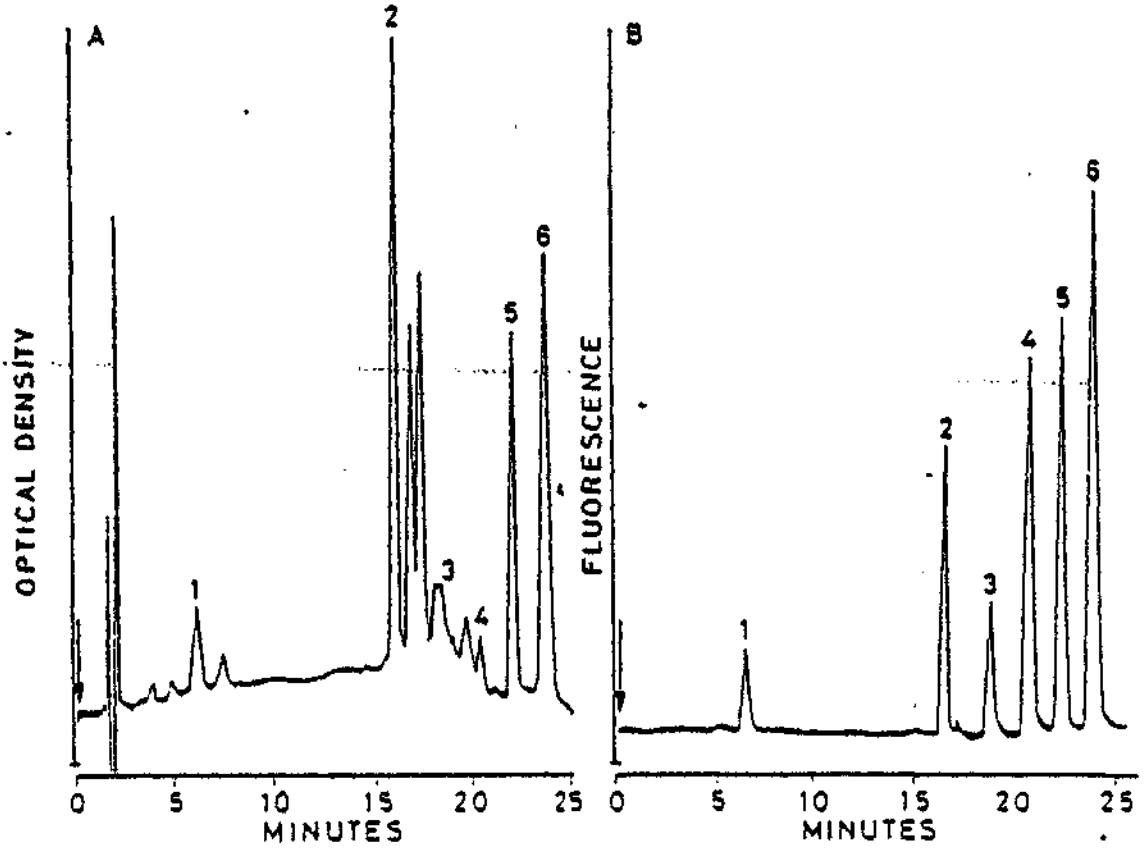

PORPHYRIN PEAK AREA
STANDARD PEAK AREA
4
3
2
1
0
COPRO
PENTA
HEXA
HEPTA
URO
PROTO
0 20 50 100
250
500
1000
ng/ml PORPHYRIN METHYL ESTER

FIG. 3


A
B
OPTICAL DENSITY
FLUORESCENCE
1
2
3
4
5
6
0
5
10
15
20
25
MINUTES

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ALLEGATO No. 2

# DIFFERENTIAL SUSCEPTIBILITY OF MICE TISSUES TO PORPHYROGENIC EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

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SUMMARY

The porphyrogenic effect of a chronic (25 ug/kg/week) administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to male C57Bl/6 mice was evaluated through quantitative and qualitative analysis of the porphyrins accumulated and of porphyrinogen carboxy-lyase activity in liver, kidney, spleen, brain and erythrocytes.

