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THE SITE OF INHIBITION OF PORPHYRIN BIOSYNTHESIS BY AN ISOMER OF DIAZINON IN RATS

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Abstract—2-Isopropyl-6-methyl-4-*S*-pyrimidinyl diethyl thiosulphate (isodiazinon) has been synthesized by an unambiguous route. Rats treated with isodiazinon over a 100-day period show decreased levels of liver ferrochelatase. Rats treated with diazinon and isodiazinon in combination over the same period show a more marked decrease in liver ferrochelatase activity as well as a decrease in the activity of coproporphyrinogen oxidase. Treatment of rats with stabilised diazinon over the same period is not associated with a decrease in the activity of either enzyme. Neither diazinon nor isodiazinon causes a decrease in the activity of glutamate dehydrogenase, succinate dehydrogenase or kynurenine hydroxylase, suggesting that the effect is specific to the porphyrin biosynthesis pathway and not due to mitochondrial damage.

Diazinon, an organophosphate insecticide used widely in agriculture, has been associated with some cases of porphyria cutanea tarda [1, 2]. Although pure diazinon when applied to the skin of rats over a 100-day period does not cause an increase in porphyrin excretion, technical grade material when applied for a similar period causes a significant increase in faecal porphyrin excretion [1]. When cultures of chicken embryo hepatocytes are treated with low levels of diazinon and its impurities, accumulation of coproporphyrin and, to a lesser extent, protoporphyrin occurs in the medium. The compound which causes greatest porphyrin accumulation is an isomer of diazinon, 2-isopropyl-6-methyl-4-*S*-pyrimidinyl diethyl thiophosphate. This compound has been designated isodiazinon [3]. It has been suggested that it is the presence of this compound in some diazinon preparations which is associated with disturbances of porphyrin metabolism in man [3]. The present investigation was begun with the aim of establishing the site in the porphyrin biosynthetic pathway at which isodiazinon acts.

MATERIALS AND METHODS

Analytical methods. High performance liquid chromatography (HPLC) separations were performed using a Pye Unicam LC3 pump equipped with an LC–UV detector. A 10 μ Partisil column (250 mm × 4 mm) was used. Conditions used routinely were: chloroform–cyclohexane (40:60) as solvent; a flow rate of 2 ml/min; and a detector setting of 260 nm. Solvents were HPLC grade from Burdick & Jackson. All UV–visible spectrophotometry was carried out using a Unicam SP 1800 spectrophotometer. NMR spectra were determined using a Varian 360 60 mHz instrument in $CDCl_3$ as solvent and using tetramethylsilane as internal standard.

Chemicals. Diazinon, pure by HPLC, was a gift from Ciba–Geigy as was 2-isopropyl-6-methylpyrimid-4-one.

2-Isopropyl-6-methylpyrimidine-4-thione was prepared as follows: A commercial diazinon preparation (Nucidol, 100 g) was subjected to vacuum-distillation (2 mm Hg) until the temperature of distillation rose to 120°. The residue (45 g) was mixed with an equal volume of dry pyridine. Hydrogen sulphide was bubbled through the stirred solution for a period of 5 hr. After standing for a further 12 hr under nitrogen, the mixture, which had separated into two phases, was adjusted to pH 1 by adding 10 M HCl. Following the addition of an equal volume of water, the mixture was extracted with ethyl acetate (30 ml). The upper phase was washed with a small volume of 2 M HCl. The two lower phases were combined and adjusted to pH 4.5 with 5 M ammonium hydroxide. A crystalline precipitate formed which was filtered off and recrystallised from aqueous methanol to yield 2-isopropyl-6-methylpyrimidine-4-thione (8.7 g, m.p. 155–156°, yield 35%). The spectroscopic properties of this compound were identical with those of the thione prepared earlier by an unambiguous method [3].

