Biochemical Pharmacology, Vol. 33, No. 24, pp. 4011–4015, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

CENTRAL SIDE EFFECTS OF PENTAMETHYLMELAMINE: BIOCHEMICAL AND BEHAVIOURAL STUDIES

CARLO PEREGO, MASSIMO BROGGINI, SILVIO GARATTINI, MAURIZIO D'INCALCI, GUIDO ACHILLI, MARIA LIPARTITI and FRANCA PONZIO
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy

(*Received* 3 *October* 1983; *accepted* 26 *June* 1984)

Abstract—The central side effects of pentamethylmelamine (PMM), an antitumoral agent, were studied on brain neurotransmitters from the biochemical and behavioural points of view. PMM causes a dose-related reduction in the body temperature and motility of mice. 100 mg/kg of PMM lowers the levels of noradrenaline (NA) and raises 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) in the telencephalon. A similar dose increased striatal levels of dopamine (DA) metabolites, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), at earlier times (30 min), reducing their levels at 2 hr. These effects disappear at longer times (4 hr). No changes were observed in the levels of 3-methoxytyramine (3-MT), the extraneuronal metabolite of DA. The serotonin metabolite 5-hydroxyindolacetic acid (5HIAA) was almost not affected. PMM and its metabolites do not displace [^{3}H]-spiroperidol from mouse striatal binding sites.

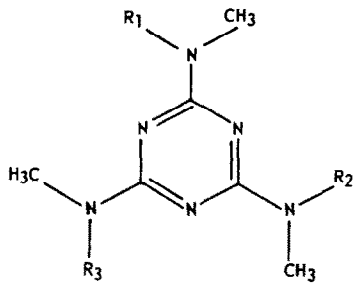
These data show that some of the neurological effects induced by PMM are associated with changes in the metabolism and/or release of brain catecholamines but are not mediated by direct action on DA receptors.

Pentamethylmelamine (PMM), the *N*-desmethyl metabolite of hexamethylmelamine (HMM), shows similar activity to the parent compound against several experimental tumours [1], with no hemopoietic inhibitory effect [2]. Being much more water-soluble than the parent compound, PMM can be administered i.v., thus possibly overcoming the problems connected with the poor and variable oral bioavailability of HMM. Phase I studies, however, showed severe CNS side effects that discouraged further clinical investigations [3–7].

We therefore explored the CNS activity of PMM in mice, finding a drastic reduction of locomotor activity and marked hypothermia. This suggested the possibility of impaired neurotransmission in the central monoaminergic system. to investigate this, we measured the levels of the monoamines noradrenaline (NA), dopamine (DA) and serotonin (5HT) and of their metabolites, 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG), 3-methoxytyramine (3MT), homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindolacetic acid (5HIAA) in several brain regions after PMM administration. We also examined the possibility of direct action at the receptor sites.

MATERIALS AND METHODS

Animals and treatment. C57BL/6J female mice (20 ± 2 g body weight) from Charles River, Italia, Calco, were used. Animals were housed under standard controlled conditions with free access to food and water. For the *in vivo* and *in vitro* experiments the following drugs were used: pentamethylmelamine (PMM);N_2, N_2, N_4, N_6-tetramethylmelamine (TMM); N_2, N_4, N_6-trimethylmelamine (TriMM) and *N*-methylol-pentamethylmelamine (*N*-methylol) (Fig. 1). All drugs were kindly provided by Drug Development Branch, Division of Cancer Treatment, National Cancer Institute Bethesda, MD, U.S.A., except *N*-methylol which was a gift from the Cancer Chemotherapy Group, Pharmacy Department, University of Aston in Birmingham. PMM was injected i.p. in water solution at doses from 50 to 200 mg/kg. Animals were killed



	R_1	R_2	R_3
HMM	CH_3	CH_3	CH_3
PMM	H	CH_3	CH_3
$N_2N_2N_4N_6$ TMM	H	H	CH_3
$N_2N_4N_6$ TriMM	H	H	H
N-methylol	CH_2OH	CH_3	CH_3

Fig. 1. Structural formula of hexamethylmelamine (HMM), pentamethylmelamine (PMM), $N_2N_2N_4N_6$-tetramethylmelamine (TMM), $N_2N_4N_6$-trimethylmelamine (TriMM) and *N*-methylol-pentamethylmelamine (*N*-methylol).

either by decapitation or, when striatal 3-methoxytyramine (3MT) was to be detected, by microwave irradiation (1.3 kW at 2.45 GHz for 2.1 sec) at the times reported under Results, striata, hippocampus, telencephalon and hypothalamus were immediately dissected, frozen on dry ice and kept at −80° until biochemical assay.