Liver was the principal site of action, both for porphyrin accumulation and for enzyme inhibition, with kidney next in decreasing order, while brain and erythrocytes were unaffected. In spleen were observed both an activation and an inhibition of the enzyme concomitant to an increase of porphyrin accumulation, with the inhibition corresponding to a higher accumulation.

In the responding tissues, all the decarboxylation steps from uroporphyrinogen III were differently affected by TCDD.

The pattern of enzyme inhibition paralleled data reported in the literature regarding tissue distribution of TCDD and also indicated that TCDD porphyria is a suitable experimental model for the human "Sporadic type" of Porphyria Cutanea Tarda.

INTRODUCTION

The human syndrome Porphyria Cutanea Tarda (PCT) has been known since 1937 when it was first described by Waldenström (1). Only recently the biochemical mechanism which underlies the overproduction of porphyrins at high number of carboxyl groups characteristic of these patients, has been explained as a result of the inhibition of the hepatic enzyme porphyrinogen carboxy-lyase(EC 4.1.1.37) (2,3,4).

It has now been suggested that in the human population these are two varieties of the disease (5): one ("familial type") in which the defect of the porphyrinogen carboxy-lyase is inherited as an autosomal dominant trait and, as a consequence, can be detected both in the liver and in other tissues like the erythrocytes, and the other ("sporadic type") in which the enzymatic defect is acquired.

In this latter case, porphyrinogen carboxy-lyase inhibition could be induced in the liver through an interaction with exogenous toxic factors, whose mechanism is not yet understood, but not in the erythrocytes.

A good resemblance of this type of PCT is obtained with hexachlorobenzene (HCB) whose chronic administration produced, in animals, increased excretion of porphyrins and a diminished activity of the hepatic and kidney porphyrinogen carboxy-lyase, while the erythropoietic tissue was unaffected (6,7,8).

However with HCB a fairly high dosage and a chronic treatment had to be used.

Compounds like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are much more potent than HCB in causing porphyria in laboratory animals so that only a single low lethality oral dose is efficient in provoking hepatic porphyrinogen carboxy-lyase inhibition in mice (9).

This result raises the possibility that, as well as the degree of the enzyme defect in relation to the administered dose, also the induction of the enzyme defect in other tissues than liver could be related directly to the potency of the porphyrogenic compound.

This effect could be particularly important since a compound like TCDD, which is known to be a widely spread environmental contaminant (10), has been already shown to cause disturbances of porphyrin metabolism in contaminated people (11,12,13). Moreover, in a previous investigation (14) we have shown that in TCDD acquired porphyria in rats, a marked accumulation

of porphyrins can be observed not only in liver but also in kidney and spleen.
The finding of a widespread disturbance of porphyrin metabolism could also help in the understanding of the mechanisms underlying the establishment of the enzyme inhibition since quite different situations as regard factors indicated as etiologic or exacerbating like extent of metabolism or of iron stores (15,16,17,18) are present in different organs. Purposes of this study were then to investigate the distribution of porphyrins and porphyrinogen carboxy-lyase activity of different tissues of normal and TCDD porphyric mice in order to determine which were affected by TCDD and particularly if liver and erythropoietic tissue behaved the same way after intoxication.
Moreover the assays of the enzymatic activity were performed measuring each single decarboxylating step in order to understand at which of them TCDD inhibition is effective.

## MATERIALS AND METHODS

### Chemicals

2,3,7,8-tetrachlorodibenzo-p-dioxin was purchased from Kor Isotopes (Boston, Massachussetts).
Pure porphyrin methyl ester standards were purchased from Porphyrin Products (Logan , Utah) and from Calbiochem (La Jolla, Ca. 92037).