Isodiazinon could not be prepared by direct reaction of the thione or its potassium salt with diethyl chlorophosphate. The major product of this reaction was diazinon itself. A successful preparation was achieved through the use of the sulphenyl chloride as an intermediate and reaction with triethyl phosphite as follows:

2-Isopropyl-6-methylpyrimidine-4-thione (2 g) was dissolved in chloroform (ethanol-free, 30 ml). *p*-Toluene sulphonic acid (2.26 g) was added and after complete solution had taken place the mixture was cooled to 0°. Chlorine (0.96 g) in solution in dry carbon tetrachloride (approximately 15%) was added dropwise with stirring over a 5-min period. A pale yellow precipitate formed during this addition but redissolved following complete addition of the chlorine. Following the addition of triethyl phosphite

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(2.06 g), a precipitate again appeared. The mixture was stirred for 15 min, following which it was washed five times with 1% aqueous potassium carbonate. After drying the colourless solution over anhydrous K_2CO_3 for a period of 5 min, the solution was passed rapidly through a short column of alumina (activity I, 1 cm × 6 cm) to effect complete drying. The solution was evaporated to dryness *in vacuo* at a temperature not greater than 40°. The residue showed three components by HPLC. Using chloroform–cyclohexane (40:60) as solvent and a flow rate of 2 ml/min, the components had retention times of 1.6, 2.6 and 6.0 min. These retention times corresponded to diazinon, isodiazinon and an unknown component, respectively. The yield of the unknown component, possibly a phosphonate, was much higher if *p*-toluene sulphonic acid was not included in the reaction mixture. Further purification of the crude reaction mixture was achieved by dissolving it in cyclohexane (5 ml) and chromatographing the mixture on a column of alumina (activity III, 13 cm × 2.5 cm) using cyclohexane as eluant. The composition of the fractions was checked by HPLC. Fractions containing pure isodiazinon were combined and evaporated to dryness at a temperature not greater than 40°. The product (0.8 g) was allowed to stand in high vacuum over paraffin flakes for 12 hr before it was stored in liquid nitrogen (see below). Isodiazinon prepared by this method showed NMR, IR and UV spectra identical with those of isodiazinon isolated from technical grade diazinon [3]. Isodiazinon could not be purified by vacuum-distillation, which resulted in its decomposition to 2-isopropyl-4-ethylthio-6-methylpyrimidine. Storage at room temperature resulted in its decomposition to the same compound. For this reason, isodiazinon to be used in the rat trials was stored as a 10% (w/v) solution in xylene in liquid nitrogen. Under these conditions no change detectable by HPLC was observed over a period of 3 months.

Treatment of animals. Female DA (Dark Agouti) rats (120–150 g body weight) were housed in individual metal cages and were fed on a diet of Allied Feeds rat cubes. Each individual experiment was carried out using a group of six rats. The rats were shaved just above the tail and the compound being tested was applied daily to the shaved area of skin using a Gilson pipette. Rats used as controls were treated with 20 μl of xylene daily; rats treated with isodiazinon received 20 μl of a 10% (w/v) solution of isodiazinon in xylene; rats treated with diazinon received 20 μg of diazinon daily; rats receiving the isodiazinon–diazinon mixture were treated with 20 μg of a 10% (w/v) solution of isodiazinon in diazinon. Total faecal porphyrins were estimated weekly following collection of a 24-hr sample of faeces. The method used was that of Lockwood [4]. Thin layer chromatography of the faecal porphyrins was carried out by the method of Smith [5]. After 100 days of treatment the rats were sacrificed by decapitation, exsanguinated and the livers removed. The livers were roughly minced and washed in 0.15 M KCl and homogenised using a Potter homogeniser in three volumes of 0.25 M sucrose.

Enzymatic methods. Unless otherwise stated, reagents were from Sigma.

Cytochrome P-450 was measured in the homogenates by the method of Joly *et al.* [6]. δ-Aminolaevulinic acid synthase was measured by the method of Marver *et al.* [7] as modified by Yoshida *et al.* [8]. The addition of sodium succinate (10 μmole) and ATP (2 μmole) was found to be necessary for maximal production of ALA.

ALA-dehydratase was measured by the method of Shemin [9]. Porphobilinogen deaminase was measured by the method of del C. Batlle *et al.* [10] using 0.2 ml of liver homogenate in place of 0.5 ml of red cell homogenate. Uroporphyrinogen decarboxylase was measured by the method of Elder *et al.* [11] using pentacarboxylate porphyrinogen as substrate.

Coproporphyrinogen oxidase was assayed using coproporphyrinogen specifically labelled with tritium on the methylene groups of the 2- and 4-carboxyethyl substituents. Tritium released during the oxidative decarboxylation of coproporphyrinogen was counted following absorption of the porphyrinogens onto charcoal. Full details of the preparation of the substrate and the method itself are to be published separately [12].

