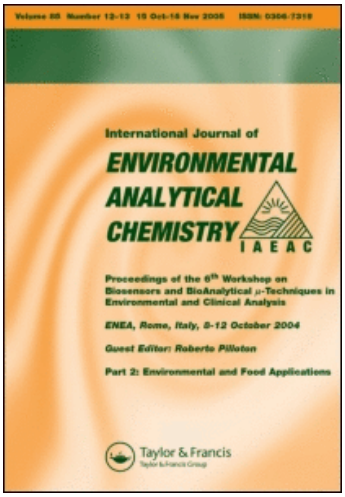

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STUDY OF BINDING OF METHYL PARATHION BY COW'S CASEIN IN MODEL SYSTEMS AND IN EWE' S MILK

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The binding of methyl parathion by cow's casein, in model systems and in sheep's milk was investigated. Casein in cow's milk binds methyl parathion to a great extent. The addition of the enzyme potato acid phosphatase (PAP) in casein solutions does not affect the release of methyl parathion from casein. Storage of casein solutions or casein solutions plus PAP at 4°C for 60 days does not affect the binding of methyl parathion to casein. The data also indicate that methyl parathion binds strongly to milk proteins and/or other substances of ewe's milk.

Keywords: Methyl parathion; Casein; Ewe's milk

INTRODUCTION

Organophosphate pesticides bind to milk proteins [1, 2]. They also bind to cholinesterase, resulting in its inhibition [3, 4]. Experiments with milk proteins, i.e., casein and whey proteins, have indicated that the pesticides parathion, malathion and trithion are bound to casein to a greater extent (0.02–0.21 μg/mg of protein) than to other milk substances (i.e., other proteins) [1]. The ability of casein to bind organophosphates might be related to the presence of a great number of seryl and phosphoseryl residues in the molecule [1]. Mallatou [5] found an increase in the concentration of methyl parathion in feta cheese during manufacture, ripening and storage for up to 120 days, but decreased significantly from 120 to 240 days. Casein is one of the main constituents of milk and remains in cheese during the manufacturing process. It is the constituent of milk that is mainly responsible for the retention of methyl parathion by cheese [2, 3].

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Acid phosphatase is an enzyme that exists in small quantities in the milk. It hydrolyzes the phosphate bonds of the phosphate esters of casein to a greater extent than alkaline phosphatase [6]. Acid phosphatase is inactivated during pasteurization. Dephosphorylation of peptides by acid phosphatase is one of the major biochemical changes that occur during ripening of cheese [7, 8]. The enzyme acid phosphatase, and specifically potato acid phosphatase (PAP), has been used in a series of experiments on the enzymic hydrolysis of casein [9,10]. Dephosphorylation of casein by the addition of PAP enzyme [10] was expected to result in the rupture of the bonds formed between casein and methyl parathion.

In the present work, the binding of methyl parathion in model casein solutions and in ewe's milk was investigated in an attempt to relate the binding/release mechanism between methyl parathion and casein to the biochemical changes that occur during the curdling of the milk and ripening of the cheese. A knowledge of the distribution mechanism of methyl parathion to cheese and to whey is of great importance in the fields of public hygiene and environmental pollution.

EXPERIMENTAL

Materials

Casein was separated from fresh cow's milk according to the method described by Shammet *et al.* [11]. After separation, the casein suspension was heated at 71°C for 30 min to inactivate proteolytic enzymes, which co-precipitate with casein. Sodium caseinate was lyophilized, pulverized, dried over P_2O_5 and kept in an airtight bottle at 4°C. Methyl parathion ($C_8H_{10}NO_2PS$) 99% (Institute of Organic Chemistry Annopol, Warsaw, Poland) was added to a 2.8% solution of cow's casein (in sodium barbital buffer, pH 7) or in ewe's whole milk to give a final concentration of 2 or 10 mg/L of methyl parathion in casein solution and of ewe's milk, respectively. The enzyme potato acid phosphatase (E.3.1.3.2.) from Sigma (USA) was added to give final concentration of 1.333 g/L which corresponds to 50 mg enzyme/g of casein [10]. As a preservative for sodium barbital/HCl buffer, 0.02% NaN_3 was added at a concentration of 0.03 mol/L [10]. To samples containing casein, 2-mercaptoethanol was added such that its final concentration was 0.45% (v/v) [9]. Mercaptoethanol was added to rupture the S–S bonds of caseinate to facilitate the action of PAP on casein.