### Animals and treatment

Male C57B1/6 mice 5 weeks old were obtained from Charles River Italy (Calco, Como, Italy). Treatment was started after one week of housing. Animals were kept in cages with food and water ad libitum and were divided in two groups: the treated group received i.p. once a week a dose of TCDD (25 ug/kg) dissolved in a mixture of acetone and corn oil (1:6) for 9 weeks; the control group received the vehicle alone (2.5 ml/kg) once a week for the same time. Mice were then anesthetized with diethyl esther and blood was removed directly from the heart with a 0.45 mm ED x 10 mm L needle and collected in a tube containing 3.8%

citrate in the ratio 0.1 ml citrate: 0.9 ml blood.
The livers, kidneys, spleens and brains were then removed, rinsed in ice cold saline, blotted dry and weighed. A portion of the livers was taken for the determination of the TCDD content.

Preparation of the enzymatic fraction

Tissues (liver, spleen, kidney and brain) were immediately homogenized with a blendor (Ultra Turrax, Janke and Kunkel KG, Stauffen i.Br., Germany) in 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer containing 0.1 mM EDTA pH 6.8 (1:4 w:v) and the homogenate was centrifuged at 40.000 x g at 4°C for 30 minutes.
The supernatant, used as the enzyme source, was frozen in liquid nitrogen and stored at -80°C for the time of the experiments.
Blood plasma and buffy coat were separated by centrifugation at 1000 x g for 15 minutes and red cells were washed 3 times by suspension and centrifugation with 2 volumes of 0.9% NaCl ice cold. The cells were suspended with 1 volume of 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer containing 0.1 mM EDTA pH 6.8 and hemolyzed by sonication with a Sonifier Model B-15P (Branson Company, Danbury, Connecticut) for 10" with the % duty cycle set at 50.
The hemolyzate was centrifugated at 40.000 x g for 30' and the resulting supernatant was frozen in liquid nitrogen and stored at -80°C.

Assays

Protein content was determined by the method of Lowry (19); hemoglobin content of the blood hemolyzate supernatant was determined as cyanmeta hemoglobin, with a Eurochima Kit (Elvi S.p.A., Milan, Italy).
Porphyrinogen carboxy-lyase activity in tissues was assayed using uroporphyrinogen (isomer III 97.4% as determined by HPLC analysis) as substrate (3 mM).
Solutions of uroporphyrinogen were prepared immediately before the incubation by reducing in the dark and under nitrogen a 8 mM solution of uroporphyrin free in NaOH 4 mM with 5% sodium amalgam (Hopkin and Williams, Chadwell Heath, England) and further treated as described

Smith and J.E. Francis (20). Uroporphyrin free was prepared
oporphyrin octomethylester as described by Elder et al. ( 7) and
rations of uroporphyrin free solutions were determined as
ed by A.G. Smith and J.E. Francis (20).
ions were carried out for 15' minutes at 37°C in the dark under
n in a final volume of 1 ml containing 3-4 mg protein for liver,
, spleen and brain and 8-11 mg protein for blood.
ion medium was 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer, pH 6.8, containing
EDTA and 3 mM sodium mercaptoacetate. Substrate was added under
n and reaction was terminated by the addition of 6 ml of ice cold
. Porphyrinogens content of the incubation mixture was then
ed as previously reported (21) by a HPLC separation of the
onding extracted and methylated porphyrin mixture. At the same
ank samples were prepared in which the same amount of tissue and
trate were added in order to determine the endogenous porphyrin
of each tissue.
ntent was subtracted from the amount of porphyrins recovered in
les before calculating the enzymatic activity. Statistical
ance of differences between groups was assessed by Student's

ration of liver supernatant

minary experiments, in order to eliminate endogenous porphyrin
, liver supernatant was passed through Sephadex C-25 Medium type
ia, Uppsala, Sweden) columns.
lumns of I.D. 1 cm were filled up to 27 cm height with the resin
l in 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer pH 6.8 and stored at 4°C.
of 1.5 ml of liver supernatant were applied and eluted with
$_2HPO_4/NaH_2PO_4$ buffer pH 6.8; fractions of 0.5 ml were collected.
determinations of the enzymatic activity only those fractions
owed the lowest fluorescence under U.V. light were used.

