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Original Research

The Effectiveness of Penetration of Erythrocyte Membrane by Sodium Salt of 2,4-Dichlorophenoxyacetic Acid

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Abstract

The effectiveness of penetration of erythrocyte membrane by sodium salt of 2,4-dichlorophenoxyacetic acid was analyzed. The experiment was executed in a dependence on different doses of the herbicide and at different times of incubation of red blood cells with 2,4-D-Na. It is known that the main mechanism of detoxification of the cell from xenobiotics including 2,4-D is to bind them with proteins contained in blood plasma. In the case of exposure of blood to high doses of 2,4-D-Na, the unbound part of xenobiotics may penetrate into erythrocytes and change the activity of numerous parameters of the cells. The results obtained by the use of high pressure liquid chromatography (HPLC) revealed that 2,4-D-Na is adsorbed by erythrocytes of 5% haematocrite in the amount of ~1% of the initial concentration. Moreover, it was observed that 2,4-D-Na is capable of accumulating in erythrocyte's membrane and haemolysate in the amounts of 0.15% and 1.23% of the initial concentration, respectively.

It was also stated that penetration of 2,4-dichlorophenoxyacetic acid into erythrocytes is not associated with incubation time (the similar concentrations of 2,4-D-Na were detected after different incubation times of 0.5 to 3 hours); however, it was related with concentrations of the herbicide. We suggest that 2,4-D-Na was transported with concentration gradient in human erythrocytes.

Keywords: erythrocytes, 2,4-dichlorophenoxyacetic acid, HPLC, blood, pesticide

Introduction

The extensive use of phenoxyherbicides has generated a series of toxicological and environmental problems, particularly in developing countries. Numerous animal species are exposed to 2,4-dichlorophenoxyacetic acid and its transformation products in water [1], soil and plants as well as by agro-industrial and commercial exposure [2]. The 2,4-D easily permeates into the human organism from the alimentary tract and skin (less than 5% [3]) and is subsequently excreted in the urine in nearly unchanged form. Apart from the initial herbicide, conjugates of 2,4-D with amino acids or proteins as well as 2,4-dichlorophenol have been found [4, 5].

Persons employed in production, commercial distribution, packaging and repackaging as well as other plant protection personnel and those involved in plant spraying are chronically exposed to 2,4-dichlorophenoxyacetic acid action [6, 7]. 2,4-D is a common herbicide that is used around houses and gardens and also on golf courses, ball fields, parks, in agriculture and forestry [3, 8]. In an

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investigation of mice and rats it was observed that 2,4-D was effectively adsorbed by alimentary tract in amounts of 97% to 99% of the initial doses [9, 10]. Ingestion of a dose of 5 mg/kg of 2,4-D in male volunteers resulted in half time absorption ranging from 1.7 to 4.2 h [11]. The major part of adsorbed herbicide is transported by blood system. Van Ravenzwaay et al. [10] reported that in rats, 2,4-D was highly bound (93–97%) to plasma proteins over a broad range of concentrations. Likewise, at low plasma concentrations of 2,4-D (< 20g/ml), 97% of the herbicide was bound to plasma proteins in goats [12].

The erythrocytes constitute the largest (in respect to volume) morphological component of blood. Red blood cells are deprived of most organelles; they have characteristic shape of discocite. Biconcave shape of erythrocyte gives better ratio of area to volume, and thus determines better conditions to gas exchange and also increases deformability of red blood cells [13].

Erythrocytes membrane is composed from lipid bilayer, proteins bound with bilayer and peripheral proteins that stuff internal membrane layer. The last above-mentioned group of protein forms membrane skeleton, that consists of the only component of cytoskeleton of erythrocyte [13].

Membrane layer transports different substances such as water, ions (Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, NH^{4+}), polyamines, choline, catecholamines, aminoacids, glucose, nucleosides, urea and glutathione derivatives. In spite of very simple structure of red blood cells, it executes many metabolic processes like metabolism of adenine nucleotides (within salvage pathway) and carbohydrates transformations (glycolyse and penthaphosphoric cycles) that support cell with energy accumulated in ATP molecules. These processes are necessary to sustain the shape of erythrocyte and for induction of membrane transport [14].