Sample Preparation

The following model systems were used to study the binding of methyl parathion to casein: (i) buffer + methyl parathion (control); (ii) buffer + methyl parathion + PAP; (iii) buffer + methyl parathion + casein; (iv) buffer + methyl parathion + casein + PAP. Dissolution was helped using an ultrasonic bath. After mixing, all samples were shaken for 6 h and then for another 2 h in a shaking water bath at 37°C [12]. At the end of the shaking, the samples were adjusted to pH 4.6 by adding, drop-by-drop, 1 N HCl with vigorous stirring. A pH of 4.6 is normally the pH of ripened feta cheese. The samples with casein were filtered through a Whatman No 42 filter paper before methyl parathion determination.

In similar experiment the samples were left at 4°C for 60 days, conditions similar to those applied for the storage of feta cheese in an attempt to help the release of methyl parathion from its complex with casein. At the end of this period the pH was lowered to 4.6 and the samples were filtered before the determination of methyl parathion. The foregoing experimental trials were carried out seven times.

The following systems were used for the study of binding of methyl parathion to ewe's milk. To 100 mL of fresh ewe's milk, NaN_3 (0.02%), 0.5 mL 2-mercaptoethanol [9] and methyl parathion adjusted to a final concentration of 10 mg/L, were added. To another identical sample 240 mg of the enzyme PAP was added at a rate of 50 mg enzyme/g casein [10]. Samples were mixed vigorously by vortexing for 2–3 min and were then shaken for another 2 h at 37°C in a shaking water bath.

Chromatographic Analysis

Samples that contained casein were treated as follows: 10 mL (filtrate from insoluble casein suspension) were extracted with 2 mL *n*-hexane with vigorous shaking for 1 min. Then they were centrifuged for 8 min at 5000 rpm. The aqueous phase was separated and re-extracted twice more with 2 mL *n*-hexane. The organic extracts were combined and dried with 0.5 g anydrous Na_2SO_4, filtered and passed through glass wool, which had a layer of Na_2SO_4 [13]. Finally, the samples were condensed in a rotary evaporator at 35°C to a final volume of less than 2 mL.

Methyl parathion in milk samples was determined according to the method of the Association of Official Analytical Chemists (AOAC) [14].

To determine the concentration of the methyl parathion a gas-liquid chromatograph (Perkin-Elmer, type 8500 Beaconsfield, England) was used. The chromatographic conditions for the analysis were: glass column 1.5%, SP^{TM} 2250/1.95% SP-2401 on 100/120 supelcoport TM 2.0×4 mm ID. Flow rate 60 mL min^{-1} Ar/CH_4 90/10 (30 mL on column and 30 mL make up). Detector ECD ^{63}Ni, $T = 300$°C, IP 225°C, Col 200°C. The concentration of methyl parathion was estimated using a reference curve. All reagents used were of pesticide analytical grade.

Results of the analyses are presented in Table I.

Statistical Analysis

For statistical analysis of the results, one-way analysis of variance, using the statistical package of Statgraphics (Statistical Graphics Corporation, Rockville, MD) was applied. For finding differences ($P < 0.05$) of means, the LSD test was applied.

RESULTS AND DISCUSSION

Binding of Methyl Parathion by Casein

From the data of Table I it is clear that casein binds methyl parathion to a great extent. The significantly lower concentration (0.530 mg/L) of methyl parathion in the filtrate from the insoluble casein suspension (pH 4.6) compared to that of the blank (1.820 mg/L) can be attributed to the binding of methyl parathion by casein. The data also show that the methyl parathion residues are enclosed or bound into the casein

TABLE I Effect of the presence of potato acid phosphatase (PAP) enzyme on the recovery of methyl parathion in filtrates from insoluble casein suspensions

System	*Concentration of methyl parathion*[a] (mg/L)	
	Fresh	*After storage*[b]
Methyl parathion (blank)	1.820 ± 0.064^{c}	2.087 ± 0.018^{c}
Methyl parathion + PAP	1.458 ± 0.087^{c}	1.551 ± 0.088^{c}
Methyl parathion + casein	0.530 ± 0.053^{d}	0.471 ± 0.069^{d}
Methyl parathion + casein + PAP	0.630 ± 0.025^{d}	0.533 ± 0.012^{d}

[a]Mean values (± SD) of seven trials; [b]stored for 60 days at 4°C; [c,d]means in the same column followed by different letters are significantly different ($P < 0.05$).