Ferrochelatase was assayed radiochemically by the following method: An $^{59}Fe(II)$ stock solution was prepared by dilution of 10 μl of $^{59}Fe(II)$ citrate (Amersham, specific activity 28 mCi/mg, 1 mCi/ml) to 1 ml with 0.25 mM ferrous sulphate. Ascorbic acid (3 mg) was added followed by enough anhydrous sodium carbonate to adjust the pH to 7.0 (approximately 5 mg). The solution was stored under nitrogen.

Mesoporphyrin was used as substrate for the enzyme rather than protoporphyrin because of its greater stability. A stock solution was prepared by hydrolysis of a few mg of the dimethyl ester (prepared according to Falk [13]) using acetic acid – 10 M HCl (10:1) for 24 hr at room temperature. After evaporation of the solvent *in vacuo*, the residue was converted to the ammonium salt by exposure to ammonia vapour, and dissolved in 0.01 M KOH to give a concentration of 0.25 mM. The concentration was determined by accurate dilution with 0.1 M HCl and the use of a millimolar extinction coefficient of 445 at the Soret maximum (399 nm). The assay was carried out in 1 ml Eppendorf tubes. Ten μl of mesoporphyrin solution (2.5 nmole) was added to enough 0.2 M Tris–HCl, pH 8.2, containing 0.1% Tween 20 to give a final volume of 0.1 ml. Ten μl of homogenate which had been diluted with an equal volume of Tris–Tween buffer was added. The tube was flushed with nitrogen, sealed and pre-incubated at 37° in a shaking water bath. Ten μl of $^{59}Fe(II)$ stock solution (2.5 nmole) was added, the tube again flushed with nitrogen, sealed and incubated with shaking for 30 min. The reaction was terminated by addition of 0.1 ml of 8 M urea and 50 μl of 1 mM haematin as carrier in Tris–Tween buffer. After incubation for 5 min at 37°, 0.9 ml of ethanol–acetic acid (5:1) was added. The mixture was vortexed, centrifuged and the supernatant transferred to another tube containing 0.2 ml of a 2% suspension of activated charcoal (Norite) in ethanol. The suspension was mixed with a vortex mixer for 1 min and centrifuged. The charcoal was washed by addition

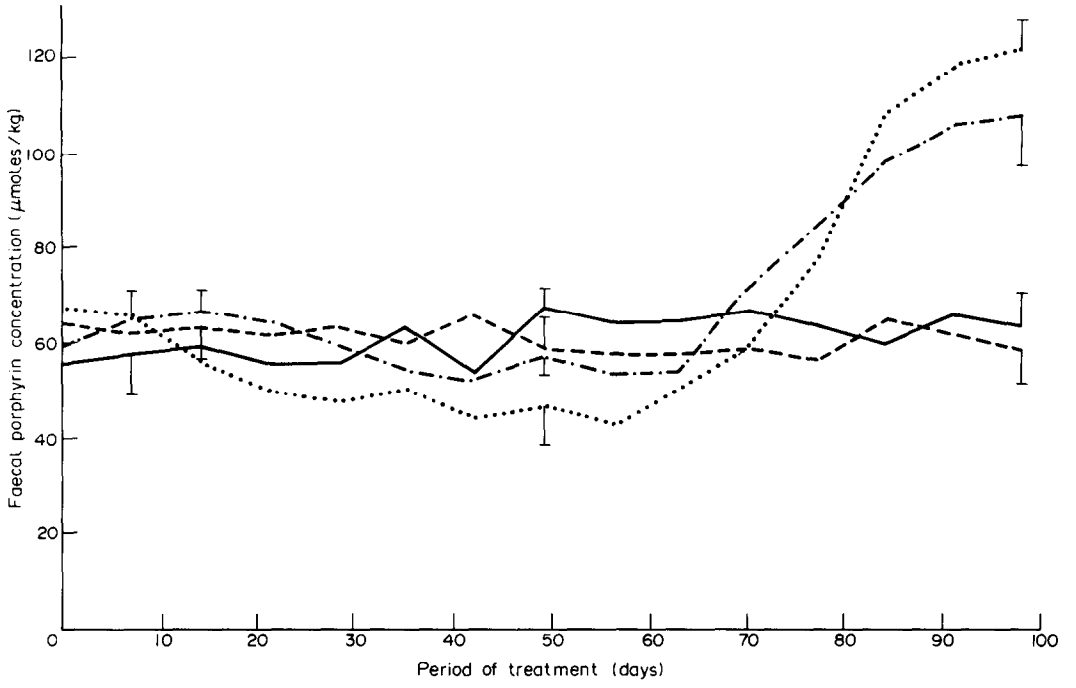

Fig. 1. Effect of faecal porphyrin excretion of treating rats with diazinon (——), isodiazinon (–·–·–), diazinon: isodiazinon mixture (····) and controls (– – –). Each point represents six animals. Representative S.D. are shown by the bars.