## RESULTS

### Porphyrogenic effect of chronic TCDD treatment in different tissues

Data reported in Table 1 and 5 show that porphyrins did not accumulate at the same extent in all the tissues of mice chronically treated with high doses of TCDD.

Liver was the most affected organ with a mean value of porphyrin content which was 104 times greater in porphyric than in control animals, then marked modifications were observed in kidney (18 times) and spleen (31 times). Apparently no effect was evident on the brain nor on the erythrocytes.

A detailed analysis of the porphyrin pattern present in the normal tissues showsthat in the liver (Table 1) all the porphyrins from 8 to 4 carboxylic side chains were detectable and the pattern was uro > hepta > copro >> penta and hexa with the percentage ratio to uroporphyrin content being 11.6 and 2.5 respectively for heptacarboxylic- and coproporphyrin.

This porphyrin pattern was markedly modified by TCDD treatemnt becoming uro > hepta>> hexa > penta >>copro and also the percentage ratio of coproporphyrin to uroporphyrin was greatly reduced (0.06). Similar modifications were observed also in kidney (Table 1) and spleen (data not reported) while no significative variation was evident in both brain and erythrocytes.

### Effect of chronic TCDD treatment on porphyrinogen carboxy-lyase activity of different tissues

Assays of the enzyme activity were performed as described in Materials and Methods using the 40.000xg supernatant without previous elimination of the endogenous porphyrin content for all the tissues, both normal and porphyric.

This procedure was used after preliminary experiments (Table 2) which showed that removal of 95% of endogenous porphyrin content did not affect porphyrinogen carboxy-lyase activity. This result obtained with liver supernatant, was considered as conclusive also for kidney and spleen since liver was the most affected organ.

The percentage distribution of porphyrinogens present in the incubation mixture at the end of the incubations and porphyrinogen carboxy-lyase activity of different tissues of control and treated mice are reported in Table 3,4,5.

Porphyrinogen carboxy-lyase activity was expressed by measuring uroporphyrinogen decarboxylation as the sum of the hepta-, hexa-, penta- and tetracarboxylic porphyrinogens produced from the substrate uroporphyrinogen III during the incubation; heptaporphyrinogen decarboxylation as the sum of hexa-, penta-, and tetracarboxylic porphyrinogens; hexaporphyrinogen decarboxylation as the sum of penta- and tetracarboxylic porphyrinogens; pentaporphyrinogen decarboxylation as the amount of coproporphyrinogen.

Results show that in all tissues considered, both in control and in TCDD treated animals, it was present a measurable porphyrinogen carboxy-lyase activity.

In control animals enzymatic activity were similar in liver, kidney and spleen while they were comparably lower in brain and, most of all, in erythrocytes. Treatment with TCDD (Table 3) induced a marked degree of enzyme inhibition first of all in liver and then in kidney; this effect was evident considering any step of decarboxylation. Furthermore, in both these organs, the percentage of inhibition was higher measuring the rate of the whole process (i.e. 93.4 and 76 respectively for liver and kidney, considering the amount of coproporphyrinogen formed) than taking into account only the rate of the first decarboxylation (i.e. 72 and 36 respectively for liver and kidney for the uroporphyrinogen decarboxylation); intermedian decarboxylation steps had corresponding intermedian percentage of inhibition.

Apparently treatment with TCDD did not affect brain and erythrocytes porphyrinogen carboxy-lyase (Table 4). In all these tissues, the different degrees of inhibition reported paralleled well the extent of endogenous accumulation of porphyrins previously described.

On the contrary, results of more difficult interpretation were obtained with spleen tissue (Table 5).