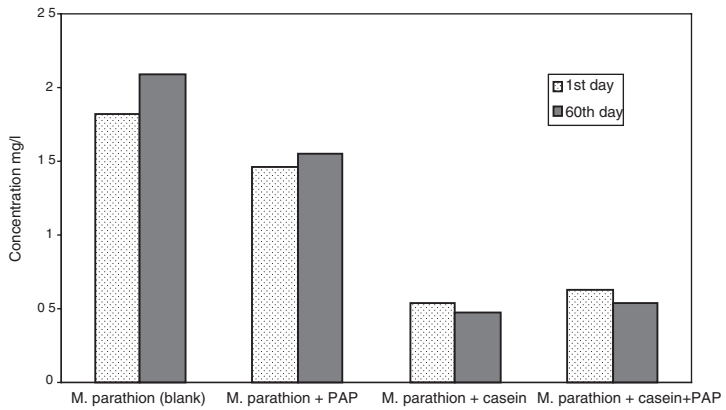

FIGURE 1 Effect of storage time (at 4°C) on the recovery of methyl parathion in filtrates from insoluble casein suspensions in the presence and absence of the enzyme PAP.

molecule. For this reason methyl parathion might be inaccessible to the extraction solvents used in this study. Methyl parathion might be bound to the seryl and phosphoseryl groups of the casein chain similarly to the way that methyl parathion is bound to the enzyme cholinesterase [4]. Racotovelo *et al.* [1] reported that binding of organophosphorus pesticides with casein is dependent on the presence of seryl and phosphoseryl groups in the protein molecule. The complex formed between methyl parathion and casein may not necessarily be due only to linkage between the phosphoseryl residues of casein and parathion. Other type of interactions, e.g., S–S exchange, hydrogen interaction, may bind methyl parathion. The binding of methyl parathion and other organophosphates by proteins and, especially by casein, has also been reported by others [2, 3].

The data of Table I also show that the addition of the enzyme PAP to the system (methyl parathion + casein) had no significant effect ($P < 0.05$) on the concentration of methyl parathion in the filtrate from an insoluble casein suspension. The inability of the enzyme PAP to affect the binding of methyl parathion by casein may be due to: (a) the phosphorus binding sites of casein not being accessible to the enzyme [15]; (b) formation of a complex between methyl parathion and casein that may affect the enzyme's action on the casein molecule (steric hindrance) [16]; (c) possible phosphorylation of the enzyme, similar to that which occurs in cholinesterases [4].

The influence of the long storage (60 d) at 4°C on the binding of methyl parathion by casein is shown in Table I and diagrammatically in Fig. 1. The results show that storage

TABLE II Effect of the proteolytic enzyme potato acid phosphatase (PAP) on the recovery of methyl parathion in ewe's milk

Sytems	*Methyl parathion*[a] (mg/L)	
	Initial concentration	*Concentration found*
Milk + methyl parathion (control)	10	4.539 ± 0.107^{b}
Milk + methyl parathion + PAP	10	3.491 ± 0.091^{c}

[a]Mean values (± SD) of seven trials; [b,c]means in the same column followed by different letters are significantly different ($P < 0.05$).

of samples that contained casein and methyl parathion and/or the enzyme PAP, at 4°C for 60 days did not affect the binding of methyl parathion by casein.

In relation to this, Mallatou (1998) [5] demonstrated – in experiments conducted to examine the stability of methyl parathion during manufacture, ripening and storage of feta cheese – that the concentration of methyl parathion found in feta cheese on the first day increased progressively with the age of the cheese up to 60 days. This suggests that methyl parathion may be bound to casein during the first stages of ripening and that later, as proteolysis progresses, part of it is released.

Binding of Methyl Parathion by Ewe's Milk

Data on the effect of the enzyme PAP on the release of methyl parathion from ewe's milk are presented in Table II. Although methyl parathion was added to milk at a concentration of 10 mg/L, the concentration determined after mixing and treatment were 4.539 and 3.491 mg/L in the absence and the presence of PAP, respectively. These results indicate that the bond between methyl parathion and casein is very strong. Table II also shows that the concentration of methyl parathion in milk in the presence of the enzyme PAP is significantly lower ($P < 0.05$) than that found when PAP was not added (control). The lower recovery of methyl parathion after the addition of the enzyme PAP in ewe's milk might be explained by the increase of the binding of the enzyme PAP with methyl parathion and/or by the hydrolysis of methyl parathion by the enzyme [17]. In the latter case, the resulting hydrolysis product could not be detected by the methods used in this study. The inability of PAP to release methyl parathion from the milk might be due to the restraining action of some milk constituents on the activity of the enzyme [18,19]. Li-Chan and Nakai [10] found that the percentage of dephosphorylation of cow's milk by the action of the enzyme of acid phosphatase was 27%. These results indicate that the bond between methyl parathion and casein or other milk constituents is very strong.

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