CHAPTER 13

Fluoroacetate

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I. INTRODUCTION

Fluoroorganic compounds attracted the attention of researchers nearly 70 years ago when among a large class of biologically inert chemicals a group of very toxic compounds was revealed, having the general formula CH_2FCOOR and the common name ''fluoroacetate'' (FA). The toxicological effects of FA do not become apparent immediately even after exposure to lethal doses, but after a latent period of half an hour to several hours for animals and humans. The level of FA in some Australian plants can reach up to 5 g/kg dry weight (Hall, 1972) and can cause death of domestic animals, sometimes with appreciable economic damage (McCosker, 1989; Minnaar *et al.*, 2000a). FA can be found in fog and rain drops in some industrial regions (Rompp *et al.*, 2001). The best known representative of FA is its sodium salt (SFA, compound 1080). This substance is used in several countries for controlling populations of some vertebrates. There are also series of fluorocompounds whose metabolism is connected with the formation of FA, these are: antineoplastic drugs (5-fluorouracil and isomers of fluoronitrosourea); N-(2-fluoroethyl) derivatives of the narcotic analgetics normeperidin and normethazocin; pesticides, 1,3-difluoro-propanol and fluoroacetamide (FAA, compound 1081); and 1-(di)halo-2-fluoroethans and fluoroethanol (Reifenrath *et al.*, 1980; Tisdale and Brennan, 1985; Feldwick *et al.*, 1998). The urgency of the problems associated with FA toxicity; and therapy for acute intoxication has greatly increased in connection with a new threat – international terrorism (Holstege *et al.*, 2007). Physicochemical features of FA, the lack of taste and odor, delayed manifestation of toxicity, and similarity of clinical signs of intoxication to some natural indispositions are all characteristics that necessitate comprehensive studies of mechanisms of action of FA and a search for effective therapeutic means for treatment of acute intoxication.

II. BACKGROUND

FA was initially synthesized in 1896 and only decades after that was found in *Dichapetalum*, *Gastrolobium*, *Oxylobium*, *Acacia* and *Palicourea* plants growing in Australia, South Africa, and South America (Oerlichs and McEwan, 1961; De Oliveira, 1963; McEwan, 1964; Aplin, 1971; Vickery *et al.*, 1973). Chemically pure FA is a very stable compound, and the energy of dissociation of the fluorocarbon bond in the molecule is regarded as one of the highest among the natural compounds (Ichiyama *et al.*, 2004). However, FA is broken down in biological preparations from plants (Minnaar *et al.*, 2000a). After inhalation or ingestion, FA is easily absorbed by tissues and its high toxicity is independent of its route of entry into organisms (Chenoweth, 1949). The mechanism of toxic action of FA is widely known as ''lethal synthesis'' (Peters, 1952; Peters and Wakelin, 1953), the essence of which is conversion of nontoxic FA to toxic fluorocitrate (FC) within the cells of an organism. The main reasons for death are considered to be disbalance of intracellular ions, osmotic disbalance, and deficit of ATP as a consequence of aconitase blockade (Buffa *et al.*, 1973). The latent period from the moment of poisoning with FA to manifestation of clinical signs is 0.5–3 h (in warm-blooded animals). This period reflects penetration of FA into blood and cells and conversion of FA to FC, with the consequent uncoupling of intracellular metabolism. Death usually occurs within 24–48 h, but can be later. At autopsy there are no specific signs of intoxication (Peters *et al.*, 1981). For warm-blooded animals unadapted to FA the lethal dose is less than 2 mg/kg (Atzert, 1971). But there is a considerable species-specific difference in clinical signs of intoxication and differences in sensitivity to the poison (Chenoweth, 1949). The mean lethal dose varies within the range from 0.05 mg/kg for dogs to 150 mg/kg for possums. The most common criterion for tolerance, or sensitivity, of animals to FA is intensity of metabolism. Thus, in the lizard *Tiliqua rugosa* the level of metabolism of FA is ten times lower in comparison with that of rat (*Rattus norvegicus*), and the lethal dose for lizard is 100 times higher than that of rat (Twigg *et al.*, 1986). Low intensity of metabolism means low conversion of FA to FC, which makes more effective excretion and detoxication possible. In the absence of specific clinical, physiological, and morphological signs of intoxication, determination of FA in tissues together with

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citrate and fluoride ions can be a diagnostic confirmation of FA intoxication (Harrison *et al.*, 1952; Schultz *et al.*, 1982; Koryagina *et al.*, 2006).

III. TOXICOKINETICS

A. Detoxification

The main pathway of detoxification of FA is its defluorination via a glutathione-dependent mechanism involving nucleophilic attack on the β-carbon atom and formation of fluoride and S-carboximethylglutathion, with subsequent cleavage of the latter into amino acids and S-(carboxymethyl) excreted in the urine as a conjugate complex (Mead *et al.*, 1979, 1985; Tecle and Casida, 1989). The highest defluorinating activity was found in liver, followed by kidney, lung, heart, and testicles in a descending line. No defluorinating activity was found in brain. The activity of enzymes responsible for defluorination depends on glutathione (GHS) concentration with a maximum above 5 mmol/l, the apparent K_m being 7 mmol/l at saturating concentrations of GHS (Soiefer and Kostyniak, 1983). Defluorination is mainly carried out by anionic proteins having glutathione transferase activity, though the anionic fraction contains nearly 10% of proteins without this activity but is also capable of defluorination of FA. Moreover, cationic enzymes were shown to be responsible for about 20% of cytosolic defluorination of FA (Wang *et al.*, 1986). The GHS-dependent enzyme defluorinating FA is not identical to GHS-dependent S-transferases; it is an FA-specific defluorinase having an acidic isoelectric point (pH = 6.4) and a molecular weight of 41 kD (27 kD for the main subunit) (Soiefer and Kostyniak, 1984). Activity of defluorinase isoenzymes varies markedly and has been the subject of recent research (Tu *et al.*, 2006).

B. Analytical Procedure

Analysis of biological samples of FA is problematic because of the high polarity of the fluorine–carbon bond in the molecule. Liquid chromatography (LC) has been applied for analysis of FA in different media (Livanos and Milham, 1984; Allender, 1990), and analysis of FA in plants and gastric contents by HPLC with UV detection has also been described (Ray *et al.*, 1981; Minnaar *et al.*, 2000b). Being a nonvolatile substance, FA was commonly analyzed by gas chromatography (GC), as a methyl derivative (Stevens *et al.*, 1976), ethyl or *n*-propyl derivatives (Peterson, 1975) and as pentafluorobenzyl esters (Okuno *et al.*, 1982; Vartiainen and Kauranen, 1984). Derivatization with 2,4-dichloroaniline in the presence of N,N-dicyclohexylcarbodiimide was used for GC analysis of SFA in water (Ozawa and Tsukioka, 1987) and blood serum (Demarchi *et al.*, 2001). A modified procedure by Eason *et al.* (1994) achieved low detection limits for FA at the level of 0.01 μg/g in plasma and urine and 0.002 μg/g in tissue and feces of sheep and goats. However, this procedure is labor and time consuming, and the GC-electron capture detection procedure applied is considered to be unreliable at this level of sensitivity.

The main problem for GC analysis of FA in biological samples is coelution of the matrix components. This can be overcome by sampling the analyte from an equilibrium vapor phase. Static head-space analysis of SFA as ethyl fluoroacetate, with a linear range for SFA in water of 5–200 μg/ml and a detection limit of 0.5 μg/ml has been reported (Mori *et al*, 1996). Solid-phase microextraction (SPME) from an equilibrium vapor phase has all the advantages of head-space analysis, while being a much more sensitive technique. We reported on a novel procedure for determination of FA in water and biological samples, involving ethylation of FA with ethanol in the presence of sulfuric acid, SPME of the ethyl fluoroacetate formed with subsequent analysis by GC-MS (Koryagina *et al.*, 2006). To overcome the problem of the presence of the components coeluting with FA derivatives we made use of GC-MS in the SIM (selective ion monitoring) mode. To avoid a partial overlapping of the internal standard's peak with the sample matrices' components, quantification was performed with the use of two internal standards, carbon tetrachloride and toluene. GC-MS was performed on a Shimadzu QP5000 GC-MS system, using a Supelco SPB-5 capillary column. A stable PDMS-Carboxen-Stable-Flex microfiber (75 μm) was used. The GC-FID, combined with SPME under the optimal conditions, achieved reliable determination of FA in water in the concentration range 0.001–10 μg/ml. The calibration plot for the determination of SFA in biological samples was linear in the SFA concentration range 0.01–5.0 μg/ml for both internal standards, and a linear relationship in blood plasma was observed in the range 0.01–5.0 μg/ml ($r = 0.95$). With toluene as internal standard, the linear regression equation was $Y = 0.014\ X$ [Y was a ratio S(EthylFA)/S(toluene); X was the concentration of SFA, μg/ml]. The RSD (relative standard deviation) for fluoroacetate quantification at 0.1 μg/ml was 12% ($n = 5$). With carbon tetrachloride as internal standard, a linear relationship in plasma was observed in the range 0.01–5.0 μg/ml ($r = 0.98$). The linear regression equation was $Y = 0.1656\ X$ [Y was the ratio S(EthylFA)/S(CCl4); X was the concentration of FA, μg/ml]. The RSD for FA quantification at 0.1 μg/ml was 6% ($n = 5$), and the detection limit was 0.01 μg/ml ($S/N = 3$). The calibration characteristics of rat organ homogenates were identical to those of plasma.

C. Tissue Distribution and Elimination

The data on toxicokinetics of FA are rather contradictory, apparently depending on analytical procedures and dose of the poison; also, there is evidence for animal species specificity. The first data on toxicokinetics of FA demonstrated its rather uniform distribution between organs, with some

predominance in heart, brain, and kidneys (Hagan *et al.*, 1950; Gal *et al.*, 1961). The half-life was calculated to be not less than 2 days, and this could cause secondary toxicity arising from ingestion of meat from the poisoned animals (Aulerich *et al.*, 1987). For the purposes of risk assessment for humans in case of secondary poisoning with meat, sheep and goats were given FA (0.1 mg/kg) and their tissues were analyzed for FA content. The half-life of FA was shown to be 10.8 h for sheep and 5.4 h for goats; maximal concentration of FA 2.5 h after the poisoning was revealed in blood plasma (0.098 μg/ml), followed by kidneys (0.057 μg/g), skeletal muscles (0.042 μg/g), and liver (0.021 μg/g). Only traces of FA were found in all the tissues examined 96 h after the poisoning (Eason *et al.*, 1994). At 1 and 12 h after introduction of SFA (0.2 μg/kg) to rats a similar ratio of FA was found in rat plasma (0.26 and 0.076 μg/ml, correspondingly) (Eason and Turck, 2002), the half-life period being 2.9 h. On the other hand, for rabbits under subacute intoxication with FA the half-life was found to be 1.1 h, and the level of FA in rabbit muscles, kidneys, and liver was much higher than in blood plasma (Gooneratne *et al.*, 1995).