Analysis of the determinations of the activity of porphyrinogen carboxy-lyase showed that, surprisingly, there was a wide variability in the

answer to TCDD intoxication; furthermore, data could be divided into two groups, TCDD-1 and TCDD-2, in which the enzyme was found, respectively, significatively induced and inhibited (109% and 78% expressed coproporphyrinogen formation).
Both these effects were evident when comparing the rate of all the decarboxylations except the first, from uro- to heptacarboxylic porphyrinogen.
A significative difference was also obtained comparing TCDD-1 and TCDD-2 values.
Division of the data obtained with tissues from TCDD treated animals was also suggested by the finding that the two groups were significatively different as extent of endogenous porphyrin accumulation, a higher porphyrin content being observed in the "inhibited" group, TCDD-2. However also the "activated" group, TCDD-1, had a significatively enhanced tissue porphyrin content in comparison to controls.
It could be important to remind here also that each enzyme preparation was obtained from pools of two organs from two animals randomly chosen in the same group and that the porphyrinogen carboxy-lyase activity of the corresponding livers was always markedly reduced in comparison to control values (data not reported).
Analysis of the porphyrinogens percentage distribution, present at the end of the incubation, show that in all control tissues the 4 successive decarboxylations performed by the enzyme had a different velocity one from the other, this difference being most of all present between the first and the three other successive decarboxylations.
In particular, the high accumulation of heptacarboxylic porphyrinogen indicates that the first decarboxylation of uroporphyrinogen was apparently the fastest step. The successive decarboxylations appeared less differentiated; however it was found in all tissues that a smaller amount of pentacarboxylic porphyrinogen accumulated in comparison to hexacarboxylic- and copro porphyrinogen.
This finding suggests that the formation of the intermediate with 5 carboxylic groups was likely the slowest step of the whole process.
In unaffected tissues of TCDD treated animals, erythrocytes and brain (Table 4), porphyrinogens percentages were never different from those of controls.
On the contrary, in liver and kidney (Table 3), a higher percentage

of the unmetabolized substrate uroporphyrinogen and lower percentages of all the decarboxylations products were found, due to the TCDD induced enzyme inhibition. Accumulation of the heptacarboxylic intermediate was still higher in comparison to the other porphyrinogens, but the relative ratios of percentage formation of the various intermediates were also reduced. This effect was especially evident considering the step pentacarboxylic→ coproporphyrinogen formation, which was markedly slowed, particularly in the liver.

## DISCUSSION

Chronic TCDD administration to male mice had a porphyrogenic effect not only in liver, as already reported (22,23), but also in other tissues, i.e. kidney and spleen. In all these tissues, in agreement with a previous study performed in female rats (14), derangement of porphyrin metabolism was evident first of all from a marked porphyrin accumulation, now characterized as mostly due to uro- and heptacarboxylic porphyrin. This pattern of porphyrin accumulation has been shown with other polyhalogenated compounds such as HCB (8) to be the result of an inhibition of the enzyme prophyrinogen carboxy-lyase. The same correlation was obtained also in the present study for the liver and kidney and extended to some extent, to spleen.

Liver was the principal site of porphyrogenic action of TCDD, with this resembling the form of porphyria produced by HCB (8,24). Kidney was next in decreasing effect and this result indicates an active role also for this organ in the pathobiochemical changes observed in the course of the intoxication, like the overproduction of urinary porphyrins, widely documented in other studies (14,25). A similar suggestion was also done by Day et al. (26) who analyzed data from clinical studies in patients with PCT.

Despite, the use of a fairly potent compound like TCDD, we did not find any alteration in the activity of the enzyme porphyrinogen carboxy-lyase of the erythrocytes.