Biochemical determinations. Monoamines and their metabolites were determined in mouse brain tissue as previously described [8–10]. Tissues were homogenized either in 0.4 N perchloric acid for catecholamines and their metabolites [8–10] or in acidified butanol for serotonin and 5HIAA [9]. After centrifugation, samples were extracted from the clear supernatant according to our previously described methods [9–10].

Liquid chromatography with electrochemical detection (LCED) (Bioanalytical Systems Inc., West Lafayette, IN) was used for all biochemical determinations.

The effects of PMM and its derivatives TMM, TriMM and *N*-methylol on [^{3}H] spiroperidol binding to dopaminergic receptors were determined in a receptor preparation from mouse striata [11]. Increasing concentrations of the drugs were added to these samples and the drugs' ability to displace [^{3}H] spiroperidol specifically bound to receptors was assessed.

Behavioural determinations. Body temperature was measured 0, 15, 30 and 60 min after drug administration by an electric thermometer, inserting the thermistor 0.5 cm into the rectum. The mice's ability to remain balanced on a rotating bar (rotarod test) was assessed before and after PMM treatment. The number of times PMM treated mice fell, as a percentage of the number for controls was taken as a parameter of muscle relaxant activity.

RESULTS

Effect of PMM on body temperature and on rotarod performance

As indicated in Fig. 2, PMM induces a dose-dependent reduction of body temperature, starting from the dose of 25 mg/kg i.p. The dose of 100 mg/kg i.p. caused a severe reduction in body temperature, maximal 30 min after drug administration. In the rotarod behavioural test (Fig. 3) doses of 25 and 50 mg/kg of PMM had no effect while 100 mg/kg induced complete muscle relaxation 15 min after administration.

Effect of PMM on central monoaminergic system

In order to explore whether PMM affected monoaminergic neurotransmission in the mouse brain, we investigated the changes induced by this drug in DA and its metabolites 3MT, HVA and DOPAC in striata, in 5HT and its metabolite 5HIAA in hippocampus, and in NA and its metabolite MHPG in telencephalon (Table 1). Thirty and 60 min after 100 mg/kg of PMM, striatal HVA levels were significantly elevated, while DOPAC was significantly reduced 60 min after drug administration (Table 1). Other dopaminergic parameters such as DA and 3MT were not modified by the treatment. PMM also had no effect on 5HT and its metabolite 5HIAA in

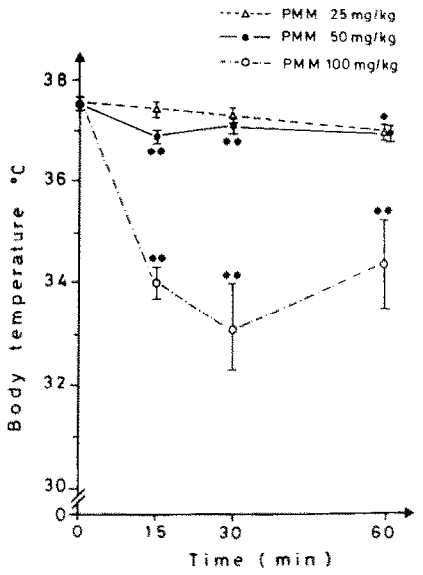


Fig. 2. Effect of PMM on body temperature. PMM was administered at doses of 25, 50 and 100 mg/kg. Body temperature was measured by an electric thermometer, inserting the thermistor 0.5 cm into the rectum, 0, 15, 30 and 60 min after drug administration. Data are the mean ± S.E. of six determinations. Statistical significance was analysed by a modification of Duncan's new multiple test [24] using an SPBS computer program [25]. < P 0.05 controls.

