from the [2-^{3}H]cycloartenol and [2-^{3}H]lanosterol (sp. act. 2.55×10^6/dpm/500 µg/flask) feeds to *P. cactorum* and *L. giganteum*. However, the ^{3}H sample, eluted from the plates and diluted with cholesterol and a mixture of phytosterols, was found to elute much earlier then with the carrier sterols on HPLC. When similar experiments were performed with *S. ferax* significant activity was evident in the 4,4-desmethyl TLC band. In other work on the sterol composition of pollen we had recently found that certain cyclosteroids, e.g. dehydropollinastanol, co-chromatographed with 24-methylenecholesterol on GLC (3% SE-30) and may co-chromatograph with 24-methylenecholesterol, as the acetates when chromatographed on silver nitrate impregnated AL_2O_3 columns but eluted earlier on HPLC than either desmosterol or 24-methylenecholesterol. The radioactive material from the TLC 4,4-desmethyl zone injected into the HPLC from the [2-^{3}H]cycloartenol feed coincided with an authentic sample of dehydropollinastanol (fig. 1). In order to verify cycloartenol metabolism by *S. ferax*, we performed another feed on a large scale. From this latter feed we isolated a few µg of radioactive compound which corresponded to dehydropollinastanol. This material was diluted with 10 mg of dehydropollinastanol producing an initial sp. act. of 471 dpm/mg: recrystallization from acetone-water, methanol-water, hexane-dichloromethane, yielded a compound having a sp. act. of 332 dpm/mg, 323 dpm/mg and 319 dpm/mg. When 1.0×10^7 dpm/500 µg/flask [2-^{3}H]dehydropollinastanol was incubated with *S. ferax*, a single radioactive 4,4-desmethylsterol was recovered from the mycelia (fig. 1). While no metabolism of the dehydropollinastanol was obvious, some ($< 1\%$) esterification occurred. The other fungal groups, many of which synthesize ergosterol[2], presumably metabolize lanosterol and cyclosteroids in a similar manner. We conclude that the Oomycetes may have evolved in a polyphyletic manner[16,17] with respect to the other fungi. Their lack of having a cycloartenol-based pathway (fig. 2) is consistent with the view[18] previously advanced that the Oomycetes may have evolved through a nonphotosynthetic, lanosterol-biosynthesizing lineage which has its origins with the prokaryotes, rather than a cycloartenol-biosynthesizing photosynthetic eukaryote, such as, the brown or golden-brown algae.

1 Acknowledgment. *Saprolegnia ferax*-ATCC 3605 (1), *Lagenidium callinectes* ATCC 24973 (2), *Apodachlyella completa* (3) were obtained from Dr J. Aronson, Arizona State Univ. (3), *Lagenidium gigateum* was obtained from a drainage ditch in California (4), and *Phytophthora cactorum* was obtained from the U.C. Berkeley Fungal Collection (5). The fungi were cultured as previously described: Cultures 1,2,3: Berg, L.B., Ph. D. dissertation, Univ. of MD., College Park (1983); culture 4: Kerwin, J.L., and Washino, R.K., Exp. Mycol. *7* (1983) 109; culture 5: Nes, W.D., and Stafford, A.E., Lipids *19* (1984) 544. Preliminary observations involving the labeled substrates were presented by Le, P.H., Nes, W.D., and Parish, E.J, J. Am. Oil Chem. Soc. *62* (1985) 655(A). Please address all correspondence to W.D. Nes, Plant Physiology and Chemistry Research Unit, ARS-US Dept of Agriculture, Berkeley, CA 94710, USA.
2 Nes, W.R., and Nes, W.D., Lipids in Evolution. Plenum Press, New York 1980.
3 Klein, R.M., and Cronquist, A., Q. Rev. Biol. *42* (1967) 105.
4 Whittaker, R.H., Science *163* (1969) 150.
5 McMorris, T.C., Lipids *13* (1978) 716.
6 Bú Lock, J.D., and Osagie, A.U., Phytochemistry *15* (1976) 1249.
7 Warner, S.A., Eierman, D.F., Sovocool, G.W., and Domnas, A.J., Proc. natn. Acad. Sci. USA *79* (1982) 3769.
8 Vishniac, H.S., J. gen. Microbiol. *12* (1955) 464.
9 Bú Lock, J.D., and Dawson, C.J., J. Lipid Res. *17* (1976) 565.
10 Berg, L., Patterson, G.W., and Lusby, W., Lipids *18* (1983) 448.
11 Warner, S.A., Sovocool, G.W., and Domnas, A.J., Mycologia *75* (1983) 285.
12 Nes, W.D., and Heupel, R.C., Archs Bioichem. Biophys., *244* (1986) 211.
13 Bladocha, M., and Benveniste, P., Pl. Physiol. *71* (1983) 756.
14 Anding, C., Parks, L.W., and Ourisson, G., Eur. J. Biochem. *43* (1974) 459.
15 Buttke, T.M., and Bloch, K., Biochem. biophys. Res. Commun. *92* (1980) 229.
16 Nes, W.D., Heupel, R.C., and Le, P.H., in: Structure, Function and Metabolism of Plant Lipids, p.207. Eds P. Siegenthaler and W. Eichenberger. Elsevier Press, New York 1984.
17 Nes, W.D., and Stafford, A.E., Proc. natn. Acad. Sci. USA *80* (1983) 3227.
18 Nes, W.D., in: Isopentenoids in Plants, p.267. Eds W.D. Nes, G. Fuller and L.Tsai. Marcel Dekker Press, New York 1984.