This suggests that the porphyrogenic effect of an exogenous compound cannot be evidentiated in this tissue and that this type of experimental model is effective only for the "sporadic type" of human PCT.
Also brain did not respond to the porphyrogenic effect of TCDD and this finding could correlate with the lack of neurological symptoms usually reported in the human forms of PCT (27).
This study also showed that spleen tissue does not respond unequivocally to a porphyrogenic compound, as far as the behaviour of the porphyrinogen carboxy-lyase is concerned; contraddictory results were also present in the literature since both enzyme activation and depression, although not statistically significant, have been reported concomitantly to a significative increase of tissue porphyrin content after the less potent compound HCB (8,24). However we observed a possible correlation between degree of porphyrin accumulation and enzyme activity, with an inhibition corresponding to a higher accumulation and this suggests an evidentiation of the enzyme defect delayed with time and also a lower efficacy of porphyrogenic activity of TCDD in this tissue.
The variations observed in the enzyme activity could be related to other aspects of the TCDD induced toxicity in spleen (28,29) in particular although it is known that spleen weight is reduced by treatment with this compound (29,30), it has not yet been investigated whether the relative proportions of the different populations of cells of this organ are specifically altered, as it has been reported with other compounds (31). It can be concluded from our results that TCDD effect on porphyrinog carboxy-lyase is fairly specific for each tissue. A first correlation could be found between extent of TCDD accumulation and degree of effect since liver, following repeated and single oral doses, was shown the site of major accumulation in the body followed by kidney and spleen (32).
This tissue specificity would also be in agreement with the hypothesis (33) that in C57Bl/6 mice TCDD toxic effects would be dependent by the saturation of the stereospecific receptor present at various extent in the cytosol of various tissues (34,35,36).

However at present it is not known which is the direct cause of the enzyme inhibition. Furthermore it has also been shown that, unlike other effects of TCDD, like AHH induction, other requirements like tissue iron are necessary to produce a decrease in the porphyrinogen carboxy-lyase activity (25).

Both this requirement and other similarly important aspects like extent and type of metabolism and availability of intracellular antioxidants compounds (15,16,37) were certainly different in the various organs investigated in this study.

On the contrary it can be excluded, as also reported by Smith et al. (9) that porphyrins accumulation in the affected tissues could, by itself, reduce the activity of the porphyrinogen carboxy-lyase since removal of the excess porphyrins did not change the enzymatic activity.

Measurements of all the different reaction products of porphyrinogen carboxy-lyase permitted to show that quite likely each of the steps of decarboxylation was affected and in a different way. This suggestion is mainly due to the finding that modifications were observed in all the relative proportions of accumulation of the intermediate porphyrinogens. Furthermore, like uroporphyrinogen, added at the beginning as substrate, also heptacarboxylic porphyrinogen accumulated so that it is unlikely that the diminished rate of hepta-carboxylic decarboxylation could be due to a substrate deficiency. This was particularly evident in the kidney, where the percentage of inhibition was lower.

In agreement with studies relative to the response of the enzyme both in vitro to various agents (38) and in vivo to HCB (8), also porphyrinogen carboxy-lyase inhibition induced in vivo by TCDD was found higher in the second stage of the reaction, measured from the amount of copro-porphyrinogen formed (39) than in the first (uroporphyrinogen decarboxylation; 39). In conclusion we have shown that TCDD produces porphyrogenic effects of different degree in various organs; in particular a pattern of distribution of porphyrinogen carboxy-lyase activity markedly different from that of controls is observed in TCDD animals, with liver enzyme functioning at a comparable rate with that of erythrocytes and kidney enzyme with that of brain. Furthermore the type of alterations induced seems a suitable experimental model for the human "sporadic type" of PCT.

Table 1 - Porphyrin accumulation in tissues of control and TCDD treated mice[a]

| Tissue | Group | Total porphyrin content (nmol/g tissue or ml blood) | Distribution of porphyrins (nmol/g tissue) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 8-COOH | 7-COOH | 6-COOH | 5-COOH | 4-COOH |
| Liver | Control | 0.894 ± 0.524 | 0.77±0.46 | 0.09±0.004(11.6) | <0.01 | <0.01 | 0.02±0.00(2 |
| | TCDD | 93.385 ± 26.099* | 82.85±23.72 | 9.97±2.33 (12) | 0.38±0.06(0.4) | 0.12±0.00(0.14) | 0.05±0.00(0 |
| Erythrocytes | Control | 0.020 ± 0.001 | 0.02±0.00 | n.d. | n.d. | n.d. | n.d. |
| | TCDD | 0.028 ± 0.003 | 0.02±0.00 | < 0.01 | n.d. | n.d. | n.d. |
| Kidney | Control | 0.212 ± 0.154 | 0.19±0.15 | 0.01±0.00 | n.d. | n.d. | < 0.01 (~5.: |
| | TCDD | 3.867 ± 1.397* | 4.23±1.76 | 0.72±0.50 (17) | n.d. | n.d. | 0.01±0.00(0. |
| Brain | Control | 0.041 ± 0.012 | 0.04±0.01 | n.d. | n.d. | n.d. | < 0.01 |
| | TCDD | 0.097 ± 0.024 | 0.08±0.02 | 0.02±0.00 | n.d. | n.d. | n.d. |