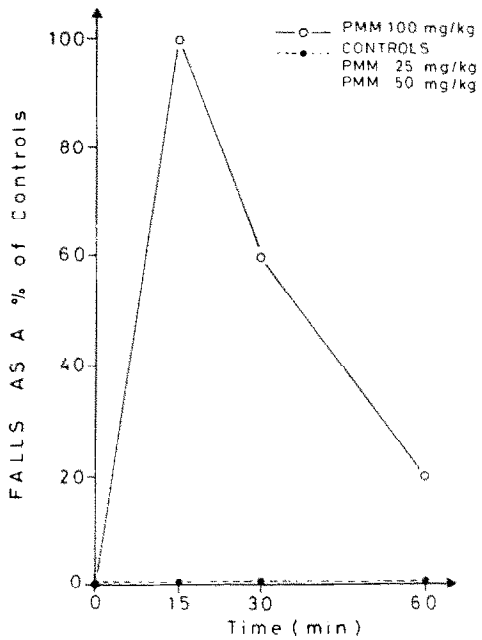


Fig. 3. Effect of PMM on rotarod test performance. PMM was administered at doses of 25, 50, 100 mg/kg. Rotarod performance was tested before 15, 30 and 60 min after PMM. Data are the mean of six determinations and are expressed as % of falls from the rotating bar in respect to controls. Time 15 is significantly different respect to controls; statistical significance was analysed by binomial test ($P = 0.031 < 0.05$).

Table 1. Effect of PMM on monoamines and their metabolites in several mouse brain areas.

		Controls	PMM 100 mg/kg 30 min	PMM 100 mg/kg 60 min
Striata	DA (ng/g)	25600 ± 2233	31944 ± 2930	29624 ± 1437
	3MT (ng/g)	65.9 ± 3.9	66.5 ± 7.8	n.d.
	HVA (ng/g)	1061 ± 41	1347 ± 64*	1249 ± 50*
	DOPAC (ng/g)	798 ± 44	840 ± 45	551 ± 49*
Hippocampus	5HT (ng/g)	443 ± 23	463 ± 19	488 ± 10
	5HIAA (ng/g)	451 ± 22	414 ± 24	402 ± 9
		Controls	PMM 100 mg/kg 30 min	PMM 100 mg/kg 120 min
Telencephalon	NA (ng/g)	696 ± 53	504 ± 13*	552 ± 12*
	MHPG (ng/g)	93.9 ± 7.4	175 ± 10*	99.6 ± 2.5

* $P < 0.01$ vs controls.

PMM was administered at a dose of 100 mg/kg i.p. and animals were killed by decapitation or μ wave irradiation 30, 60 and 120 min later. Data are the mean ± S.E. of six determinations.

Statistical significance was analysed by a modification of Duncan's new multiple test [24] using a SPBS computer program [25].

hippocampus (Table 1) and in other brain areas (striata, brainstem and telencephalon) not reported here in detail. NA levels were significantly reduced in telencephalon 30 and 120 min after PMM 100 mg/kg, while MHPG was elevated in the same area 30 min after drug administration (Table 1).

Dose–response and time curve: effect of PMM on striatal dopamine metabolites

Since PMM seems to affect striatal DA metabolites, the dose–response (Table 2) and the time course of the effect of this drug (Table 3) were explored.

PMM, 50–200 mg/kg, caused a dose-related rise in HVA 30 min after injection (Table 2), while DOPAC levels were lowered at a dose of 50 mg/kg, the dose of 100 mg/kg apparently being ineffective (Table 2). The time course of the effect of PMM was analysed using 100 mg/kg PMM (Table 3). HVA levels were significantly raised 15, 30 and 60 min after drug administration but significantly lowered 2 hr after treatment.

Striatal DOPAC was elevated 15 min after PMM, at 30 min the values were back to the normal and at 60 and 120 min they were significantly lowered.

In vitro *effects of PMM on binding of [^{3}H] spiroperiodol to striatal dopaminergic receptors*

The effects of PMM in reducing motility and body temperature and increasing HVA levels might be explained as direct antagonistic action at the DA receptor. Therefore the drug's ability to displace ^{3}H-spiroperidol from its binding sites was investigated. We tested the two *N*-demethylated bioproducts of PMM, TMM and TriMM (Fig. 1), known to be present in large amounts in mouse brain and in CSF of patients treated with the drug [12, 13]. *N*-methylol is not a metabolite of PMM, as it is an intermediate formed in the *N*-demethylation of HMM to PMM [14], but we tested it because no other methylol likely to be formed during the oxidative *N*-demethylation of PMM (e.g. *N*-methyloltetramethylmelamine) has ever been synthesized.