The erythrocytes execute an important function as they transport and metabolize xenobiotics. Drugs that are adsorbed by blood-vascular system from alimentary tract and are transported by blood may be simultaneously be oxidized by cytochrome P 450 reductase and protein S (hemoproteine 559) that are localized in erythrocytes [15].

In consideration to essential role of erythrocytes in transport of different substances, it seems to be important to analyze the penetration of commonly used herbicide – 2,4-D-Na to the interior of red blood cell.

In work the penetration of 2,4-D-Na into erythrocytes in dependence on incubation time (0.5 to 3 hours) and doses of the herbicide (10 to 500 ppm) was investigated. Moreover the comparison of the content (accumulated concentrations) of 2,4-D in membrane and inside the erythrocyte was analyzed.

Materials and Methods

Sodium salt of 2,4-dichlorophenoxyacetc acid (purity 97.5%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Human erythrocytes were obtained from whole blood taken from healthy donors at the Blood Bank of Łódz. Erythrocytes were centrifuged (3000 rpm/min)/10 min and washed three times with phosphate-buffered saline (PBS), pH 7.4.

Erythrocytes at 5% haematocrite were incubated with 2,4-D at 37°C. Samples of erythrocytes and sodium phosphate buffer without 2,4-D were used as controls.

Sample Preparation

The erythrocytes haematocrite at 5% (1.5 ml) were incubated with 100 ppm of sodium salt of 2,4-D in dependence on the time incubation (0.5-3 hours). In the second scheme erythrocytes were incubated in dependence of concentrations of 2,4-D (10-250 ppm). The pesticide at the range of concentrations was added to the erythrocyte for 3 h. 2,4-D stock solution was prepared in phosphate-buffered saline (pH 7.4).

The erythrocytes were separated by centrifugation (3000 rpm) and supernatant (in which unbound 2,4-D was present) was eliminated. Erythrocytes (about 200 µl Packet Cells) were treated with 1 ml of PBS and centrifuged (3000 rpm), then supernatant was eliminated (this step was repeated twice). Washed erythrocytes were treated with 0.5 ml of 30% trichloroacetic acid and shaken for 10 minutes. The samples were centrifuged (15000 rpm) for 10 minutes, supernatant was collected and analyzed using the HPLC method.

Haemolysate

After the erythrocytes were washed off twice, they were treated with 250 µl of water and centrifuged. Haemolysate was collected in tube, to residue 250 µl of water was added and the sample was centrifuged. Then haemolysate was collected and this step was repeated. Next to 500 µl of haemolysate 250 µl of 30% TCA was added, the sample was centrifuged and clarified supernatant was taken to analysis.

Membranes

After the erythrocytes were washed off twice, the residue was treated with 250 µl of water and the sample was centrifuged. Hemolysate was collected and this step was repeated once again. Next, to membranes 250 µl of 30% TCA and 500 µl of water was added. The sample was shaken 10 minutes and clarified supernatant was taken to analysis.

High Performance Liquid Chromatography

The sample (50 µl) was injected onto a 150 x 4.6 mm, 5 µl Zorbax SB C18 column (Agilent). The mobile phase (flow rate 1.2 ml/min, temperature 25°C) consisted of 0.02

M trichloroacetic acid in water solution and acetonitrile in the ratio of 50:50 (v/v). The detector was set to measure peaks at 284 nm.