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A new selective insecticidal uncoupler of oxidative phosphorylation

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Summary. A new aryl hydrazone structure with high insecticidal activity against the Australian sheep blowfly, *Lucilia cuprina*, was shown to have a higher activity as an uncoupler of oxidative phosphorylation in insect compared to mammalian mitochondrial preparations. This compound possesses the requirements of other uncouplers in its measured pKa and lipid solubility. However, when compared to a closely related structure with similar physicochemical properties, its insecticidal and insect mitochondrial uncoupling activities are greater and it exhibits decreased mammalian toxicity corresponding to this differential biochemical selectivity.
Key words. Lucilia cuprina; sheep blowfly; insecticides; aryl hydrazone; oxidative phosphorylation; mitochondrial uncoupling.

Mitochondrial oxidative phosphorylation which produces the energy storage molecule ATP, is a ubiquitous process throughout the animal kingdom. Any attempt to use uncouplers or inhibitors of this process as insecticidal agents must take into account that high activity of any active structures in insects may also be accompanied by high toxicity in the mammal[3]. We therefore report the finding of a selective new aryl hydrazone (**I**), with high uncoupling and insecticidal activity against the Australian sheep blowfly *Lucilia cuprina* (Wiedemann), but with a lower uncoupling activity in rat liver mitochondrial preparations.

A wide variety of structures are known which uncouple oxidative phosphorylation and these compounds generally possess a readily dissociable proton and are highly lipid soluble structures[4,5]. Some of the most active uncouplers previously reported are cyano-alkoxy carbonyl derivatives of phenylhydrazones and these agents are usually highly active in mammalian systems[5,6]. We synthesized a series of novel arylhydrazones[7] following on earlier work on phenyltriazole[8] uncouplers of oxidative phosphorylation in rat liver and yeast cell mitochondria. Structurally, the new compounds contain a phenyl ring substituted by electron withdrawing groups, NO_2 and/or CF_3. These groups were used to obtain an increase in the ionization of the proton attached to the N^1 nitrogen of the hydrazone, thus increasing the acidity of the compounds as measured by their pKa. This in-

Compounds and relevant physicochemical and biological data

	pKa	LogP octanol/water	I_{50} *L. cuprina* μM	I_{50} rat liver μM	LD_{50} *L. cuprina* mg/kg	LD_{50} mouse mg/kg
I	8.10	4.18	0.0026 (0.0024–0.0028) (4)*	0.019 (0.018–0.020) (2)*	1.11 (0.91–1.40) (4)*	19.7
II	5.90	5.50	0.140 (0.118–0.16) (3)*	0.054 (0.051–0.061) (1)*	3.23 (2.92–3.70) (3)*	6.0
III	6.60	5.16	0.27 (0.255–0.28) (2)*	0.04 (0.035–0.045) (1)*	12.66 (11.07–14.26) (2)*	20.1