Figure 4 shows that PMM and its derivatives TMM, TriMM and *N*-methylol did not displace [^{3}H] spiroperidol binding to mouse striatal membrane preparation compared to haloperidol. Even at high concentrations (10^{-4}, 10^{-5} M) PMM and its derivatives did not affect DA receptors *in vitro*.

Effect of PMM on dopamine metabolites in striata and hypothalamus of mice kept at 37°

A reduction in body temperature could as a compensation induce activation of central dopaminergic mechanisms [15, 16]. In order to explore whether the changes in striatal HVA and DOPAC levels were caused by the loss of thermoregulation induced by PMM, we performed an experiment in which the loss

Table 2. Dose–response effect of PMM on striatal dopamine metabolites

	Controls	PMM 50 mg/kg	PMM 100 mg/kg	PMM 200 mg/kg
HVA (ng/g)	856 ± 24	852 ± 35	918 ± 39*	1212 ± 72*
DOPAC (ng/g)	612 ± 21	469 ± 30*	569 ± 22	1209 ± 103*

PMM was injected at doses of 50, 100 and 200 mg/kg i.p. and animals were killed by decapitation 30 min later. Data are the mean ± S.E. of six determinations.

Statistical significance was analyzed by a modification of Duncan's new multiple test [24] using a SPBS computer program [25].

* $P < 0.01$ vs controls.

Table 3. Time course of the effect of PMM on striatal dopamine metabolites

	Controls	15 min	30 min	60 min	120 min	240 min
HVA (ng/g)	1061 ± 14	1287 ± 86*	1347 ± 64*	1249 ± 50*	823 ± 41*†	1128 ± 65
DOPAC (ng/g)	798 ± 44	963 ± 70*	840 ± 45	551 ± 49*‡	502 ± 42*†	704 ± 49

PMM was injected at a dose of 100 mg/kg i.p. and animals were killed by decapitation 15, 30, 60 and 120 min later. Data are the mean ± S.E. of six determinations.

Statistical significance was analysed by a modification of Duncan's new multiple test [24] using a SPBS computer program [25].

* $P < 0.01$ vs controls and 240 min.

† $P < 0.01$ vs 15 min.

‡ $P < 0.01$ vs other times.

of body temperature was compensated by keeping the mice at a temperature of 37°. Animals were divided into 3 groups, two of which were kept in a hot room at 37 ± 1° for 1 hr before drug or vehicle administration, and throughout the experiment, and the third group was kept at room temperature and received vehicle only (Table 4). No loss in body temperature was found in PMM treated mice kept at 37 ± 1° at any time considered. HVA and DOPAC were determined in striata and in hypothalamus, a brain area considered important in thermoregulation [15, 16]. In both areas 30 min after 100 mg/kg of PMM, HVA levels were raised (Table 4). This was similar to the effect observed in animals treated with PMM and kept at room temperature. The exposure of mice to a temperature of 37° did not modify basal HVA and DOPAC levels (Table 4).

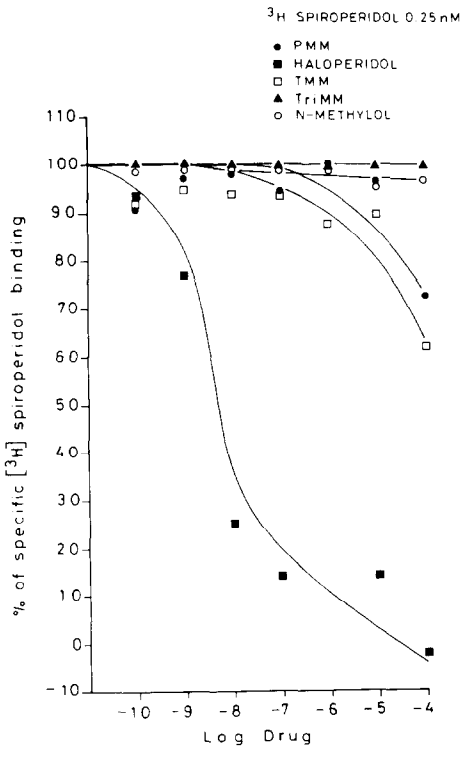


Fig. 4. Displacement of [^{3}H] spiroperidol binding by PMM or its metabolites. % of inhibition of stereospecific [^{3}H] spiroperidol (0.25 nM) binding to mice striatal membranes by PMM, TMM, TriMM, *N*-methylol and haloperidol.