Results

1. **The changes in the concentrations of 2,4-D in human erythrocytes in dependence on the time of incubation.**
   None statistically significant differences between the content of the herbicide in erythrocytes and time of incubation (0.5 to 3h) was observed. In every sample (after incubation 100 ppm of 2,4-D with erythrocytes of 5% hematocrite) the concentration of 2,4-D was about ~1% of the initial dose (respectively 23.6 ± 3.6 μg/ml PC (Fig. 1).
2. **The concentrations of 2,4-D in human erythrocytes in dependence on the incubation doses.**
   Statistically significant dependence was observed between the content of 2,4-D in erythrocytes and the initial concentrations of the herbicide used (Table 1; Fig. 2).
3. **The concentrations of 2,4-D in erythrocytes' membrane and inside the erythrocyte.**
   After incubation 100 ppm of 2,4-D with erythrocytes (5% haematocrite) the concentration of this pesticide inside the cells (respectively in haemolystate) was about ~1.23% of the initial dose, respectively 24.55 ± 8.54 μg/ml PC (Fig. 3).
   However, in samples from membranes the concentration of 2,4-D was about ~ 0.15% of the initial dose (respectively 3.08 ± 0.91 μg/ml PC).
   The concentration of the herbicide in supernatant derived from haemolysate was 5-fold higher in comparison to concentration of 2,4-D determined in supernatant derived from membranes (Fig. 3).

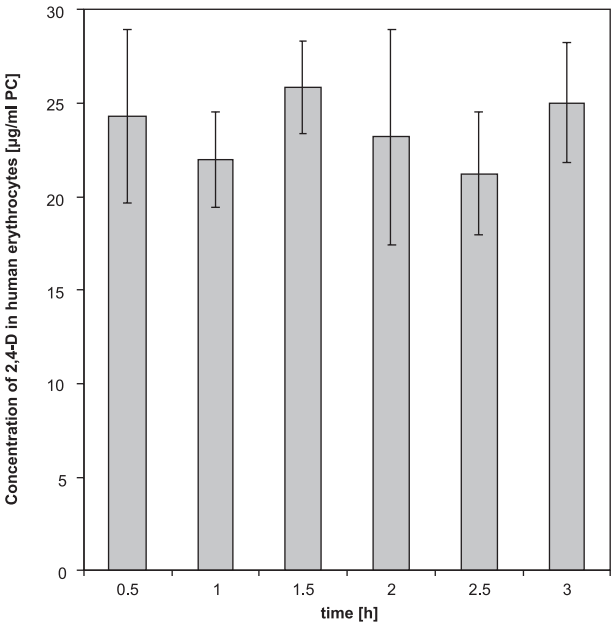

Fig 1. The concentrations of 2,4-D in human erythrocytes in dependence on incubation time.

Discussion

2,4-D-Na has amphiphilic properties, it is reasonable to think that the mechanisms of herbicide transport in blood is one or a combination of several possible carriers and/or reservoirs like lipoproteins, red cells or serum albumin. Rosso and co-workers [16] suggested that 2,4-D binds to albumin in blood and this complex is the main

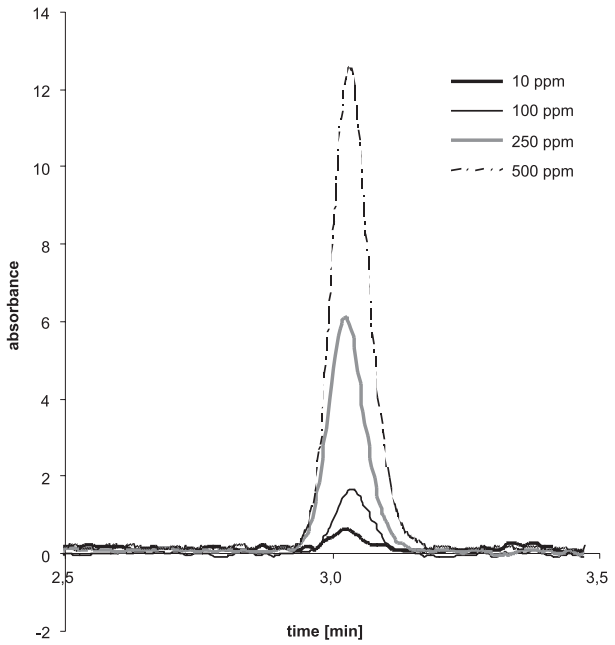

Fig. 2. Chromatograms of 2,4-dichlorophenoxyacetic acid determined in haemolysate in different concentrations (in dependence on initial concentration of the herbicide in sample).