pKa: Dissociation constant of compounds in 50:50 ethanol:water; LogP: Partition coefficient octanol/water; I_{50}: Concentration of compound required for 50% uncoupling of mitochondria as determined by method of Holan et al.[8]. Mean and confidence limits estimated from graphical representation of mean and SEM of % uncoupling for a minimum of three determinations over the relevant uncoupling range; LD_{50} insect: Median dose causing 50% mortality in adult males of *L. cuprina* at 48 h following topical application of compound in 1 μl of acetone, 60 flies per dose rate. LD_{50} and 95% confidence limits calculated by probit analysis; LD_{50} mouse: Median dose causing 50% mortality in 20 g female white mouse after 12 h following i.p. injection in olive oil (five animals per dose). * Number of separate preparations on different days on which these determinations were made.

crease in ionization had the undesirable effect of decreasing the lipophilicity of the compounds. This loss was compensated by substitution with trifluoromethyl groups in the ketone part of the molecules. In this way a balance between dissociation and lipid solubility was achieved, fulfilling the criteria postulated by Mitchell[9] as essential for properties of an oxidative phosphorylation uncoupling agent.

Material and methods. Mitochondria from the flight muscle of adult male *L. cuprina* were prepared using the methods of Bygrave et al.[10] and rat liver mitochondria were prepared by the methods of Hodgeboom[11]. Uncoupling activity was determined by measuring the stimulation of the state IV respiration rate[5]. Respiration was measured polarographically in a final volume of 2 ml as described by Estabrook[12]. The incubation media used and final concentrations were KCl (100 mM), KH_2PO_4 (25 mM), tris-base (10 mM), sodium pyruvate (20 mM), proline (20 mM), ATP (0.5 mM) and K_2CO_3 (2 mM) for *L. cuprina* mitochondria and sucrose (250 mM), K_2HPO_4 (100 mM), $MgCl_2$ (5 mM), sodium EDTA (2 mM) and triethanolamine (2 mM) for rat liver preparations. Final pH was 7.4 in both cases and the incubation temperature was 25 °C and 30 °C respectively. In all preparations 0.1 ml of mitochondrial suspension was added to the incubations.

Results and discussion. The arylhydrazone **I** was the most active member of our series of related compounds, both as an uncoupler and as an insecticide in *L. cuprina*. When compared to the previously reported carboxymethyl, cyanophenylhydrazone **III**[3], it was 104 times more active as an uncoupler and 11 times more active as an insecticide. Of 27 hydrazones, eight with similar structures, tested under the same conditions, structure **I** was the only compound which gave 7.3 times higher uncoupling activity in *L. cuprina*, than in rat liver mitochondrial preparations. Although there is little comparative data in the literature, salicylanilide uncouplers of oxidative phosphorylation[13] were reported to give similar levels of activities when tested in housefly and rat liver mitochondria, as determined by P_i-ATP exchange.

In contrast, hydrazone **II** taken as a representative example from a series of related structures[7], showed reduced uncoupling (54 times) and insecticidal activity (30 times) in *L. cuprina*, compared to hydrazone **I**. Conversely this hydrazone gave a 2.6-fold greater uncoupling activity in the mammalian rat liver, compared to the insect mitochondrial preparation. The structure **III** previously reported by Draber et al.[5], was 6.75 times more active as an uncoupler in rat liver than in *L. cuprina* preparations. Therefore, the difference in the activity of **I** in the uncoupling of oxidative phosphorylation in the two species, appears to be selective when compared with the results for the closely related hydrazone **II** and the carboxymethyl, cyano hydrazone **III**. This selectivity was also reflected in the insect mortality.

The mammalian (mouse) toxicity of compound **II** was approximately 3 times higher than either compounds **I** or **III**, and it was a poorer uncoupler of rat liver mitochondria than both of the latter structures. In the absence of species selectivity in the biochemical tests, the unexpected decreased toxicity of hydrazone **III** compared to that of **II**, can be explained by a probably facile degradation of the cyano carboxylate by esterases in vivo. This would reduce its toxicity to the mammal.