DISCUSSION

Hexamethylmelamine is used clinically for the treatment of ovarian cancer [17]. Because of its gastrointestinal toxicity and its variable absorption [18], a more water-soluble, i.v. injectable analog is needed. Its *N*-demethylated metabolite PMM is soluble in physiological solvents and equally active on some experimental tumours and was given by i.v. injection in phase I trials. Unfortunately, in some patients it induced marked neurological side effects [3–7]. The distribution curve of HMM and PMM showed higher concentrations of PMM and metabolites in brain [12] and CSF [13] after PMM than after HMM. This might justify the hypothesis that PMM and its metabolites play an important role in the neurological side effects of this drug [3–7].

High doses of PMM (i.p.) corresponding to those effective in the treatment of murine tumours (LD_{50} 200–220 mg/kg i.p.) [1, 12] induce hypomotility in mice as a sign of neurological toxicity. The reduction in motility was behaviourally tested in mice by parallel wires, cross limb, stick with string and rotarod. The results, not reported here, indicated a severe reduction in motility 15 and 30 min after PMM in all the behavioural tests used with the exception of cross limb. The rotarod test was selected rather than the others because it provides a more specific assessment of the hypomotility related to muscle relaxation [19]. In the present study we also quantified the effect of PMM on body temperature. The muscular impairment of hypothermia was dose-dependent and lasted over one hour.

The effect of PMM was also investigated on brain monoamines. 5HT and its main metabolite 5HIAA were not modified by PMM treatment in the hippocampus or other brain areas. Brain catecholamines, however, showed marked changes. PMM reduced NA in the telencephalon and raised MHPG; HVA and DOPAC in the striatum were markedly affected by PMM, with elevation at earlier times (30 min) followed by a drop at 2 hr. This effect ended 4 hr after PMM administration. DA and 3MT, a metabolite which reflects release of DA in the synaptic cleft [20–22] were not modified by PMM. Therefore, intraneuronal synthesis of DA appears to be enhanced as a result of PMM administration.

Table 4. Effect of PMM on dopamine metabolites in striata and hypothalamus of mice kept at +37°

		Controls room temp.	Control 37°	PMM 37°	PMM room temp.
Striata	HVA (ng/g)	1062 ± 46	1020 ± 30	1345 ± 20*	1342 ± 36*
Hypothalamus	HVA (ng/g)	233 ± 13	244 ± 16	332 ± 27*	n.d.

PMM was administered at a dose of 100 mg/kg i.p. to mice kept at 37 ± 1° and at room temperature.

Animals were killed by decapitation 30 min thereafter. Control groups were kept at room temperature and at 37 ± 1°

Statistical significance was analysed by a modification of Duncan's new multiple test [24] using an SPBS computer program [25].

* $P < 0.01$ vs controls.

A direct effect of PMM on the inactivation or on the active transport of acidic metabolites across the blood–brain barrier can be excluded by the fact that the acidic metabolites of DA, NA and 5HT were differently affected by PMM; 5HIAA was not modified, MHPG increased, HVA and DOPAC were either raised or reduced. In the case of a direct effect of PMM on these mechanisms the modifications could be expected all to be similar.

Since DA metabolism was most affected by PMM, we examined the possibility of direct action of PMM on DA receptor activity. Neither PMM nor its *N*-dimethylated metabolites act directly on the dopaminergic system since they are inactive on dopamine postsynaptic receptors, judging by the fact that ^{3}H-spiroperidol binding to striatal membranes was not affected even at high concentrations of the tested compounds. Therefore a direct effect of PMM on DA receptors can be excluded, at least as regards *in vitro* studies. The effect of PMM on the dopaminergic system was not caused by hypothermia, since it persisted even when the fall in body temperature was prevented by keeping the mice at 37°.