In our laboratory, the use of the above-mentioned SPME method in combination with GC-MS produced the following results (Figure 13.1): maximal concentrations were found in rats 1 h after the poisoning, 2.2 μg/ml in blood plasma and 1.89 μg/g in brain; there was 3–4 times less FA in rat kidneys, liver, and heart (from 0.64 to 0.50 μg/g). After a further 2 h the distribution between the tissues was more equal, resulting from a prominent decrease of FA in plasma and brain and a small decrease or even elevation of FA in other organs. A further decrease of FA was found in all the tissues, except for heart, 24 h after the poisoning. After 72 h, no FA was detected in plasma; we did not measure FA in rat organs at this point. The half-life was calculated to be 3.6 h.

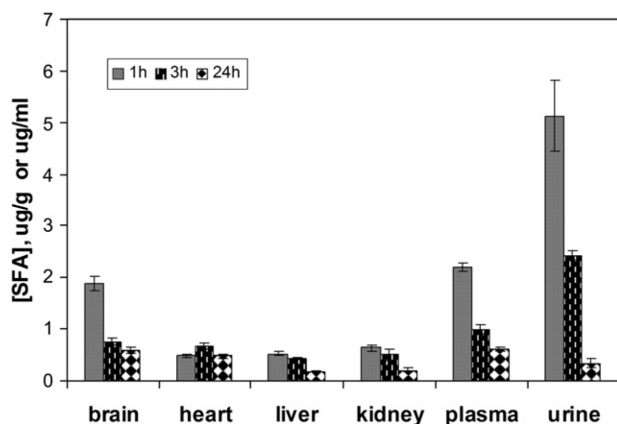

FIGURE 13.1. Data on determination of FA (recounted as SFA) in rat organ homogenates and body fluids, at times following poisoning with SFA at a peroral dose of 2 mg/kg (1/2LD$_{50}$). Standard deviations (shown) were based on 4–6 replicate analyses.

IV. MECHANISM OF ACTION

A. Molecular Mechanism of Aconitase Inhibition

The mechanism of the inhibitory effect of FA on aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] is one of the most interesting in biochemistry. Upon entering an organism, nontoxic FA undergoes a series of metabolic conversions the result of which is synthesis of highly toxic fluorocitrate (FC); this process was termed ''lethal synthesis'' (Peters, 1952). FC is formed by the enzymatic condensation of fluoroacetyl-CoA with oxaloacetate, catalyzed by citrate (*si*)-synthase (EC 4.1.3.7) (Eanes and Kun, 1974; Kirsten *et al.*, 1978). FC was initially considered to be a competitive aconitase inhibitor, but in the early 1990s it was suggested that FC acts as a ''suicide substrate'', because it has a high affinity for aconitase at any concentration of the competitive citrate (Clarke, 1991). Aconitase effects conversion of citrate to isocitrate through an intermediate, *cis*-aconitate, which binds with aconitase in two different ways, swung 180° to the C^{α}—C^{β} bond (Gawron and Mahajan, 1966). Aconitase includes a [4Fe-4S] cluster and the catalytic conversion involves substrate coordination to a specific iron atom in this cluster, Fe_a (Lauble *et al.*, 1992). The single inhibitory isomer was shown to be (–)-*erythro*-2-fluorocitrate (2R, 3R) (Carrell *et al.*, 1970), from which aconitase removes fluoride ion with a stoichiometry of 1 F^- per enzyme molecule (Kent *et al.*, 1985; Tecle and Casida, 1989). The defluorination results in generation of an actual aconitase inhibitor, 4-hydroxy-*trans*-aconitate (HTA), which binds very tightly – though not covalently – with aconitase (Kent *et al.*, 1985; Lauble *et al.*, 1996). The natural aconitase substrate isocitrate should be at a 10^6-fold excess in order to slowly displace HTA from its complex with aconitase. The HTA–aconitase complex involves four hydrogen bonds, which hold together HTA, a water molecule, Asp165, and His167 (Lauble *et al.*, 1994, 1996). In contrast, isocitrate has only one such bond.

B. Physiological and Biochemical Effects of FA

1. Effects of FA and FC on Mitochondria and Other Intracellular Organelles

Functional disturbances of mitochondria (MCh) precede the appearance of structural anomalies (Buffa and Pasquali-Ronchetti, 1977) and consist of their decreased capacity to oxidize the substrates introduced. Within the mitochondrial matrix, FA induces changes which develop in several minutes resulting in its swelling and loss of electronic density. These changes are explained by accumulation of citrate, rise of osmotic pressure, and decrease of energy production (Corsi and Granata, 1967; Buffa and Pasquali-Ronchetti, 1977). Change in the level of ATP is not caused by uncoupling of respiration and phosphorylation (Fairhurst *et al.*, 1958; Corsi and Granata, 1967). Mitochondrial

volume changes are accompanied by their conformational reorganizations: these are displacement of granules and disintegration of cristae, and extension and rupture of their membranes. Axonal cylinders stretch in 3–4 h after small doses of the poison and in 1–2 h after lethal doses. The cylinders are filled with MCh (most of which are being swelled and degenerated), multilamellar lisosome-like bodies, vesicules, and neurofibrils. In the Golgi complex, a condensation of cisternae takes place (McDowell, 1972). Concurrently, a disruption of endoplasmic reticulum, swelling of nucleus, and reduction of aggregated chromatin can be seen.

Having studied *in vitro* effects of FC on rat liver MCh we revealed that maximal inhibition of respiration was registered when MCh were uncoupled (Figure 13.2). The level of alkalinization of the medium at addition of ADP was much lower in the presence of FC, thus evidencing an inhibition of ATP synthesis. The amplitude of alkalinization was also decreased, which could be caused by incomplete ATP synthesis, an additional transmembrane redistribution of protons, and/or change of the binding constant of ADP. FC induced a leak of Ca^{2+} from MCh, which was consistent with the observed inhibition of oxygen consumption in respiratory state 1. Addition of the substrates caused re-entry of Ca^{2+} into MCh. In the presence of FC, the MCh only partially took up the Ca^{2+} ions added to the medium, followed by their spontaneous efflux through an electroneutral $2H^+/Ca^{2+}$ exchanger with $K_{1/2} = 10\ \mu mol/l$ (Teplova *et al.*, 1992).

The effects observed under exposure of MCh to SFA developed at much higher concentrations (from 4 mmol/l), as compared to FC, and greatly depended on respiratory substrates. With pyruvate as substrate, the time period of oxidative phosphorylation (OP) and the level of NADH oxidation increased linearly at increasing SFA concentration in the medium (Zinchenko *et al.*, 2007). However, with

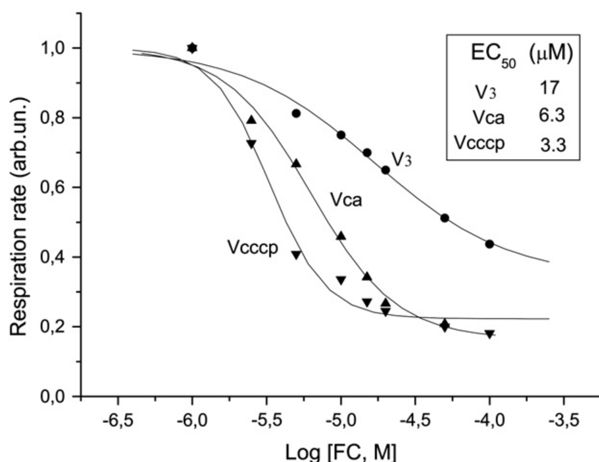

FIGURE 13.2. Effects of FC on respiration of rat liver MCh. Dependence of respiration rate activated by ADP (V_3), calcium transport (V_{Ca}), and protonophore CCCP (Vcccp) upon concentration of FC. Substrates: pyruvate *plus* malate.

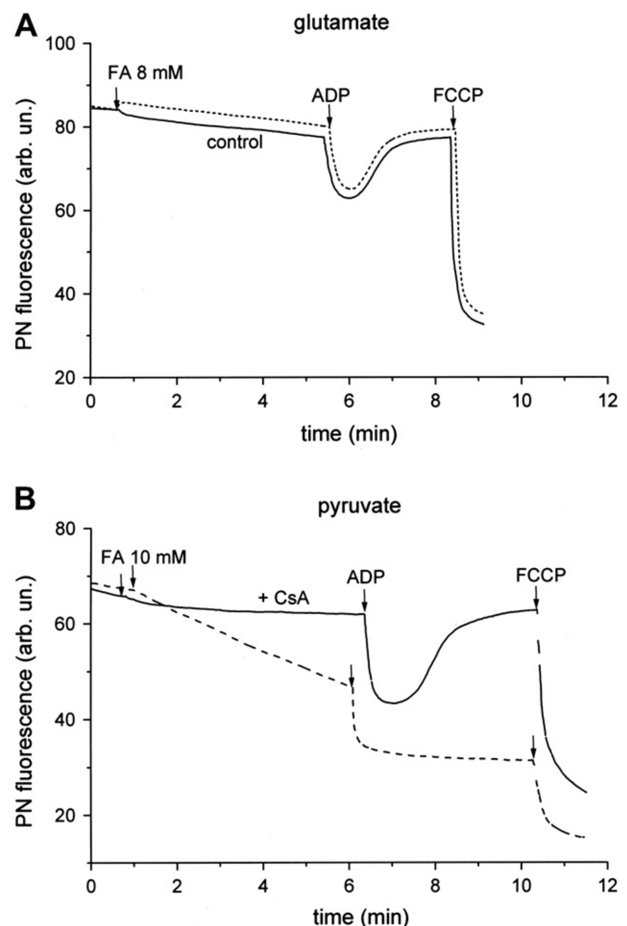

FIGURE 13.3. Effects of FA on redox state of pyridine nucleotides (PN) of rat liver MCh. (A) Glutamate as respiratory substrate. (B) Prevention of PN oxidation and/or leakage by cyclosporin A (CsA) when pyruvate used as respiratory substrate. Additions: (A) SFA 8 mmol/l (dots) or sodium acetate 8 mmol/l (control line), ADP 120 μmol/l, FCCP 1 μmol/l; (B) SFA 10 mmol/l (dots) or SFA 10 mmol/l plus CsA 1 μmol/l (line).

utilization of succinate and especially glutamate, SFA had no effect on OP in concentrations as high as 8 mmol/l (Figure 13.3A) and even 16 mmol/l (not shown here). Moreover, the effect of SFA with pyruvate as respiratory substrate can be prevented by incubation of MCh with cyclosporine A, a known inhibitor of the mitochondrial transition pore (Figure 13.3B). This means that under exposure to FA development of mitoptosis and apoptosis is possible, but opening of the pore is reversible in nature and preventing oxidation or leak of NADH from MCh can turn them back to normal functional state.