The high insecticidal activity and selectivity in the uncoupling of oxidative phosphorylation by the hydrazone **I** indicates that structures capable of transport of protons through mitochondrial membranes in addition to specific lipid partitioning and ionization requirements also require a steric conformation which may not be equal for the mitochondrial membranes of all species. Therefore there exists a possibility for a new rationale in the design of insecticidal respiration uncouplers with decreased mammalian toxicity.

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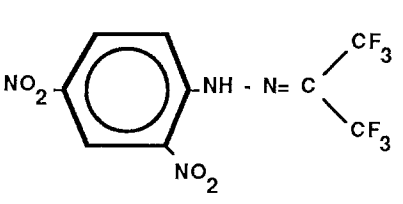
3 Büchel, K.H., and Draber, W., Adv. Chem. Ser. *114* (1972) 141.
4 Wilson, D.F., Ting, H.P., and Koppleman, M.S., Biochemistry *10* (1971) 2897.



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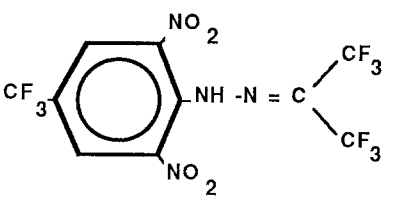

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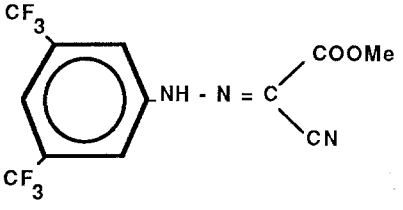

III

5 Draber, W., Büchel, K. H., and Schafer, G., Z. Naturf. (B) *27b* (1972) 159.
6 Heytler, P. G., Biochemistry *2* (1963) 357.
7 Holan, G., Eur. Patent Publ. EPA I 0001019.
8 Holan, G., Evans, J. J., Haslam, J. M., and Roulston, W. J., in: Environmental Quality and Safety Supplement, vol. 3, Pesticides, p. 450. Eds F. Coulston and F. Korte. George Thieme, Stuttgart 1975.
9 Mitchell, P., Biol. Rev. *41* (1966) 445.
10 Bygrave, F. L., Daday, A. A., and Doy, F. A., Biochem. J. *146* (1975) 601.
11 Hogeboom, G. H., Meth. Enzym. *1* (1955) 16.
12 Estabrook, R. W., Meth. Enzym. *10* (1967) 41.
13 Williamson, R. L., and Metcalf, R. L., Science *158* (1967) 1694.

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Diabetes alters drug metabolism – in vivo studies in a streptozotozin-diabetic rat model

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Summary. The influence of experimental streptozotozin-induced diabetes on hepatic drug metabolism in vivo has been studied in rats, using $^{14}CO_2$-exhalation after ^{14}C-aminopyrine injection. Male diabetic rats showed a decreased (−18%), females an increased (+19%) $^{14}CO_2$-exhalation compared to controls, indicating altered hepatic drug metabolism due to diabetes.
Key words. Aminopyrine breath test; diabetes; in vivo; NADPH; phenobarbital induction; streptozotozin.

Several publications have demonstrated altered oxidative drug metabolism in vitro in experimentally induced diabetes. These studies involved microsomes[1–6], isolated hepatocytes[7,8] and extrahepatic tissue[9–11]. The extent of the diabetic alteration in drug metabolism was dependent on sex and substrate[1,3,7,12]. The relevance of these in vitro studies for the in vivo situation, however, is ill-defined and controversial. Some workers have suggested a good correlation between in vitro and in vivo studies[13], whereas others found discrepancies between findings in microsomes and isolated hepatocytes[7]. In order to evaluate the influence of diabetes on drug metabolism in the intact animal, the oxidative demethylation of aminopyrine, labeled with ^{14}C at its two methyl groups, was measured. In this model, the cleaved methyl groups are metabolized to $^{14}CO_2$ and eventually exhaled in the breath. After trapping the CO_2 in ethanolamine, the radioactivity is counted[14]. This 'breath test' has been shown to be a good indicator of altered liver function in experimental animals and in man[14–16]. Studies were performed, therefore, in male and female control and diabetic rats. In order to get some insight into possible mechanisms of the effect of diabetes on hepatic aminopyrine demethylation, similar studies were also performed in rats whose drug metabolizing enzymes, the cytochrome P-450, had been induced by phenobarbital administration. The results showed alterations in aminopyrine demethylation in vivo qualitatively similar to the ones previously obtained in vitro.