Table 1. The content of 2.4-D in human erythrocytes in dependence on the initial concentration of this herbicide.

	Concentration of 2.4-D incubated with erythrocytes			
	10 ppm	100 ppm	250 ppm	500 ppm
Determined concentration of 2.4-D after its penetration into cell in μg/ml PC	2.1 ± 0.48	20.02 ± 2.49	47.60 ± 16.25	96.40 ± 15.19
Per cent of dose which penetrated the erythrocytes of 5% haematocrite in relation to incubation dose (initial concentration of 2.4-D) in %	1.05%	1.0%	0.95%	0.96%

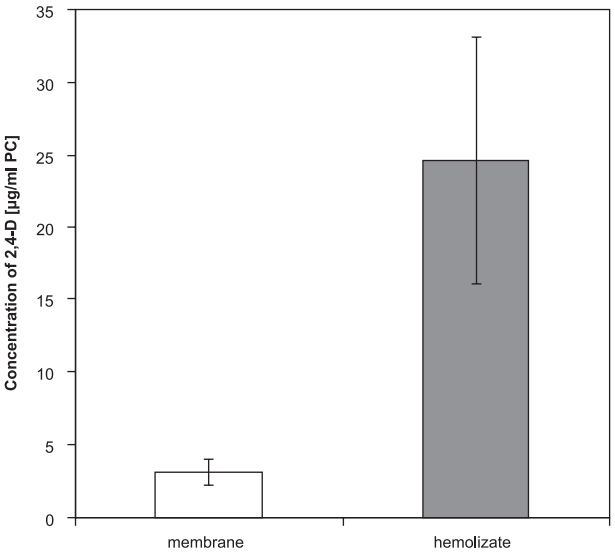

Fig. 3. The concentrations of 2,4-D determined in membranes and in interior of erythrocytes after 1hour incubation with 100 ppm of 2,4-D.

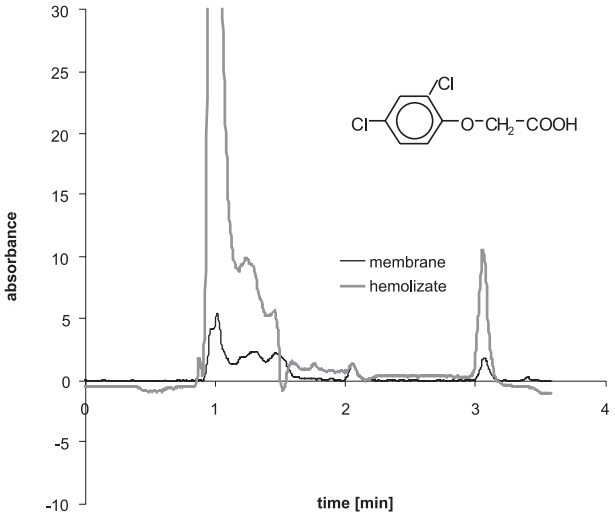

Fig. 4. Chromatograms of 2,4-dichlorophenoxyacetic acid determined in erythrocytes' membrane and haemolysate.

blood transport system for 2,4-D. The essential item is the possibility of binding of 2,4-D with proteins and changing their capacity. On the one hand it may make detoxification easier (conjugation with aminoacids and plasma albumins) and on the other it may cause essential disturbances in cell function and finally its death.

2,4-D may also penetrate into erythrocytes and disturb their structures. Recently, it has been reported that 2,4-D induces alteration in human erythrocytes and in membrane model system at relatively high concentrations (higher than 100 µM). Suwalsky and co-workers [17], showed that 2,4-D disturbed the phospholipids bilayer structure of human erythrocytes, which integrity is essential for the proper functions of this cell. The authors postulated that the morphological transformation of erythrocytes may be a result of specific oxidative transformation of their skeleton that may lead to echinocyte formation [17, 18].