2. Effects of Fluoroacetate on Isolated Cells

The effects of FA on the physiological and biochemical status of cells and tissues are tightly dependent upon the level of their oxidative metabolism. Thus, FA does not inhibit phagocytosis because of the low level of TCA cycle

activity within macrophages (Cifarelli *et al.*, 1979). We investigated a series of cell types, transformed lines and those obtained from animals, under exposure to FA or FC. The level of NAD(P)H in Ehrlich ascite tumor (EAT) cells slowly decreased and the level of Ca^{2+} increased when the cells were incubated with SFA (Zinchenko *et al.*, 2007). SFA could induce depletion of intracellular calcium stores and activation of influx of extracellular Ca^{2+} ions through the store-operated calcium (SOC) channels. Discovery of other calcium channels such as TRPV5 and TRPV6 (Hoenderop *et al.*, 2003; van de Graaf *et al.*, 2006), which remain inactivated when $[Ca^{2+}]_i$ is increased and become activated when $[Ca^{2+}]_i$ is decreased, stimulated the investigation of the level of calcium ions in endoplasmic reticulum (ER) with chlortetracycline (CTC). Under exposure to ATP, FC does not affect the velocity of calcium efflux from ER, so the signal transmission from P2Y receptor via G-protein is not inhibited in EAT cells (Zinchenko *et al.*, 2007). However, FC induced a growth in both amplitude of Ca^{2+} leakage and velocity of its influx into ER. A rather long period (8–10 min) of Ca^{2+} influx into ER was observed, which indicated efflux of intracellular Ca^{2+} from cells by plasma membrane Ca-ATPase immediately after mobilization and leaving ER. This greatly reduces $[Ca^{2+}]_i$ for transport back to ER. It was demonstrated earlier (Zinchenko *et al.*, 2001) that the velocity of return transport of Ca^{2+} into ER depends upon activity of plasma membrane SOC channels. Therefore, we suggest that FA (or FC) can induce entry of calcium ions into cells through SOC channels.

The toxic effects of FC on endothelial cells have been shown to be similar to its effects on other energy-dependent tissues: a reduction of ATP level and oxygen consumption but accumulation of lactate and considerable decrease of protein synthesis (Rist *et al.*, 1996). We have demonstrated a gradual decrease of the mitochondrial membrane potential and elevation of $[Ca^{2+}]_i$ under exposure to SFA (unpublished data). Conversely, in cardiomyocytes SFA induced a slow enhancement of the mitochondrial membrane potential together with a rise of basal $[Ca^{2+}]_i$; propagation of calcium waves along the surface of sarcoplasmic reticulum, or visible elevation and velocity of spreading of the pre-existing waves, was also observed (Zinchenko *et al.*, 2007). Probably the increased level of $[Ca^{2+}]_i$ is the reason for its transport into MCh with a subsequent inhibition of the proton ATPase and rise of the membrane potential. Mechanistically, this phenomenon could be explained by the existence of a Ca^{2+}-dependent protein inhibiting H^+-ATPase (Hubbard and McHugh, 1996).

We have also studied the kinetic parameters of platelet aggregation in experiments with rats *in vitro* and *ex vivo* (Mindukshev *et al.*, 2006). Aggregation of platelets was induced with ADP over the concentration range 10–100 nmol/l. The median effective concentrations (EC_{50}) of ADP for the cells exposed to SFA, 10 and 5 mmol/l, were calculated to be 25 nmol/l and 35 nmol/l, correspondingly, and these platelets can be characterized as hypersensitive to ADP. Studying the kinetic parameters of platelet aggregation under intoxication of rats with SFA, we encountered a problem of spontaneous aggregation of the platelet-rich plasma, which was in agreement with the data on primary transition of the platelets to the hypersensitive state. However, the cells that avoided aggregation demonstrated an extremely high level of desensitization. In some experiments, ADP could not induce platelet aggregation at very high (nonphysiological) concentrations near 10 μmol/l.

Under intoxication with SFA a significantly reduced thymus, as well as a prominent quantity reduction of freshly obtained thymocytes and elevation of apoptosis, were revealed (Figure 13.4). SFA also caused an acceleration of apoptosis of control and dexamethazone-treated human lymphocytes *in vitro*, although spontaneous apoptosis of

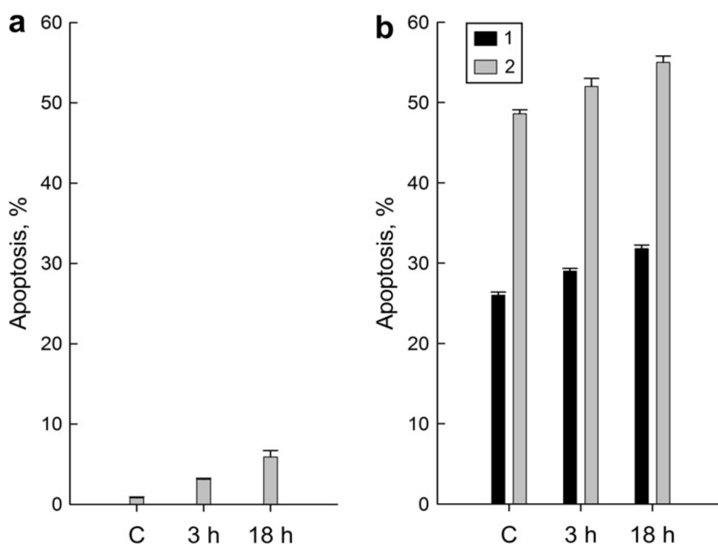

FIGURE 13.4. Effect of SFA on development of apoptosis of rat thymocytes 3 and 18 h after administration of SFA at $1/2LD_{50}$. Registration of apoptosis with Hoechst-33258. (A) Apoptosis in freshly isolated thymocytes; (B) Apoptosis in thymocytes cultivating for 20 h after isolation in the absence (1, black) and presence (2, gray) of dexamethasone.

human neutrophils was inhibited (not shown here). Moreover, SFA practically had no effect on reactive oxygen species (ROS) production by peritoneal macrophages of mice. One can suggest that the inhibitory effect of FA on neutrophil apoptosis is realized through ROS, whereas the enhanced apoptosis and depression of the cells responsible for adaptive immunity is a nonspecific reaction under SFA intoxication, reflecting a general decline and redistribution of energy resources of the organism.

3. Biochemical Parameters Under Intoxication with Fluoroacetate

Among the biochemical effects caused by FA are: accumulation of citrate and disturbance of its transport from MCh; elevation of lactate and disturbances in carbohydrate metabolism; decrease of free fatty acids (FFA) concentration; elevation of adenosine and ammonia; disbalance of bivalent cations and acid–base equilibrium; changes in GABA balance in brain; and rise in phosphates and various enzymes in blood plasma (Buffa and Peters, 1950; Engel *et al.*, 1954; Elliott and Phillips, 1954; Maytnert and Kaji, 1962; Williamson, 1967; Stewart *et al.*, 1970; Bgin *et al.*, 1972; Eanes *et al.*, 1972; Buffa *et al.*, 1973; Liang, 1977; Kirsten *et al.*, 1978; Taitelman *et al.*, 1983a; Bobyleva-Guarriero *et al.*, 1984; Bosakowski and Levin, 1986; Szerb and Redondo, 1993). However, among such a variety of biochemical changes citrate seems to be the only parameter whose qualitative (but not quantitative) trends are not a matter of controversy. In rat hearts under acute intoxication with FA, concentration of citrate can exceed control values 8–15 times (Bosakowski and Levin, 1986). Elevation of citrate concentration is in direct proportion to respiratory activity of a tissue: metabolically active tissues – such as heart, kidneys, and spleen – maximally accumulate citrate, though in liver, which is also characterized by high respiratory level and metabolic activity, a small accumulation of citrate has been observed (Cole *et al.*, 1955; Twigg *et al.*, 1986). In our experiments with rats poisoned with SFA at a dose of $1/2LD_{50}$, concentration of citrate in blood plasma and organs increased within 1 h (Figure 13.5). The most prominent elevation of citrate was revealed 6 h after the poisoning in heart (5×), kidneys (3×), and brain (2.5×). There was doubling of the level of citrate in blood plasma after 1 h, though it was the only biochemical parameter of plasma that remained elevated for 3 days.