Material and methods. Male and female Sprague-Dawley rats (150–210 g b.wt) were used. Diabetes was induced by i.v.-injection of streptozotozin (Sigma, St. Louis, USA), 75 mg/kg freshly dissolved in 0.3 ml of citric buffer pH 4.5[1]. Control animals received buffer alone. Only diabetic animals exhibiting urine glucose concentrations > 100 mmol/l and blood glucose levels > 30 mmol/l at the time of the experiments were used. Ketonuria could not be detected (Keto-Diastix, Ames, GB). All diabetic rats exhibited polydipsia, growth retardation, and increased liver to body weight ratio. These parameters indicating severity of diabetes were not different in males and females. A group of rats received phenobarbital-Na 80 mg/kg i.p. on days 7, 8 and 9 after streptozotozin. Experiments were performed on day 10 after induction of diabetes.

Aminopyrine breath test: (Dimethylamine-^{14}C)-aminopyrine (Amersham, Buckinghamshire, GB), specific activity 120 mCi/mmol, was used. The dose was 1 µCi/kg i.v., dissolved in 3 ml NaCl. To prevent adsorption to the tubing, 0.2 mg/ml of unlabeled aminopyrine was added. The breath test was performed as described earlier[14] by quantitatively collecting exhaled CO_2 in 10-min.-portions, in the unanesthetized animal.

Calculations. Since it has been demonstrated that peak exhalation rate as well as the $^{14}CO_2$ elimination rate constant in breath correlate well with in vitro aminopyrine demethylase activity[15], these two parameters were chosen to characterize the amino-

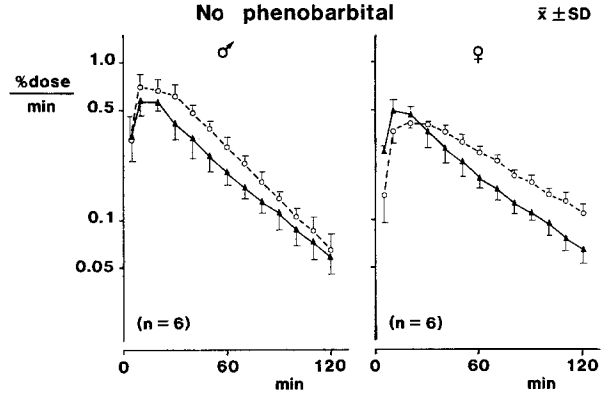

Figure 1. $^{14}CO_2$ exhalation after i.v. injection of a tracer dose of ^{14}C-aminopyrine (1 µCi/kg b.wt) in control and streptozotozin-diabetic rats not receiving phenobarbital. Diabetes decreased peak exhalation rate in male rats by 18%, whereas in females, there was an increase by 19%.
○---○ Controls; ▲———▲ Diabetics.

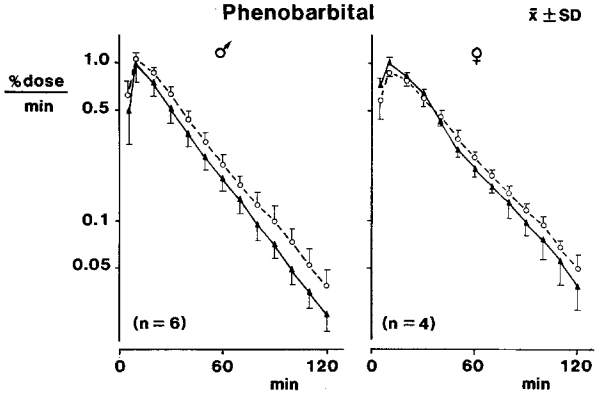

Figure 2. $^{14}CO_2$ exhalation after i.v. injection of a tracer dose of ^{14}C-aminopyrine (1 µCi/kg b.wt) in phenobarbital treated rats. Diabetes altered peak exhalation rate in a similar direction as in non-induced animals.
○---○ Controls; ▲———▲ Diabetics.