[a]Each value represents the mean ± S.E. of 4 different experiments in which tissues from 2 animals were pooled.

n.d. = not detectable

* $p \leq 0.05$ by Student's t test in comparison to control value

Table 2 - Porphyrin content and hepatic porphyrinogen carboxy-lyase activity of control and TCDD treated mice before and after gel filtration

| Tissue | Group | Porphyrin content (nmol/g tissue) | | Porphyrinogen carboxy-lyase activity[a] (pmol/min/mg$^{p}$) | |
|---|---|---|---|---|---|
| | | Before Sephadex treatment[b] | After Sephadex treatment | Before Sephadex treatment | After Sephadex treatment |
| Liver | Control | 0.45 ± 0.16 | 0.02 ± 0.00 (95)[c] | 16.779 ± 1.631 | 15.880 ± 1.540 |
| | TCDD | 77.24 ± 46.25 | 1.84 ± 0.79 (97) | 3.606 ± 0.163 | 3.520 ± 0.239 |

[a]Enzyme activity is expressed as uroporphyrinogen decarboxylation i.e. as the sum of hepta-, hexa-, penta-, and tetra-carboxylic porphyrinogens produced during the incubation. Corrections were made for endogenous or remaining porphyrin content in the enzyme preparation.

[b]Percentage of prophyrins eliminated by the Sephadex treatment.

Each value represents the mean ± S.E. of three different experiments in which livers from 2 animals were pooled.

Table 3 - Porphyrinogen carboxy-lyase activity in liver and kidney of control and TCDD treated mice with uroporphyrinogen III as substrate

| Tissue | Group | Porphyrinogens (%)[a] | | | | | Enzyme activities (pmol/min/mg P)[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 8-COOH | 7-COOH | 6-COOH | 5-COOH | 4-COOH | Uroporphyrinogen decarboxylation | Heptaporphyrinogen decarboxylation | Hexaporphyrinogen decarboxylation | Pentaporphyrinoge decarboxylation |
| Liver | Control | 61 | 23 | 5.8 | 2.8 | 6.6 | 17.196±1.463 | 6.816±0.703 | 4.222±0.497 | 2.952±0.371 |
| Liver | TCDD | 93 | 4.9 | 0.8 | 0.2 | 0.2 | 4.904±0.326* (72)[c] | 1.055±0.076* (85) | 0.359±0.067* (91.5) | 0.195±0.023* (93 |
| Kidneys | control | 62 | 24 | 5.5 | 2.4 | 5.6 | 17.455±0.763 | 6.150±0.379 | 3.639±0.243 | 2.555±0.195 |
| Kidneys | TCDD | 78 | 16 | 3.1 | 0.9 | 1.2 | 11.241±0.739* (36) | 2.736±0.273** (56) | 1.117±0.121** (68) | 0.618±0.080** (7 |

[a] Percentage distribution of porphyrinogens present in the incubation mixture at the end of the incubation. Endogenous porphyrin content was subtracted.

8-COOH = uroporphyrinogen; 7-COOH = heptaporphyrinogen, 6-COOH = hexaporphyrinogen; 5-COOH = pentaporphyrinogen; 4-COOH = tetraporphyrinogen.

[b] Uroporphyrinogen decarboxylation was measured as the sum of hepta-, hexa-, penta- and tetracarboxylic porphyrinogens produced during the incubation; heptaporphyrinogen decarboxylation as the sum of hexa-, penta-, and tetracarboxylic porphyrinogen decarboxylation as the sum of penta- and tetracarboxylic porphyrinogens; pentaporphyrinogen decarboxylation as the amount of tetracarboxylic porphyrinogen conditions of incubation were as described under Materials and Methods.