Transfer of citrate through the inner membrane of MCh is provided by a tricarboxylate transporter (m.w. 32.5 kD), which also catalyzes transport of *treo*-D_s-isocitrate, *cis*-aconitate and other tricarboxylates (LaNoue and Schoolwerth, 1979; Kaplan *et al.*, 1990). This is electroneutral exchange for either another tricarboxylate or dicarboxylate (e.g. malate or succinate), or for phosphoenolpyruvate. Formation of glutathione-crityl thioester is irreversibly inhibited by (–)erythrofluorocitrate ($IC_{50} = 25$ pmol FC/mg protein), which makes a stable adduct with the synthase (Kun *et al.*, 1977). However, the block of citrate transport

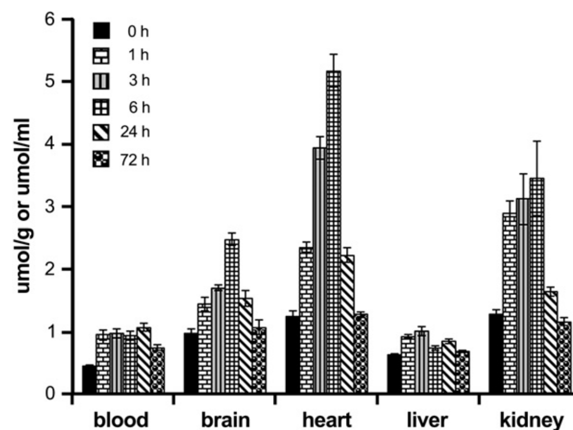

FIGURE 13.5. Concentration of citrate in blood and organs of rats under intoxication with SFA at $1/2LD_{50}$.

is not absolute and universal for all the organs and tissues. There are data on citrate transfer from MCh to cytosol with its subsequent utilization by cytoplasmic aconitase (c-aconitase), which is virtually not affected under FA intoxication, and then by cytoplasmic NADP-dependent isocitrate dehydrogenase (cICDH) (Max and Purvis, 1965). Around 32% of citrate produced in MCh can be transported to cytosol (Buffa *et al.*, 1972). These processes should be regarded as being adaptive and positive, they lead to reduced oxygen consumption because the NADPH generated does not require further oxidation in the respiratory chain and can be utilized in other metabolic pathways.

Among the negative consequences of citrate accumulation is a change of electrolyte composition and acid–base disbalance in the organism. Moreover, elevation of citrate level in cells leads to disturbance of glucose metabolism due to inhibition of the key glycolytic enzyme phosphofructokinase (Bowman, 1964; Peters, 1972). Hyperglycemia during intoxication with FA can be very prominent, in spite of inactivation of gluconeogenesis (Godoy *et al.*, 1968; Bobyleva-Guarriero *et al.*, 1983, 1984). Nevertheless, we could not find significant changes in rat blood glucose level throughout the periods of intoxication with FAA or SFA at a dose of $1/2LD_{50}$; at the same time there was a significant increase of glucose level in liver, heart, and brain (unpublished data). This may signify a utilization of glucose by other tissues and first of all by skeletal muscles, as a result of which the local increase of glucose in organs is not reflected by the level of glucose in blood. Thus, glucose cannot serve as a reliable criterion of intoxication.

Some researchers considered the elevated glucose level to be a result of decreased insulin secretion by pancreatic β-cells due to their damage by FA (Cole *et al.*, 1955; Karam and Grodsky, 1962). Along with hyperglycemia there was hyperketonemia, observed characteristically for the diabetic state, caused by inhibition of TCA cycle and depletion of oxaloacetate (Williamson, 1967; Buffa *et al.*, 1973; Taitelman *et al.*, 1983a). Also consistent with diabetes is

inhibition of hormone-induced lipolysis in adipose tissue (Taylor *et al.*, 1977). Moreover, FA increased glucose conversion to fatty acids, and such coincidence of anti-lipolytic and lipogenetic effects of FA provides a basis for suggesting a relation in effects of FA and insulin. However, injection of insulin does not alleviate FA intoxication in general and ''FA diabetes'' in particular (Reichelt, 1979). During FA intoxication, the initial hyperglycemia can even be reversed into hypoglycemia (Boquist *et al.*, 1988), so this effect of FA was considered to be an insulin-like phenomenon (Zieve *et al.*, 1983). The principal distinction, however, should be depletion of glycogen stores in different tissues under intoxication with FA (Godoy *et al.*, 1968; Boquist *et al.*, 1988). After poisoning with FA, glycogen levels in animal tissues may decrease by 75% in 1 h and by 90% in 2 h (Buffa *et al.*, 1973; Zhou *et al.*, 1984). According to our data, during SFA intoxication ($1/2LD_{50}$) glycogen levels are maximally decreased after 6 h in both liver (by 55%) and brain (by 40%), and the dynamics of the glycogen levels was similar in these organs. Such a decrease could result from the indirect action of adrenalin or sympathetic regulation (Buffa *et al.*, 1973). In addition, inhibition of *de novo* glycogen synthesis has been reported (Zhou *et al.*, 1984).

Nevertheless, disturbances in hormonal regulation during the FA intoxication can also take place: reduction of calcium concentration in blood plasma could be caused by a poor reabsorption of calcium ions in kidneys due to a decrease of parathyroid hormone level; an excess of Ca^{2+} excretion up to 0.173 mg/min (the control rate being 0.06 mg/ml) has been registered (Perez and Prindt, 1977). Decrease of calcium level could be the reason for the so-called ''hypocalcemic tetanus'' (Roy *et al.*, 1980), manifesting itself as typical convulsions, disturbances of blood clotting, and hypotension leading to vascular attacks. The level of decrease of calcium correlates with extension of the Q-T interval on ECG, which is a consequence of broad spectrum of cardiac arrhythmia (Buffa and Peters, 1950; Arena, 1970).

ATP level is usually reduced, though ADP and AMP levels can be elevated in the first hours of the FA intoxication, with subsequent decrease (Bowman, 1964; Stewart *et al.*, 1970). There are other reports of a constant level of ATP in some organs and tissues. For example, FA did not affect ATP and GTP, as well as cyclic nucleotides and levels in hepatocytes *in vitro* (Dohi and Murad, 1981). When dogs were intoxicated with sublethal doses of FA there was no observable decrease in oxygen consumption and ATP level; this was explained by utilization of glutamate and aspartate which can enter the TCA cycle distally of aconitase (Liang, 1977). The inversion of reactions at glutamate dehydrogenase (GDH) is a simple and effective compensative mechanism during blockade of the TCA cycle in kidney cells: instead of glutamine synthesis, glutamate is deaminated to form 2-oxoglutarate to support the flow of reducing equivalents in the TCA cycle and ATP synthesis, while the ammonia produced neutralizes local tissue acidosis (Yu *et al.*, 1976). Such utilization of glutamate may account for the significant reduction of glutamate level in rat organs, beginning at the first hour after intoxication with FA. The data obtained according to the GC method of Matsumura *et al.* (1996) have shown a decrease of glutamate, aspartate, and some other amino acids in rat brain (Figure 13.6), as well as a decrease of glutamate and nearly complete absence of glutamine in blood plasma of rats and rabbits (not shown here) 3 h after poisoning with SFA. The levels of amino acids in blood plasma of animals indicate the extent of protein breakdown in muscles, on the one hand, and the level of their utilization by other organs and tissues, on the other hand. Under intoxication with FA, glutamate and its precursor glutamine are probably the main nutrients. Elevation of amino acid levels in blood plasma of rats within 3 h after poisoning signifies an elevation of protein breakdown. Furthermore, this indicates that other amino acids – because of their transport, catabolism, etc. – are not nutrients of primary importance under energetic deficit conditions.

Elevation of lactate level in the blood of animals poisoned with FA has been reported (Engel *et al.*, 1954; Taitelman *et al.*, 1983a). In agreement with these workers, we observed a prominent rise in lactate levels in blood just after convulsions (unpublished data). In rat heart and brain, lactate levels decreased under intoxication with SFA or FAA, irrespective of convulsions. During SFA intoxication the decrease in lactate level (and increase of glucose level) in rat heart takes place earlier and to a greater extent than for FAA intoxication: 38% decrease in 3 h for lactate, as compared with 25% in 6 h in the case of FAA; 100% increase in 3 h for glucose, as compared with 67% in 6 h in the case of FAA. Also, the maximal increase of citrate was registered at 24 h after poisoning with FAA, but at 6 h after poisoning with SFA. These and other biochemical data are

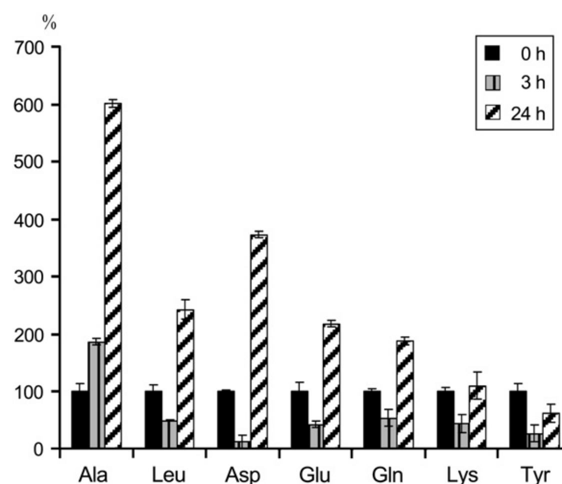

FIGURE 13.6. Changes of some amino acids in rat brain 3 and 24 h after administration of SFA at $1/2LD_{50}$.

consistent with clinical pictures of intoxication with equipotential doses of SFA and FAA: intoxication with SFA is generally more violent and takes a shorter period of time.

4. Effects of FA on the Cells of the Nervous System: Interaction of Glia and Neurons

Acetate is metabolized in astrocytes nearly 18 times faster than in cortical synaptosomes, though activity of acetyl-CoA synthase in synaptosomes is almost double that in astrocytes (5.0 and 2.9 nmol/min per mg of protein, respectively). The principal difference in the acetate metabolism rates is explained by differences in the kinetics of its transport, which is mediated by a monocarboxylate carrier (Hosoi *et al.*, 2004); acetate uptake by astrocytes, unlike synaptosomes, rapidly increases and follows saturation kinetics (V_{max} = 498 nmol/mg protein/min, K_M = 9.3 mmol/l) (Waniewski and Martin, 1998). Having penetrated into astrocytes at one site, FA can diffuse into other cells through gap junctions (Ransom, 1995). Citrate accumulating in astrocytes is readily released from cells and effectively penetrates other astrocytes (Westergaard *et al.*, 1994). The TCA cycle in nerve tissues is blocked by FA but not completely, only by 35–55% (Patel and Koenig, 1968). This leads to decreased consumption of glucose and increased consumption of glutamine (if the latter is available); no reduction of ATP was observed (Hassel *et al.*, 1994). The natural metabolic pathway is switched over to utilization of glutamine, glutamate, and 2-oxoglutarate in the TCA cycle. GDH of astroglia plays a big role in this switching over, promoting the ATP-independent utilization of glutamate (Plaitakis and Zaganas, 2001). The absence of an aspartate/glutamate mitochondrial exchanger (the key component of the malate/aspartate cycle) in astrocytes also plays in support of this (Xu *et al.*, 2007). There is little GDH in neurons as compared to astrocytes, with activity of GDH depending not only upon proximity to glutamatergic fibers and terminals, but also upon activity of neighboring neurons regardless of their functional specialization; a deficiency of GDH activity in astroglia may be a cause of cytotoxic effects of glutamate and aspartate (Aoki *et al.*, 1987).