of 1 ml of 0.1 M HCl, vortexing and centrifugation. This procedure was repeated twice before absorbed radioactivity was counted on the charcoal using an Abbott ABA 100 gamma counter. Results were corrected for non-enzymic formation of haem by subtracting blank values obtained from incubation mixtures containing buffer in the place of homogenate. Using this method, reaction rates were constant for up to 30 min incubation and were linear with respect to the protein concentration of the liver homogenate. Non-enzymic reaction rates were negligible.

Estimations of succinate dehydrogenase and glutamate dehydrogenase were by standard methods [14]. Kynurenine hydroxylase was estimated by the method of Nisimoto *et al.* [15].

Liver porphyrins. Liver porphyrin concentrations were measured fluorimetrically following mixing of 1 part of homogenate with 9 parts of methanol, 10% perchloric acid and centrifugation. An excitation wavelength of 403 nm and an emission wavelength of 598 nm were used.

RESULTS

Faecal porphyrins

Faecal porphyrin analyses were carried out in order to determine the point at which the rats should be sacrificed for the enzymic studies. Previous work [1] had shown that the excretion of urinary porphyrin does not change significantly when the rats are treated with diazinon preparations. The results of faecal porphyrin estimations are shown in Fig. 1. Pure diazinon did not cause a significant increase in faecal porphyrin excretion during the course of treatment. Significant changes were seen with rats treated with isodiazinon and with the isodiazinon–diazinon mixture. In these rats the increase in faecal porphyrin is primarily due to increasing excretion of protoporphyrin and coproporphyrin, although small amounts of isocoproporphyrin and pentacarboxylate porphyrins are also seen (Table 1).

Table 1. A typical analysis by thin layer chromatography of porphyrins from pooled faeces of six rats after 100 days of treatment

Porphyrin	Controls	Rats treated with diazinon : isodiazinon (9:1)
Protoporphyrin	39.1	72.0
Coproporphyrin	19.5	36.8
Isocoproporphyrin	ND	5.4
Pentacarboxylate porphyrin	ND	7.2
Other porphyrins (6–8 carboxylate)	ND	2.6

Units are micromole/kg dry weight.

As noted earlier [1], diazinon–isodiazinon causes a decrease in faecal porphyrin excretion in the early phase of treatment.

Enzyme analyses

Results of enzyme analyses are shown in Table 2. Significant differences from controls are seen in the level of coproporphyrinogen oxidase in rats treated with the isodiazinon–diazinon mixture, and in the levels of ferrochelatase in rats treated both with isodiazinon and with the isodiazinon–diazinon mixture.

Table 2. Enzyme analyses on homogenates of rat liver following 100 days of treatment with diazinon and isodiazinon

	Cytochrome P-450	ALA synthase	ALA dehydratase	Uroporphyrinogen synthase
Controls	126 ± 21	2.74 ± 0.62	32.9 ± 1.32	1.87 ± 0.12
Diazinon	134 ± 10	3.49 ± 1.09	31.7 ± 1.37	1.99 ± 0.23
Isodiazinon	135 ± 17	2.57 ± 1.32	29.8 ± 8.70	1.86 ± 0.23
Diazinon: isodiazinon (9:1)	134 ± 14	2.48 ± 1.15	28.1 ± 4.95	2.06 ± 0.26

	Uroporphyrinogen decarboxylase	Coproporphyrinogen oxidase	Ferrochelatase (haem synthase)
Controls	41.1 ± 3.3	44.6 ± 3.8	136 ± 14.2
Diazinon	37.1 ± 3.6	48.8 ± 5.5	143 ± 18.3
Isodiazinon	39.9 ± 2.6	40.0 ± 3.8	118 ± 12.8*
Diazinon: isodiazinon (9:1)	42.2 ± 6.8	32.1 ± 5.7*	79 ± 6.6*

	Succinate dehydrogenase	Glutamate dehydrogenase	Kynurenine hydroxylase
Controls	11,710 ± 4530	6250 ± 1850	195 ± 41
Diazinon	9400 ± 2510	7680 ± 760	167 ± 39
Isodiazinon	11,560 ± 3590	5220 ± 1230	177 ± 56
Diazinon: isodiazinon (9:1)	11,460 ± 3920	5650 ± 1040	165 ± 27

Results are means ± S.D.