It is known that xenobiotics/drugs may bind to the membrane and/or to haemoglobin, carboanhydrase, and binding proteins in the cytosol of the erythrocytes [19].

Our results showed that 0.9% of the initial concentration of 2,4-D-Na penetrated in 0.5 h into erythrocytes and 0.15% may be bound with erythrocyte membrane (Fig. 3).

The capacity of a compound to penetrate cell membrane depends on its hydrophobicity (log P) and ionization level at relevant pH (pK_a). Generally, the lower value of pK_a of a compound is, the higher toxicity it reveals. Both 2,4-D is lipophilic weak acids log P = 2.75 for 2,4-D with a low pK_a = 2.73 [20].

Bergesse and Belgano [21] studied the influx of undissociated form of 2,4-dichlorophenoxyacetic acids into Chinese hamster ovary cells. They observed a rapid uptake of 2,4-D, which reached a stationary state in 30 min. and the influx was determined in relation to concentration of 2,4-D. Bergesse and Belgano [21] also emphasized the fact that cells appear to have a preference for the uptake of the undissociated form of the herbicide. There is a decrease in the intake when the dissociated form increases. This phenomenon could be the consequence of alterations at the carrier level by electrical charge changes in the cell membrane of the dissociated form of 2,4-D. They observed also that undissociated form of 2,4-D was transported against a concentration gradient. The 2,4-D concentration was 6.7 times higher in the cell's interior than in the extracellular incubation mixture.

It was also stated that penetration process of the dissociated form of 2,4-D is independent at the time of incubation (0.5-3 h), but it is related with doses of 2,4-D-Na (Fig.2, Table 1). We observed the similar effect to Bergesse and Belgano [21], the uptake of 2,4-D stationary state in 30 min. In our experiments dissociated forms of 2,4-dichlorophenoxyacetic acid-Na penetrated erythrocyte in the amount of ~1% of the initial concentration of 2,4-D-Na during 0.5 hours of incubation (in all doses -10 -500 ppm). These findings revealed that 2,4-D-Na was transported with concentration gradient (the amount of 2,4-D was much higher outside in comparison to the interior of the cell).

Lipid bilayer of erythrocyte membrane due to its hydrophobic capacities is poorly permeable for most polar and charge-endowed compounds. The role of transporters is played by specialized proteins that are responsible for transport of the specified molecule or group of compounds. These proteins may be divided to carrier and channel transporters. The proteins that actively transport molecules across membrane against electrochemical gradient execute active transport. Channel proteins and many carrier proteins transport molecules without energetic effort according to their electrochemical gradient, in a process of passive transport [22].

The mechanism of penetration of 2,4-D into cell has been previously described. Most results of the investigations have revealed that 2,4-D is transported. Phenoxyherbicides are transported from the cerebrospinal fluid also *via* the organic anion transport system, and inhibitors of this transport may block their elimination from the brain *in vivo*, just as they block their transport by the isolated choroids plexus [23, 24]. Suwalsky et al. [17] suggested also that 2,4-D interfered with Cl^- transport and total transport across the neuroepithelial membrane.

Investigations of the erythrocytes partitioning of relatively small organic cationic, anionic, and nonelectrolytic molecules have shown that lipophilicity, molecular size, and chiral characteristics are important. Lipophilic organic compounds penetrate the erythrocytes by dissolving into the lipid bilayer membrane. Small size hydrophilic compounds enter the erythrocytes through aqueous channels. The erythrocytes partitioning by passive diffusion has been reported for organic cationic and anionic drugs, as well as for nonelectrolytes [19].