The taking of glutamate by astrocytes is an electrogenic process in which one molecule of glutamate is cotransported with three sodium ions (or $2Na^+$ and $1H^+$), being exchanged for $1K^+$ and $1OH^-$ or $1HCO_3^-$ (Bouvier *et al.*, 1992). To re-establish the ionic balance, Na^+/K^+-ATPase would work with ATP provided by phosphoglycerate kinase bound to plasma membrane. This stimulates glycolysis and lactate production in astrocytes. Lactate is released from astrocytes and then taken by neurons to be further oxidized. Pyruvate, which is also produced in astrocytes, can be utilized in the TCA cycle to form 2-oxoglutarate or transaminated to form alanine; the latter can also enter neurons (Tsacopoulos and Magistretti, 1996; Tsacopoulos, 2002). However, the rate of alanine metabolism through alanine transaminase (ALT) in synaptosomes is much less than the rate of its uptake; moreover, neuronal ALT and AST work mainly to synthesize alanine and aspartate (Erecinska and Silver, 1990), hence a stable elevation of alanine level, in contrast to that of other amino acids, in brain of rats poisoned with SFA (Figure 13.6). The role of alanine as a source of glutamate is increased during the restoration period after ischemia/hypoxia, when alanine concentration is elevated and glutamate concentration is reduced.

Inhibition of glutamate uptake by astroglial cells can be one of the causes of convulsions observed under intoxication with FA (Szerb and Issekutz, 1987). The toxic effect is governed mainly by citrate, which chelates calcium ions (Fonnum *et al.*, 1997). Intrathecal injection of FC in mice caused convulsions in about 15 s, while in about 37 min by intracerebroventricular injection (Hornfeldt and Larson, 1990). Moreover, intrathecal injection of sodium citrate caused the same effect. This means that the main target of FC and citrate, and the area for generation of convulsions, should be the spinal cord. Convulsions could also be generated by other compounds having the common property of chelating calcium ions; these are EDTA, EGTA, glutamate, and lactate (Hornfeldt and Larson, 1990). Thus, along with elevation of citrate level, activation of anaerobic oxidation of glucose in neurons followed by accumulation of lactate in cerebrospinal fluid could also lead to coma and convulsions (Stewart *et al.*, 1970). Chelation of zinc and other divalent cations by citrate enhances the signaling activity of NMDA receptors (Westergaard *et al.*, 1995). In addition, disturbances of GABA metabolism were revealed as a result of the TCA cycle blockade: after injection of FA, there was initially an elevated level of GABA registered in different regions of the brain, followed by its reduction concurring with the beginning of clonicotonic convulsions (Maytnert and Kaji, 1962; Stewart *et al.*, 1970).

The convulsive state is aggravated by increasing the concentration of ammonia ions (Raable, 1981), an excess of which can lead to redistribution of K^+ and Cl^- ions, disturbances of neuronal depolarization and hyperpolarization, and impairment of post-synaptic inhibition. The neuron dysfunctions observed result in encephalopathy, ataxia, convulsions, and coma (Iles and Jack, 1980; Raable and Lin, 1983, 1984; Xiong and Stringer, 1999). On the other hand, FC affecting astroglia cause a drop in membrane potential and depolarization, and decrease of $[K^+]_i$ (Largo *et al.*, 1997); this should lead to compensatory transport of bicarbonate ions into astrocytes and acidification of the extracellular medium. Together with the natural carbonate acidification of the medium close to chemoceptors of the retrotrapezoid nucleus, this activates the diaphragmal nerve and increases the expired minute ventilation (Erlichman *et al.*, 1998; Holleran *et al.*, 2001): maximum ventilation is attained at 4% CO_2 against 8–10% in control hypercapnic trials. Control of extracellular pH in nervous tissue is coupled with functioning of the Na^+/HCO_3^- cotransporter, existing in plasma membrane of astrocytes but lacking in that of neurons (Deitmer, 1992; Romero and Boron, 1999). This transport has an electrogenic character, because two or

even three bicarbonate ions are transferred per one sodium ion. Again, however, a continuous supply of glutamine to the glutamatergic nerve terminals is the necessary condition of respiratory rhythm generation; blockade of the TCA cycle in astroglial cells with FA can impair the respiratory activity (Hulsmann *et al.*, 2000).

5. Physiology of Blood Vessels Under Intoxication with FA

FA does not affect circulation in resting organs, but a significant increase of blood flow can be seen in working respiratory muscles (Johnson and Reid, 1988). Conversely, a reduction of blood was registered in hepatic artery, and contractive activity of isolated portal veins was suppressed after introduction of FA into the medium (Liang, 1977). These data, along with data on the effects of FA on endothelial cells *in vitro*, suggested that endothelium of blood vessels could be one of the primary targets for FA. If so, the endothelium-dependent relaxation of blood vessels would be affected. To test the hypothesis, we administered SFA to rats subcutaneously at a dose of 2–3 mg/kg (LD_{50}–LD_{84}), and investigated endothelium-dependent relaxation of rat aorta 3 and 24 h after the poisoning. Norepinephrine in saturating concentrations induced a rapid constriction of aorta followed by a smooth transition to plateau; in contrast, vasoconstricting hormones angiotensin II, vasopressin and 5-hydroxytryptamine induced a bell-shaped vasoconstricting response of aorta. To assess the functional state of endothelium, carbocholine was introduced at 10^{-5}mol/l. Acting on muscarinic receptors of endothelial cells, it induced generation of nitric oxide and release of endothelium-derived hyperpolarizing factor (McCulloch *et al.*, 1997). All the agonists applied had similar effects on contraction of aortas obtained from control and poisoned animals (not shown here). The experiment clearly demonstrated that FA has no influence upon the contractile properties of isolated rat aorta at 3 and 24 h after poisoning. This endothelial function is not affected, at least directly, under intoxication with FA.

6. Body Temperature of Rats and Rabbits Under Intoxication with FA

One of the main pathophysiological features of intoxication with FA is decrease of the body core temperature of endotherms, which indicates a disturbance of heat production and/or regulation (Brockmann *et al.*, 1955; Taitelman *et al.*, 1983b; Misustova *et al.*, 1980). It is interesting to note that the effects of FC are comparable with those of selective inhibitors of p38 MAP-kinases (activation of which precedes production of pyrogens) and antagonists of cytokines TNF, IL-1, and IL-6 (Milligan *et al.*, 2001, 2003). In our experiments, following administration of lethal doses of SFA to male rats, a marked decrease of rectal temperature was registered beginning from 1 h after the poisoning and gaining minimal levels in 6 or 24 h depending on doses. Then a gradual increase in temperature took place in surviving rats, returning to normal in 2–7 days. For example, under intoxication with SFA at a dose LD_{50}, a minimal rectal temperature of the surviving male rats (31.5°C as compared to 38.5°C in control animals) was registered in 6 h, and 7 days after the poisoning the temperature was 1°C below the control level. Under intoxication with FAA at equipotential doses, a minimal rectal temperature of the surviving male rats (32.6°C as compared to 38.9°C in control animals) was registered in 2 days, and even 7 days after the poisoning the temperature was 3°C below control level. But we observed fewer changes of the rectal temperature in rabbits after s.c. administration of SFA at a dose LD_{50}: maximal decrease was only 1°C (38°C as compared to 39.1°C) in 6 h. According to our observations, decrease of the temperature below 38°C in 3 h can serve as a reliable sign forecasting the lethal outcome of the poisoned rabbits.

7. Electrophysiological Studies of FA Intoxication

Clinical analysis of ECG of rats poisoned with SFA or FAA (Kuznetsov *et al.*, 2007) revealed a similar dynamic of the temporal parameters of ECG, with slowing down and delay of the repolarization processes being the most important (Table 13.1). A drop in amplitudes of the atrial and ventricular ECG complexes can be observed within an hour after poisoning, followed by decrease of the systolic index in 24 h thus indicating an impairment of the contractile capacity of myocardium. Registration of ECG of rats which died in 2 days of intoxication with SFA revealed a sharp drop of heart rate (down to 120–180 per min) 24 h after poisoning, together with complete absence of the P wave which reflects atrial depolarization (Figure 13.7A). Simultaneous reduction of both amplitude and duration of the T wave can be seen. An upward shift of the ST segment, though not accompanied by growth of the T wave amplitude, was registered in 70% of rats (Figure 13.7B). The cumulative evidence of the shape and amplitude changes of the ECG waves indicates a development of acute myocardial ischemia, though a transient one and maximally expressed 24 h after poisoning. Reduction of the S wave amplitude could be caused by disturbances of excitation processes in basal ventricular regions and in some areas of the right vetricle. Taking into account an increase in duration and shape distortions of the ventricular complex, one cannot exclude an incomplete right bundle-branch block. A significant extension of the T wave during the course of examination is indicative of deceleration of the fast repolarization of myocardium, though the process of slow repolarization (the QT interval in ECG, corresponding to the systole of ventricles) is accelerated within 3–24 h after poisoning.