Units for cytochrome P-450 analysis are pmole of P-450 haem per mg of protein; the units for all other enzymes are pmole of product formed per min per mg of protein except for coproporphyrinogen oxidase where units are pmole of substrate transformed per min per mg of protein.

* $P < 0.01$.

Liver porphyrins

Results of liver porphyrin analyses are shown in Table 3. As with the results of faecal porphyrin analysis, a significant increase in porphyrin concentration is seen only in the livers of rats treated with the isodiazinon and the isodiazinon–diazinon mixture. Consistent with the results of enzymic analyses, the porphyrin accumulating in the livers of both of these groups of rats was protoporphyrin with only traces of coproporphyrin being present.

DISCUSSION

The results show that the activity of the enzyme ferrochelatase is lowered significantly by prolonged treatment of rats with isodiazinon. Mixtures of isodiazinon and diazinon cause an even more marked lowering of the level of this enzyme, as well as a lowering of the level of coproporphyrinogen oxidase (Table 3). Possible mechanisms which would explain these effects are: direct inhibition of the enzymes by diazinon–isodiazinon or by their metabolites; production of inhibitory porphyrins such as *N*-alkylporphyrins [16]; or interference with the synthesis of ferrochelatase and coproporphyrinogen oxidase, possibly by a non-specific effect on mitochondrial protein synthesis. Although gross damage to mitochondria is eliminated by the absence of any diminution in the activity of the mitochondrial marker enzymes succinate dehydrogenase and glutamate dehydrogenase, and the outer mitochondrial membrane enzyme kynurenine hydroxylase, more specific effects on mitochondrial structure cannot be excluded. The slow production of *N*-alkylporphyrins is a more attractive hypothesis. These compounds act as powerful non-competitive inhibitors of ferrochelatase and are produced by other porphyrogens which cause protoporphyrin accumulation in the livers of treated animals. Possibly the most studied of these compounds is 3,5-diethoxycarbonyl-1,4-dihy-

Table 3. Porphyrin accumulation in livers of rats treated for 100 days with diazinon and isodiazinon

	Mean	S.D.
Controls	4.41	0.53
Diazinon	5.05	0.87
Isodiazinon	7.09*	1.05
Diazinon : isodiazinon (9:1)	8.10*	0.81

Results are expressed in pmole per mg of protein.

* $P < 0.01$.

drocollidine (DDC) [17]. The alkylating ability of organophosphorus compounds is well known [18] and is associated with increasing acute toxicity of these compounds. This hypothesis is especially attractive as preliminary results suggest a correlation between alkylating properties and ability to cause porphyrin accumulation in tissue culture for a range of organophosphorus compounds. Nonetheless, direct inhibition of ferrochelatase and coproporphyrinogen oxidase remains a possibility to be considered even though such a mechanism might be expected to operate more quickly than observed in this work. Davies and Holub [19], in a study of the effect of diazinon on plasma and brain acetylcholinesterase, observed that brain acetylcholinesterase is not reduced until the ninety-second day of treatment of female Wistar rats. This would suggest the presence of a 'detoxification pool' which is not exhausted until about the period of treatment used in the present study.

The observation that serum cholinesterase and erythrocyte acetylcholinesterase activities are rapidly reduced by diazinon treatment [19] lends support to this suggestion. Indeed, it provides an explanation for the marked synergism observed with mixtures of diazinon and isodiazinon. Rats treated with the mixture showed a much greater reduction in the activity of ferrochelatase than is seen in rats treated with isodiazinon alone. Because of the greater toxicity of isodiazinon, dose rates used with this compound were much lower than with diazinon. It is possible that this compound reacts preferentially with cholinesterases rather than with other enzyme systems. In the presence of large amounts of diazinon the 'cholinesterase pool' may be saturated with diazinon, making at least a portion of the more reactive compound available for reaction with liver protein systems.