The organic anion transport system is regulated by band III protein in human erythrocytes. Band 3 protein is an integral membrane protein in human erythrocytes, which catalyzes the one-for-one exchange of anions across the membrane**.** The anions bind to specific anion binding sites on the band III protein for which saturation kinetics are observed at high substrate concentrations**.** Various specific inhibitors of band 3, which can bind covalently or noncovalently to the anion binding site are known, such as 4.4'-diisothiocyanostilbene-2.2'-disulfonate (DIDS'). The normal mechanism of action involves facilitative transport of anions in which electro-neutrality is preserved. While $C1^-$ and HCO^- exchange is the most important physiological process, other anions such as sulphate and inorganic phosphate can also be transported by band 3 [25].

Bergesse et al., [21] suggested that the herbicide influx was an active, energy dependent process and metabolic toxins such as sodium azide and dinitrophenol produced a strong inhibition of 2,4-D uptake by the cells. The investigation, however, was led on nucleolus cells that contained mitochondria and exhibited respiratory chain reactions that produced ATP. Erythrocytes do not have mitochondria and respiratory chain. The erythrocytes draw energy from glucose metabolism *via* direct glycolysis and the hexose monophosphate shunt [26].

In contrast to the results described above, Castro and co-workers [27] and also Arias and Fabra de Peretti [28] found that 2,4-D reduced the number of viable cells and transport of 2,4-D was energy-independent, since it was not affected by metabolic inhibitors such as 2,4-dinitrophenol and sodium arsenate, which competes with phosphate in the synthesis of ATP.

Summing up, we observed that the uptake of dissociated form of 2,4-D into human erythrocytes was stabilized after 30 min. of incubation with the pesticide. We also observed that 2,4-D-Na influx into human erythrocytes agreed with concentration gradient of the compound (~1% of the initial concentration of 2,4-D).

Abbreviations

2,4-D-Na – sodium salt of 2,4-dichlorophenoxyacetic acid, PC – Packet Cells = 100% hematocrite

References

1. MICHAŁOWICZ J., The occurrence of chlorophenols, chlorocatechols and chlorinated methoxyphenols in drinking water of the largest cities in Poland. Polish Journal of Environmental Studies **14**, 327, **2005**.
2. BUKOWSKA B., 2,4-dichlorophenoxyacetic acid and their influence on humans and animals. Polish Journal of Environmental Studies **15**, 365, **2006**.
3. GARABRANT D.H. PHILBERT M.A., Review of 2,4-dichlorophenoxyacetic acid (2,4-D) epidemiology and toxicology. Crit. Rev. Toxicol. **32**, 233, **2002.**
4. WAITE D.T., CESSNA A.J., GROVER R., KERR L.A., SNIHURA A.D., Environmental concentrations of agricultural herbicides: 2,-4-D and triallate. J. Environ. Qual. **31**, 129, **2002**.
5. TIMCHALK C., Comparative inter-species pharmacokinetics of phenoxyacetic acid herbicides and related organic acids. Evidence that the dog is not a relevant species for evaluation of human health risk. Toxicology **200**, 1, **2004**.
6. BURNS C.J., BEARD K.K., CARTMILL J.B., Mortality in chemical workers potentially exposed to 2,4-dichlorophenoxyacetic acid (2,4-D) 1945-1994 an update. Occup. Environm. Med. **58**, 24, **2001**.
7. PHILLIPS P.J., BODE R.W., Pesticides in surface water runoff in south-eastern New York State, USA: seasonal and stormflow effects on concentrations. Pest. Management Science **60**, 531, **2004.**
8. COX C., 2,4-D toxicology. J. Pest. Ref., **19**, 14, **1999.**
9. EISEMAN J.L., The pharmacokinetic evaluation of 14C-2-,4-dichlorophenoxyacetic acid (2,4-D) in the mouse. Final Report, Industry Task Force on 2,4-D Research Data, Midland, MI **1984.**
10. VAN RAVENZWAAY B., HARDWICK T.D., NEEDHAM D., PETHEN S., LAPPIN G.L., Comparative metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and dog. Xenobiotica **33**, 805, **2003.**
11. SAUERHOFF M.W., BRAUN W.H., BLAU G.E., GEHRING P.J., The fate of 2.4 dichlorophenoxyacetic acid (2,4-D) following oral administration to man. Toxicology **8**, 3, **1977**.
12. ORBERG J., Effects of low protein consumption on the renal clearance of 2,4-dichlorophenoxyacetic acid (2,4-D) in goats. Acta Pharmacol. Toxicol. **46**, 138, **1980**.
13. DĄBROWSKI Z. Eds. Blood physiology. Bartosz, G., pp. 72–167G., Copyright by PWN Sp. Z o.o. Warszawa. **2000. [**in Polish]
14. BARTOSZ G., The other face of oxygen: Copyright by PWN Sp. Z o.o. Warszawa**. 2003. [**in Polish]
15. COSSUM P.A., Role of the red blood cell in drug metabolism. Biopharm Drug Dispos. **9**, 321, **1988.**
16. ROSSO S.B., GONZALEZ M., BAGATOLLI L.A., DUF-