Neurological side effects present in patients were generally Parkinson-like symptoms such as ataxia and tremors [3–7, 23] typically related to dysfunction of the extrapyramidal system. The biochemical effect of PMM in mice, associated with changes in the metabolism and/or release of brain catecholamines, could help explain the central side-effects of PMM in patients. If this is a major mechanism it should be possible to counteract the side effects of PMM by using appropriate drugs with opposite action on the catacholaminergic system.

Acknowledgements—This work was partially supported by Public Health Service grant (No. ROI1-CA-2904303) from the National Cancer Institute, National Institudes of Health, Department of Health and Human Service, and by CNR (National Research Council, Rome, Italy) Project Oncology-neurotoxicology 1984.

REFERENCES

1. National Cancer Institute, *Clinical Brochure—Pentamethylmelamine*. Cancer Therapy Evaluation Program, June (1978).
2. M. Morimoto, D. Green, A. Rahman, A. Goldin and P. S. Schein, *Cancer Res.* **40**, 2762 (1980).
3. R. T. Eagan, E. T. Creagan, J. Rubin, M. J. O'Connell and J. S. Kovach, *Cancer Treat. Rep.* **64**, 993 (1980).
4. R. S. Goldberg, J. P. Griffin, J. W. McSherry and I. H. Krakoff, *Cancer Treat. Rep.* **64**, 1319 (1980).
5. D. A. Van Echo, D. F. Chiuten, M. Whitacre, J. Aisner, J. L. Lichtenfeld and P. H. Wienrik, *Cancer Treat. Rep.* **64**, 1335 (1980).
6. D. C. Ihde, J. S. Dutcher, R. C. Young, R. S. Cordes, A. L. Barlock, S. M. Hubbard, R. B. Jones and M. R. Boyd, *Cancer Treat. Rep.* **65**, 755 (1981).
7. J. A. Ajani, F. F. Cabanillas and G. P. Bodey, *Cancer Treat. Rep.* **66**, 1227 (1982).
8. R. Keller, A. Oke, I. Mefford and R. N. Adams, *Life Sci.* **19**, 995 (1976).
9. F. Ponzio and G. Jonsson, *J. Neurochem.* **32**, 129 (1979).
10. F. Ponzio and G. Jonsson, *Develop. Neurosci.* **1**, 80 (1978).
11. D. R. Burt, I. Creese and S. H. Snyder, *Science* **196**, 326 (1977).
12. M. Broggini, C. Rossi, T. Colombo and M. D'Incalci, *Cancer Treat. Rep.* **66**, 127 (1982).
13. M. D'Incalci, C. Sessa, G. Beggiolin and C. Mangioni, *Cancer Treat. Rep.* **65**, 350 (1981).
14. A. Gescher, M. D'Incalci, R. Fanelli and P. Farina, *Life Sci.* **26**, 147 (1980).
15. H. Thoenen, *Nature, Lond.* **228**, 861 (1970).
16. M. T. Lin, J. J. Wu and B. L. Tsay, *Naunyn-Schmiedebergs Arch. Pharmac.* **322**, 271 (1983).
17. J. T. Wharton, F. Rutledge, J. P. Smith, J Herson and M. P. Hodge, *Am. J. Obstet. Gynecol.* **133**, 833 (1979).
18. M. D'Incalci, G. Bolis, C. Mangioni, L. Morasca and S. Garattini, *Cancer Treat. Rep.* **62**, 2117 (1978).
19. K. Kolasa, R. Fusi, S. Garattini, S. Consolo and H. Ladinsky, *J. Pharm. Pharmac.* **34**, 314 (1982).
20. F. Ponzio, G. Achilli and S. Algeri, *J. Neurochem.* **36**, 1361 (1981).
21. F. Ponzio, G. Achilli, C. Perego and S. Algeri, *Neurosci. Lett.* **27**, 61 (1981).
22. B. H. C. Westerink and S. J. Spaan, *J. Neurochem.* **38**, 342 (1982).
23. R. S. Kaplan and P. H. Wiernik, *Semin. Oncol.* **9**, 103 (1982).
24. C. Y. Kramer, *Biometrics* **12**, 307 (1956).
25. M. Rocchetti and M. Recchia, *Comput Programs Biomed.* **14**, 7 (1982).