Respiratory rhythm was gradually increased in rats under intoxication with SFA, and there were additional respiratory components in 50% of animals 24 h after administration of the poison (Figure 13.8) that may indicate disturbances of innervation of respiratory muscles. Spectral analysis of the

TABLE 13.1. Parameters of ECG (averaged cardiocycle) of adult rats in normal state and different terms after introduction of SFA at $1/2LD_{50}$

	Parameters										
	Amplitude (mV)				Duration (s)						
Terms	**P**	**R**	**S**	**T**	**P**	**T**	**PQ**	**QRS**	**QT**	**RR**	**SI**
Background	0.297 ± 0.019	0.973 ± 0.131	0.723 ± 0.137	0.747 ± 0.071	0.018 ± 0.001	0.036 ± 0.001	0.050 ± 0.001	0.019 ± 0.001	0.057 ± 0.001	0.140 ± 0.003	40.7
1 h	0.144 ± 0.016***	0.669 ± 0.083	0.235 ± 0.088*	0.469 ± 0.089*	0.018 ± 0.001	0.049 ± 0.002***	0.050 ± 0.001	0.022 ± 0.002	0.070 ± 0.002***	0.139 ± 0,005	50.4
3 h	0.170 ± 0.017***	0.826 ± 0.066	0.161 ± 0.059**	0.518 ± 0.083	0.021 ± 0.001*	0.044 ± 0.002**	0.057 ± 0.004	0.022 ± 0.001*	0.067 ± 0.002***	0.182 ± 0.011**	36.8
1 day	0.124 ± 0.030***	1.153 ± 0.171	0.089 ± 0.050**	0.538 ± 0.143	0.019 ± 0.001	0.051 ± 0.002***	0.048 ± 0.002	0.024 ± 0.002*	0.075 ± 0.004***	0.237 ± 0.036*	31.7
3 days	0.195 ± 0.023**	1.309 ± 0.122	0.102 ± 0.052**	0.403 ± 0.102**	0.019 ± 0.004	0.046 ± 0.003**	0.052 ± 0.004	0.025 ± 0.001**	0.071 ± 0.003***	0.158 ± 0.005**	44.9
7 days	0.167 ± 0.012***	1.106 ± 0.113	0.450 ± 0.088	0.458 ± 0.041**	0.018 ± 0.001	0.062 ± 0.003***	0.052 ± 0.002	0.025 ± 0.001**	0.088 ± 0.003***	0.171 ± 0.007***	51.5

*$p < 0.05$
**$p < 0.01$
***$p < 0.001$
SI – systolic index, calculated after formula SI = (QRST*100)/RR

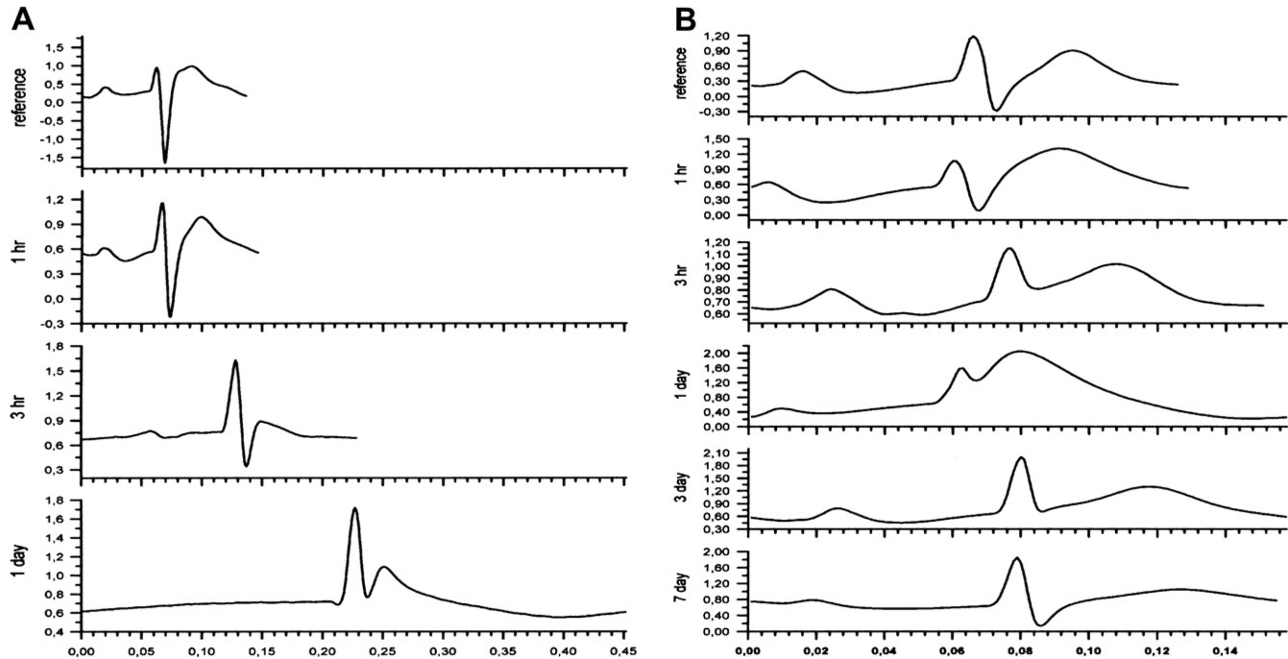

FIGURE 13.7. (A) ECG (averaged cardiocycle) of rat that died nearly 2 days after introduction of SFA at LD_{50}. (B) ECG (averaged cardiocycle) of rat that survived after introduction of SFA at LD_{50}. Along the *x*-axis – time (s), along the *y*-axis – amplitude (mV).

respiratory curve demonstrated that there was an enhanced synchronization of the respiratory rhythm observed within 3 h after poisoning. Simultaneously, the amplitude of respiration increased followed by a gradual decrease to the third day. Over the same period, a certain reduction of lability of respiratory rhythm was noted, accompanied by the appearance of two distinct peaks corresponding with frequencies of 90 and 120 cycles of respiration per minute. By the seventh day, the respiratory spectrogram was similar to the initial one, though the frequency of respiration was not completely restored. Comparison of spectrograms of respiration and ECG demonstrates disturbances of control mechanisms underlying generation of the second-order waves (respiratory arrhythmia visible at the spectrogram as a peak in the high-frequency region 0.8–2.5 Hz). One day after administration of SFA, there was a marked frequency

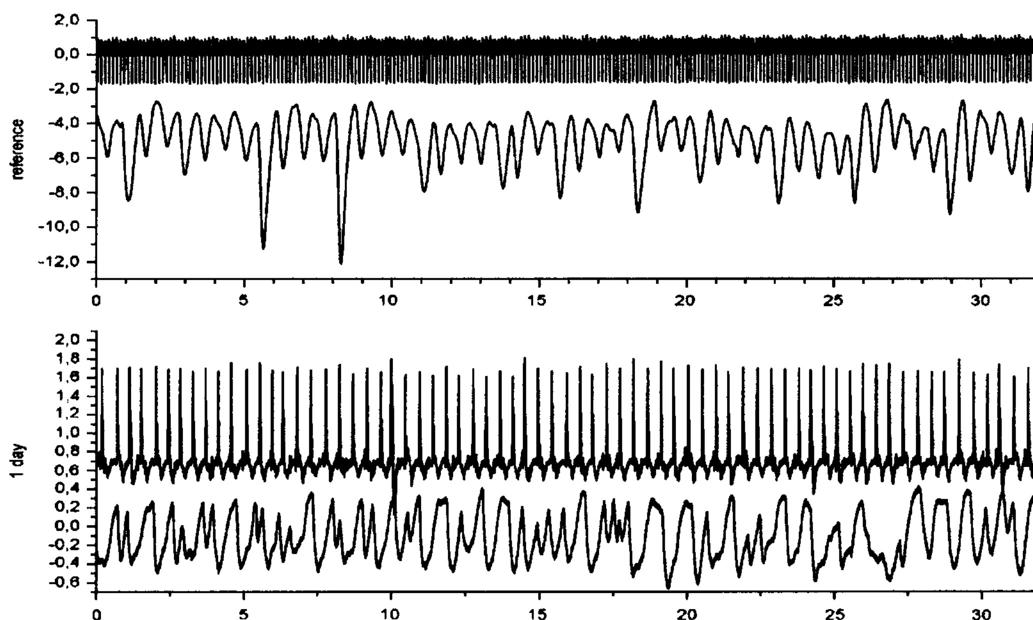

FIGURE 13.8. Records of ECG (upper) and respiratory rhythm (lower) from a narcotized rat before and 24 h after introduction of SFA.

maximum at the respiratory spectrum, in contrast to that of the ECG spectrogram.

Analysis of the heart rate variability (HRV) demonstrates that 1 h after poisoning an enhancement of parasympathetic influence took place, and this was accompanied by insignificant and paradoxical enhancement of heart and respiratory rates (Table 13.2). Then against a background of enhancement of humoral (metabolic) and sympathetic influences and simultaneous decline of parasympathetic influence, a stable decrease of heart and respiratory rates took place indicating a prominent divergence between vagosympathetic balance and resulting physiological parameters. Previously it was shown in experiments with dogs that systemic, pulmonary, and coronary hemodynamic parameters during the first hours after introduction of FA were not mediated by the autonomic nervous system and adrenergic neuromediators (Liang, 1977). This is in partial agreement with our results obtained with rats, though this cannot be extrapolated to all the periods of intoxication and all animal species.

V. TOXICITY AND RISK ASSESSMENT

A characteristic feature of the clinical picture of intoxication with FA is a latent period of 0.5 to 6 h (Egekeze and Oehme, 1979). The duration of the latent period depends on animal species' metabolism and dose administered (Chenoweth, 1949; Goncharov *et al.*, 2006). A broad variability of clinical manifestations of FA effects in different animal species is one of its characteristic features. There is a correlation between food specificity and toxic effect of FA; the cardiovascular system is mainly affected in herbivores, while the CNS is mainly affected in carnivores. According to this, four groups were recognized in terms of clinical signs of intoxication (Chenoweth and Gilman, 1946). The first comprised herbivores (rabbits, goats, sheep, cattle, and horses), in which FA induced ventricular fibrillation without notable CNS disorders (Marais, 1944; Chenoweth, 1949; Egekeze and Oehme, 1979). The second group comprised dogs and guinea pigs, in which the CNS was primarily affected. In dogs, a species highly sensitive to FA, symptoms of secondary intoxication appear after a latent period of 1 to 10 h (Chenoweth and Gilman, 1946; Egyed and Shupe, 1971). For animals of the third group the clinical pattern of intoxication is similar to that of the second group of animals, but slightly less pronounced. This group comprised rats and hamsters relatively tolerant to FA. After a latent period lasting 1–2 h, tremor and elevated excitability were common symptoms. Death usually occurred within 4–6 h as a result of respiratory depression, after exposure to high FA doses (Chenoweth and Gilman, 1946; Pattison, 1959). The surviving animals demonstrated depression, weakness, ataxia, and strongly pronounced bradycardia down to 30 heartbeats per minute. At sublethal doses of FA, a full recovery can occur in 72 h after poisoning (Chenoweth and Gilman, 1946; Pattison, 1959). A mixed response to FA exposure was described in animals of the fourth group – cats, pigs, and rhesus monkeys; it included disturbances of both CNS and cardiovascular system. On acute poisoning, adynamia, salivation, vomiting, frequent defecation, pupil dilatation, nystagmus, accelerated respiration, enhanced excitability, tremor, and clonicotonic convulsions were observed in these animals (Chenoweth and Gilman, 1946; Gammie, 1980).