FARD R.O., FIDELIO G.D., Evidence of a strong interaction of 2,4-Dichlorophenoxyaceic acid herbicide with serum albumin. Life Sciences **63**, 2343, **1998.**

17. SUWALSKY M., QUEVEDO L., NORRIS B., BENITES M., Toxic action of the herbicide 2,4-D on the neuroepithelial synapse and on the nonstimulated skin of the frog Caudierbera caudiverbera. Bull. Environ. Contam. Toxicol. **62**, 570, **1999.**
18. BUKOWSKA B., ZATORSKA A., The prehemolytical changes in human erythrocytes treated with 2,4-dichlorophenoxyacetic acid (2,4-D). Current Topics in Biophysics **27**, 11, **2003.**
19. HINDERLING P.H., Red Blood Cells: A Neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol. Rev. **49**, 279, **1997.**
20. AYDIN H., ÖZDEMIR N., UZUNÖREN N., Investigation of the accumulation of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat kidneys. Forensic Science International **153**, 53, **2005.**
21. BERGESSE J.R., RIVAROLA V.A., BALEGNO H.F., Changes in 2-Aminoisobutyric Acid and Cycloleucine Uptake Produced by 2,4-Dichlorophenoxyacetic Acid in Chinese Hamster Ovary Cells. Toxicol. Lett. **36**, 189, **1995.**
22. JÓŹWIAK Z., BARTOSZ eds. Biophysics. Selected issues with exercises. M. Bryszewska, pp. 395 – 405 Copyright by PWN Sp. z o.o., Warszawa 2005, Ist edition **2005.**
23. KIM C.S., PRITCHARD J.B., Transport of 2.4.5-trichlorophenoxyacetic acid across the blood-cerebrospinal fluid barrier of the rabbit. J. Pharmacol. Exp. Ther. **267**, 751, **1993.**
24. PRITCHARD J.B., Accumulation of anionic pesticides by rabbit choroids plexus in vitro. J. Pharmacol. Exp. Ther. **212**, 354, **1980.**
25. KAUL R.K., MURTHY S.N.P., REDDY A.G., STECK T.L., KOHLER H., Amino Acid Sequence of the Na-Termina1201 Residues of Human Erythrocyte Membrane Band 3. J. Biol. Chem. **258**, 7981, **1983.**
26. BEUTLER E., LICHTMAN A., COLLER B.S., KIPOS T.J. eds. Williams Hematology, pp. 349–363, McGraw Hill, Inc., New York, **1995.**
27. CASTRO S., FABRA A., MORI G., RIVAROLA V., GIORDANO W., BALEGNO H., Characterization of 2,4-dichlorophenoxyacetic acid transport and its relationship with polyamines in Azospirillum brasiliense. Toxicol. Lett. **84**, 33, **1996.**
28. ARIAS R., FABRA DE PERETTI A., Effects of 2,4-dichloroohenoxyacetic acid on Rhizobium sp. Growth and characterization of this transport. Toxicol. Lett. **68**, 267, **1993.**