Although many drugs which affect porphyrin biosynthesis cause stimulation of ALA-synthase activity together with changes in the level of cytochrome P-450 [20], significant changes in the levels of these two proteins were not seen in this study. It should be noted, however, that changes in the activity of ferrochelatase as a result of the treatment of rats or mice with griseofulvin and DDC are very much greater than those observed here [21]. Despite the apparent lack of induction of ALA-synthase, a moderate increase in liver porphyrin concentration is seen in rats treated with diazinon–isodiazinon. This increase is, however, much less than observed with either griseofulvin or DDC. A parallel is perhaps suggested with the asymptomatic heterozygous state of bovine protoporphyria where the activity of ferrochelatase relative to normal values is similar to that observed in this study [22]. A reduction in the level of ferrochelatase of this order may therefore not be expected to cause a measurable induction of ALA-synthase.

An additional feature of the effect of isodiazinon–diazinon on faecal porphyrin excretion is the statistically significant drop in faecal porphyrin level seen most clearly after 40 days of treatment (Fig. 1). Histological evidence in two rats killed at this point indicates that this effect is due to partial cholestasis, a phenomenon observed with other organophosphorus compounds [23].

Although earlier work suggests that some cases of porphyria cutanea tarda are associated with exposure to diazinon, this work shows that the enzyme uroporphyrinogen decarboxylase is unaffected by either diazinon or its isomer. The possibility that inhibition of ferrochelatase by diazinon may give rise to an overt porphyria in individuals with a pre-existing deficiency of uroporphyrinogen decarboxylase is under investigation.

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REFERENCES

1. P. Bleakely, A. W. Nichol and A. G. Collins, *Med. J. Aust.* **1**, 314 (1979).
2. C. Bopp and B. Kosminsky, *Med. Cutanae Ibero Lat. Am.* **3**, 271 (1975).
3. A. W. Nichol, S. Elsbury, G. H. Elder, A. H. Jackson and K. R. N. Rao, *Biochem. Pharmac.* **31**, 1033 (1982).
4. W. H. Lockwood, in *Porphyrins in Human Disease* (Ed. M. Doss) p. 486. Karger, Basel (1976).
5. S. G. Smith, *Br. J. Derm.* **93**, 291 (1975).
6. J. G. Joly, C. Dorjon and Y. Pesant, *Am. J. Pharmac.* **3**, 577 (1975).
7. H. S. Marver, D. P. Tschudy, M. G. Perbroth and A. Collins, *J. biol. Chem.* **241**, 2803 (1966).
8. T. Yoshida, Y. Suzuki and M. Uchiyama, *Biochem. Pharmac.* **24**, 1996 (1975).
9. D. Shemin, in *Methods in Enzymology* (Eds. H. Tabor and C. W. Tabor), Vol. 17, Part A, p. 205. Academic Press, New York (1970).
10. A. M. del C. Batlle, E. A. W. de Xifra and A. M. Stella, *Int. J. Biochem.* **9**, 871 (1978).
11. G. H. Elder, J. O. Evans and S. A. Matlin, *Clin. Sci. molec. Med.* **51**, 71 (1976).
12. A. W. Nichol and S. Elsbury, in preparation.
13. J. E. Falk, *Porphyrins & Metalloporphyrins*, pp. 177 and 236. Elsevier, Amsterdam (1964).
14. H. Tabor and C. W. Tabor (Eds.), *Methods in Enzymology*, Vols. I and II. Academic Press, New York (1955).
15. Y. Nisimoto, F. Takeuchi and Y. Shibata, *J. Biochem.* **78**, 573 (1975).
16. F. de Matteis, A. H. Gibbs and T. R. Tephly, *Biochem. J.* **188**, 145 (1980).
17. T. R. Tephly, B. L. Coffman, G. Ingall, M. S. Abou Zeit-Har, H. M. Goff, H. D. Tabba and K. M. Smith, *Archs Biochem. Biophys.* **212**, 120 (1981).
18. G. W. Fischer and K. Lohs, *Archs Geschwulstforsch.* **42**, 34 (1973).
19. D. B. Davies and B. J. Holub, *Archs envir. Contam. Toxic.* **9**, 637 (1980).
20. F. de Matteis, *Enzyme* **16**, 266 (1973).
21. F. de Matteis and A. H. Gibbs, *Biochem. J.* **146**, 285 (1975).
22. J. R. Bloomer, K. O. Morton, R. J. Reuter and G. R. Ruth, *Am. J. Human Genet.* **34**, 322 (1982).
23. E. A. Rodriguez Garay, J. A. Rassero and M. Palchik, *Farmaco Ed. Prac.* **34**, 157 (1979).