This classification has been revised recently (Sherley 2004). The division of animals into cardiac and neurological symptomatic groups is considered to be unnatural as it ignores common neurological signs manifested in all the groups: among these are tremor, ataxia, hypersensitivity, myotonic convulsions, weakness, and partial paralysis. The cardiac response in a pure form was not a common event and was described just for a limited number of animals, though CNS involvement is obviously widespread.

As for humans, exposure to stock solution during formulation and dermal or respiratory exposure during application of baits, as well as accidental or intentional acute intoxications, are the main human health concerns. Formulators and pest control workers are the largest occupational risk group (Norris, 2001). The clinical picture of acute intoxication of humans is similar to that of rhesus monkeys, and among the symptoms are nausea, vomiting, abdominal pains, salivation, irrational fear, weakness, tachypnoe, cyanosis, and sometimes sweating and increased temperature (Brockmann *et al.*, 1955; Pattison, 1959; Arena, 1970; Taitelman *et al.*, 1983b). Psychomotor agitation and sometimes a loss of spatiotemporal feeling can occur. In addition, tremor, nystagmus, involuntary dejection and urination, muscle spasms, hypertonus of the extremities, and even alalia, have been reported (Gajdusek and Lutheer, 1950; Harrison *et al.*, 1952; Robinson *et al.*, 2002). The most characteristic signs of intoxication involve generalized recurrent convulsions alternating with deep depression. Sudden loss of consciousness and coma may occur. These symptoms were associated with metabolic acidosis and hypotension (Pattison, 1959; Chi *et al.*, 1996, 1999), as well as cardiac rhythm disturbances, such as tachycardia, bradycardia, asystolia, and sustained ventricular fibrillations (Gajdusek and Lutheer, 1950; Reigart *et al.*, 1975; Trabes *et al.*, 1983). Death usually occurs in 3 h to 5 days of heart block, arrhythmia, or respiratory failure (Reigart *et al.*, 1975; Montoya and Lopez, 1983). Important diagnostic symptoms registered with ECG are arrhythmia, the QT and ST intervals, and the T wave (Pattison, 1959; Taitelman *et al.*, 1983b; Chi *et al.*, 1996). Kidneys are among the most sensitive organs: acute renal failure associated with uremia and increased level of creatinine in serum can be observed under acute FA poisoning (Chung, 1984; Chi *et al.*, 1996). Pathomorphological abnormalities of humans poisoned with FA are also nonspecific and similar to those of animals. In the case of lethal outcome, petechial hemorrhages and excess blood filling of internal organs (Hayes, 1982), edema

TABLE 13.2. Analysis of heart rate variability of adult rats in time and frequency domains under intoxication with SFA at $1/2LD_{50}$

	Period of examination											
	Control		**1 h**		**3 h**		**24 h**		**3 days**		**7 days**	
Parameters	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**
Heart rate, contr/min	424.6 ± 6.1	–	441.6 ± 9.4	+4.0	340.1 ± 11.4***	−20.0	291.8 ± 18.5***	−31.3	370.6 ± 6.0***	−12.7	341.0 ± 6.0***	−19.7
Coefficient of arrhythmia, rel.un.	0.049 ± 0.003	–	0.058 ± 0.005	+18.4	0.156 ± 0.042*	+218.4	0.245 ± 0.073*	+400	0.053 ± 0.003	+8.2	0.051 ± 0.006	+4.0
Value of VLF (ms^2)	0.041	8.8	0.053	7.6	0.087	11.8	0.060	9.7	0.043	10.1	0.044	10.9
Value of LF (ms^2)	0.075	16.1	0.107	15.2	0.128	17.3	0.114	18.4	0.073	17.1	0.079	19.6
Value of HF (ms^2)	0.350	75.1	0.542	77.2	0.523	70.9	0.445	71.9	0.311	72.8	0.280	69.5
Value of To (ms^2)	0.466	–	0.702	+50.6	0.738	+58.4	0.619	+32.8	0.427	−8.4	0.403	−13.5
LF/HF, rel.un.	0.214	–	0.197	−7.9	0.245	+14.5	0.256	+19.6	0.235	+9.8	0.282	+31.8
HF/To, rel.un.	0.751	–	0.772	+3.8	0.709	−5.6	0.719	−4.3	0.728	−3.1	0.695	−7.5
Respiration rate/min	94.1 ± 3.0	–	101.9 ± 3.1	+8.3	94.4 ± 3.2	+0.3	98.8 ± 3.7	+5.0	111.9 ± 6.1*	+18.9	120.2 ± 8.1**	+27.7

Coefficient of arrhythmia = $(RR_{max} - RR_{min})/RR_{mean}$
Values of shifts (%) for **VLF**, **LF**, and **HF** indices are given against **To** index of corresponding period of examination. For other parameters the shift was calculated against the initial control value

of lungs and brain, and sometimes mediastenal emphysema and acute inflammatory reaction with coagulating necrosis in esophagus were registered in humans (Brockmann *et al.*, 1955). The morphological basis of cardiotoxic effects is acute myocardial dystrophy, a characteristic of which is diffuse lesions of cardiac muscle (Pattison, 1959; Taitelman *et al.*, 1983b). Acute renal failure develops due to the influence of FA on subcellular structures of kidneys. Metabolic acidosis aggravates the clinical course of renal failure. Diffuse degeneration of renal tubules was observed (Hayes, 1982). For cases that lack clinical and morphological specificity, biochemical data and primarily citrate and fluoride levels can be used for diagnostic purposes (Pattison, 1959; Schultz *et al.*, 1982). Thus under acute intoxication with FAA, citrate (108 μg/g in heart and 23.9 μg/g in kidney) and fluoride (6.3 mg/g dry weight of heart and kidney) were found in human corpse; the dose of FAA was estimated to be near 23 mg/kg (Hayes, 1975). In addition, the indubitable diagnostic confirmation of the intoxication should be based on determination of the poison in tissues. Under acute SFA poisoning with lethal outcome, FA was found in urine (368 μg/ml), liver (58 μg/g), and brain (76 μg/g) (Harrison *et al.*, 1952).

Among the after-effects that develop after acute intoxication with FA are various neurological disturbances: impaired muscular tonus and reflex activity, and transient spasmodic and meningeal syndromes. Long after an acute poisoning (from 1.5 to 9 years) a tendency for epileptoid seizures, ataxia, extremity muscular hypertension, spastic tetraplegia, blindness of cortical origin, diffuse brain atrophy, and psychic disorders were observed (Pridmore, 1978; Trabes *et al.*, 1983). A case of chronic intoxication with FA of a farm worker has been described (Parkin *et al.*, 1977): the clinical signs were renal insufficiency and less pronounced injuries of other organs.

VI. TREATMENT

Decades of studies on the toxicology of FA have led scientists to the conclusion that treatment of intoxications can be successful only if timely general and symptomatic therapy is applied, but not specific antidotes (Dorman, 1990; Norris, 2001). Much experimental work over an extensive period has been undertaken in an effort to find effective donors of acetate groups, because of their ability to inhibit conversion of FA to FC. Ethanol, monoacetin (glycerol monoacetate), acetamide, and cortisone acetate were tested for their potency to serve as antidotes (Hutchens *et al.*, 1949; Chenoweth, 1949; Cole *et al.*, 1955; Giller, 1956; Egyed, 1971; Egyed and Shlosberg, 1977). Therapeutic effect was revealed for simultaneous introduction of ethanol and acetate (Hutchens *et al.*, 1949; Tourtelotte and Coon, 1949). Negative effects of monoacetin and acetamide were enhancement of hyperglycemia and metabolic acidosis, damage to capillaries and hemolysis of red blood cells, and increase of citrate concentration in different organs (Engel *et al.*, 1954; Egyed and Shlosberg, 1973). Administration of cortisone acetate inhibited the FC synthesis and prevented development of ketosis, though increased hyperglycemia (Cole *et al.*, 1955).

Several antidotes were tested for their capacity to activate transport of the TCA cycle intermediates through mitochondrial membranes. For this purpose fluoromalate was proposed, though any positive result was negligible (Peters *et al.*, 1972). Malate was also tested, but proved to be effective only in *in vitro* experiments (Buffa *et al.*, 1972). Also *in vitro*, glutathione and a series of SH-containing compounds (cysteamine and *N*-acetylcysteine) were tested (Mead *et al.*, 1985). However, they were incapable of replacing glutathione in enzymatic defluorination of FA and have not found practical application. TCA cycle intermediates (succinate, malate, citrate, and glutamate) were tested, but did not exhibit protective effect (Hutchens *et al.*, 1949). A positive result was observed in experiments with mice, which were administered calcium gluconate and succinate (Omara and Sisodia, 1990). This therapy was hardly more effective than ethanol. Some 16 years later another research group tested the therapy with cats, which are known to be much more sensitive to FA. Again, differences in survival between treated and nontreated animals were not significant ($p > 0.05$) (Collicchio-Zuanaze *et al.*, 2006). Administration of calcium chloride to cats under acute intoxication with FA made it possible to postpone their death by up to 166 min combination of calcium chloride with monoacetin gave a similar effect (Taitelman *et al.*, 1983a). Nevertheless, calcium chloride caused reduction of the QT interval and favored survival of humans in case of their intoxication with FAA (Taitelman *et al.*, 1983b).

Our strategy for development of therapeutic means of treating acute FA intoxication was based on a deep analysis of the biochemical literature, together with our own experimental data. Thus, a high sensitivity of aconitase to inhibition by superoxide anion and nitric oxide (Gardner *et al.*, 1994; Andersson *et al.*, 1998; Castro *et al.*, 1998) means that ROS and NO could be competitive antagonists of FC to avert its effect on aconitase. Also, as considered earlier, during FA intoxication glutamate could be utilized in the TCA cycle through GDH or transaminases (Yu *et al.*, 1976; Liang, 1977; Hassel *et al.*, 1994). Moreover, the effects of FC could be prevented by prior introduction of isocitrate (bypass of inhibited aconitase) and fructose-1,6-bisphosphate (energy substrate for neurons) (Lian and Stringer, 2004).