[c] Percentage of inhibition in comparison to the corresponding control value.

Each value represents the mean ± S.E. of 4 different experiments in which livers from 2 animals were pooled.

* $p \leq 0.01$ by Student's t test

** $p \leq 0.001$ " "

Table 4 - Porphyrinogen carboxy-lyase activity in erythrocytes and in brain of control and TCDD treated mice with uroporphyrinogen III as substrate

| Tissue | Group | Porphyrinogen (%)[a] 8-COOH | 7-COOH | 6-COOH | 5-COOH | 4-COOH | Uroporphyrinogen decarboxylation | Heptaporphyrinogen decarboxylation | Hexaporphyrinogen decarboxylation | Pentaporphyrin decarboxylatic |
|---|---|---|---|---|---|---|---|---|---|---|
| rythrocytes | Control | 84 | 11.7 | 2.2 | 0.6 | 1.17 | 2.887±0.214 | 0.653±0.027 | 0.267±0.005 | 0.163±0.006 |
| rythrocytes | TCDD | 76 | 16 | 3.25 | 0.9 | 1.73 | 3.080±0.138 | 0.667±0.014 | 0.263±0.010 | 0.151±0.010 |
| rain | Control | 82 | 12.9 | 2.6 | 0.9 | 1.23 | 10.439±0.291 | 2.783±0.151 | 1.248±0.085 | 0.726±0.056 |
| rain | TCDD | 81 | 14.1 | 2.8 | 0.9 | 1.48 | 11.901±1.050 | 3.211±0.398 | 1.457±0.243 | 0.197±0.185 |

Percentage distribution of porphyrinogens present in the incubation mixture at the end of the incubation.

ndogenous porphyrin content was subtracted.

-COOH = uroporphyrinogen; 7-COOH = heptaporphyrinogen; 6-COOH = hexaporphyrinogen; 5-COOH = pentaporphyrinogen; 4-COOH = tetra-orphyrinogen.

Enzyme activities were measured as described in the legend to Table 3

ach value represents the mean ± S.E. of 4 (brain) or 6 (erythrocytes) different experiments in which organs from 2 animals were ooled.

Table 5 - Porphyrin accumulation and porphyrinogen carboxy-lyase activity in spleen of control and TCDD treated mice with uroporphyrinogen III as substrate.

| Tissue | Group | Total porphyrin content (nmol/g tissue) | Enzyme activities (pmol/min/mg P)[2] | | | |
|---|---|---|---|---|---|---|
| | | | Uroporphyrinogen decarboxylation | Heptaporphyrinogen decarboxylation | Hexaporphyrinogen decarboxylation | Pentaporphyrinogen decarboxylation |
| Spleen | Control | 0.063±0.005 | 14.302±1.221 | 4.497±0.262 | 2.619±0.195 | 1.931±0.159 |
| | TCDD-1 | 1.188±0.238°*** | 15.334±2.289 | 6.673±0.85(48)***(b)°° | 5.038±0.682*(92)°°° | 4.037±0.623*(109 |
| | TCDD-2 | 2.767±0.688*** | 13.677±0.877 | 2.611±0.094(42)c*** | 0.956±0.118***(64) | 0.435±0.121***(7 |

Enzyme activities were measured as described in the legend to Table 3

Each value represents the mean ± S.E. of 8 (controls) and 4 (TCDD 1 group) or 3 (TCDD 2 group) different experiment in which organs from 2 animals were pooled.

Percentage of activation in comparison to control values

Percentage of inhibition in comparison to control values

$p \leq 0.05$ by Student's t test in comparison to control values

$p \leq 0.02$ " " " " "

$p \leq 0.01$ " " " " "

$p \leq 0.05$ by Student's t test in comparison to TCDD-2 values

° $p \leq 0.02$ " " " "

°° $p \leq 0.001$ " " " "

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