We have demonstrated that FA can adversely affect mitochondrial functions only if pyruvate was available as respiratory substrate, and that changes of redox-state of pyridine nucleotides (PN) or their leakage from MCh could be critical factors that impair mitochondrial respiration and lead to cell death (Zinchenko *et al.*, 2007). Opening of the mitochondrial pore is a reversible phenomenon: prevention of oxidation and/or leakage of NADPH from MCh can

restore the normal functional state of MCh. For example, when succinate or glutamate was used as a respiratory substrate, mitochondrial functions were not affected by FA (Figure 13.3A).

As for other alternative substrates, we suggest that the accumulating intracellular citrate could be one of them. As discussed earlier, blockade of citrate transport from MCh under FA intoxication is not an obligatory event, and citrate can enter cytosol to be further utilized by cICDH (Max and Purvis, 1965; Buffa *et al.*, 1972). The cICDH activity is almost equally distributed between cytosol and MCh of astroglia and microglia, whereas cICDH accounts for about 75% of activity in neurons and oligodendrocytes (Minich *et al.*, 2003). We have not found data on the ratio of mitochondrial and cytoplasmic aconitases in cells of the nervous system, but it is interesting to note that a similar ratio of m- and cICDH exists in hepatocytes (Rakhmanova and Popova, 2006), and that c-aconitase accounts for 65% of the aconitase in these cells (Konstantinova and Russanov, 1996). In rat heart a similar ratio of m- and c-aconitases has been revealed: 35 and 65%, correspondingly (Medvedeva *et al.*, 2002). Based on these data, one may suggest that an effective pathway for citrate utilization and NADPH synthesis exists in these (and other) cells in case of inhibition of m-aconitase. This alternative pathway could play a positive physiological role because NADPH might be used for anabolic reactions and heat generation, glutathione reduction and NO synthesis, and regulation of blood vessel tone by means of ROS generation (Winkler *et al.*, 1986; Bobyleva *et al.*, 1993; Lee and Yu, 2002; Gupte and Wolin, 2006). As was pointed out earlier, studies focused on the pentose cycle as the main source of NADPH need to be re-evaluated taking into consideration the metabolic activity and substrate specificity of a tissue (Winkler *et al.*, 1986). cICDH along with malic enzyme and transhydrogenase participates in NADPH regeneration to further reduce glutathione in brain mitochondria (Vogel *et al.*, 1999), but cICDH can provide a seven-fold greater generation of NADPH as compared to malic enzyme (Winkler *et al.*, 1986). The level of cytoplasmic NADPH can influence potassium channels and calcium balance (Wolin *et al.*, 2005; Gupte and Wolin, 2006). In our *in vitro* studies, FA induced a slow elevation of $[Ca^{2+}]_i$ in different cells (Zinchenko *et al.*, 2007). This could indicate an activation of the SOC channels; the process is not affected by FA and does need ATP to be implemented, at least in glial cells (Lian and Stringer, 2004). We suppose this mechanism to be common for many types of cells, and this could explain a primary hypersensitivity of platelets exposed to FA (Mindukshev *et al.*, 2006). In cardiomyocytes, elevated $[Ca^{2+}]_i$ can stimulate their functional activity observed in our experiments *in vitro* and also supported *in vivo* by a primary increase of systolic index (Table 13.1). As for modulating effects of Ca^{2+} on bioenergetics of MCh, it is pertinent to recall ''classic'' activation of the TCA cycle dehydrogenases followed by increase of mitochondrial potential and NADH generation: 2-oxoglutarate dehydrogenase (OGDH) and mICDH can be activated by calcium ions through allosteric mechanisms and pyruvate dehydrogenase is activated due to dephosphorylation by the Ca^{2+}-dependent phosphatase (McCormack *et al.*, 1990; Hansford, 1994). The exact role of these dehydrogenases in the bioenergetic status of MCh affected by FA needs to be clarified, though one can suppose that OGDH could derive a special benefit from such an activation if it is provided with exogenic or endogenic 2-oxoglutarate.

TABLE 13.3. Assessment of therapeutic effectiveness of METIS preparations under acute intoxication of rats with SFA

Therapy	Index of therapeutic efficiency: Ratio LD_{50} treated/LD_{50} nontreated
Ethanol, $n = 42$	**1.6**
METIS-1, $n = 48$	**2.5**
METIS-2, $n = 92$	**3.3**
METIS-4, $n = 39$	**4.3**

n – number of animals used in experiment to calculate the index

According to the above discussion, we have defined several directions for biochemical correction under acute intoxication with FA and suggested suitable preparations for therapeutic complexes: (1) competitive inhibition of FA and CoA interaction; (2) competitive inhibition of FC and aconitase interaction; (3) replenishment of the TCA cycle distally of aconitase; (4) utilization of accumulating citrate. In a previous publication we presented the first data on effectiveness of a therapeutic complex named METIS (Goncharov *et al.*, 2006). Further experiments proved the validitity of the therapeutic approach, and we have now appreciably enhanced the efficacy of the complex (Table 13.3). In addition to these data on the index of therapeutic effect, a spectrum of physiological and biochemical data was obtained. Animals treated with METIS complex had little changes of body weight, temperature, and oxygen consumption. Dynamics of citrate in brain, kidneys, and blood was also improved, and kinetic parameters of platelet aggregation were corrected. Comparative analysis of the FA level in tissue homogenates, blood plasma, and urea of rats revealed that the METIS complexes reduced the level of FA in brain almost two-fold, thus indicating inhibition of FA utilization first of all in the cells of the nervous system.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

The extreme toxicity of FA is determined by its similarity to acetate, which has a central role in cell metabolism. FA enzymatically condenses with CoA-SH to produce

fluoroacetyl-CoA, which replaces acetyl-CoA entering the TCA cycle and produces FC. The latter reacts with aconitase and blocks the TCA cycle. Energy production is reduced, as well as concentration of metabolites generated distally to aconitase. 2-Oxoglutarate is the most important of them, being a precursor of glutamate, which is a neuromediator in the CNS and participates in neutralizing ammonia either directly through glutamine synthase or indirectly through the urea cycle. Accumulation of citrate is one of the causes of metabolic acidosis. Chelating of Ca^{2+} is apparently one of the central events in pathogenesis of intoxication.

The first papers on toxicology of FA were published in the 1940s. The long history of investigations was fruitful, with several important discoveries: biochemical mechanism of ''lethal synthesis''; structure of aconitase; functional relations of glia and neurons; and switching of metabolic pathways. However, the main problem of toxicology (for any poison) was not solved – development of an effective therapy. Analysis of the scientific literature has demonstrated that reciprocal relations of signaling and metabolic pathways under intoxication with FA are unclear. Inhibition of m-aconitase causes blockade of TCA cycle, reduction of pyridine nucleotides, accumulation of citrate, disturbances of intracellular signaling, deenergization, and cell death. However, the dynamics and significance of these events are different depending of the type of cells and tissues, which is why it is very difficult to predict the primary reaction of different cells and more so the whole organism.

Biochemical pathways underlie the basis of physiological rhythms; they should have a certain space–time structure and presuppose coordinated interactions of different cells. Thus, one of the causes of disturbances of normal respiration under FA intoxication could be disturbances in rhythmic activity of respiratory neurons; but suppression of these neurons is a consequence of the inhibiting effect of FA on astrocytes, not neurons (Hulsmann *et al.*, 2000). We described development of cardiac and respiratory tachyarrhythmias reflecting reproduction of decasecond rhythms characteristic for immature or abnormal excitatory structures (Kuznetsov *et al.*, 2007). Previously, it was suggested that such endogenic rhythmic activity could be determined by the level of the pentose cycle activity (Kuznetsov, 1999, 2002). This cycle indeed plays an important role in neurons, protecting them from oxidative or traumatic stress (Ben-Yoseph *et al.*, 1994; García-Nogales *et al.*, 2003; Bartnik *et al.*, 2005). However, it should be noted that although the activity of NADPH-generating enzymes of the pentose cycle in astrocytes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) is 2–3 times higher than in brain as a whole, the activity of cICDH is also very high in these cells (Rust *et al.*, 1991). This metabolic pathway is interesting primarily because under FA intoxication citrate is accumulated, and the pathway may be regarded as a form of biochemical adaptation that facilitates utilization of the central metabolite. Modern data suggest that spatial and temporal division of m- and c-aconitases not only provides regulation of iron balance in cells, but actually provides regulation of balance between catabolic and anabolic processes (Tong and Rouault, 2007).

Providing the cells have utilized citrate entering cytosol, another problem should be utilization of generating NADPH. One possible and very important mechanism of PN oxidation is heat generation through shiver and nonshivering thermogenesis. Rise in activity of NADPH-generating enzymes and pathways, including c-aconitase and cICDH, is accompanied by enhanced thermogenesis (Bobyleva *et al.*, 1993). It was shown that NADPH could be used together with or even instead of NADH as a reducing cofactor for cytoplasmic glycerophosphate dehydrogenase (Bobyleva *et al.*, 1993; Fahien *et al.*, 1999). But if the role of this pathway for transferring electrons from cytoplasma to MCh in skeletal muscles is rather clear, then the level of activity and functional state of glycerophosphate shuttle in brain cells are contradictory and serve as a subject for discussion. Activity of glycerophosphate shuttle in brain is explained by the need for glycerol-3-phosphate as a substrate for phospholipid synthesis in oligodendroglia (Adler and Klucznik, 1982; Nguyen *et al.*, 2003). In neurons and astrocytes, the activity of glycerophosphate dehydrogenases is much lower than in oligodendrocytes (Rust *et al.*, 1991; Nguyen *et al.*, 2003). There are data, however, that indicate an important role of this shuttle in astrocytes, taking into consideration (1) the absence of malate–aspartate shuttle in these cells (Waagepetersen *et al.*, 2001; McKenna *et al.*, 2006), and (2) the elevated level of mRNA of cICDH in astrocytes after convulsions, under exposure to morphine, indometacine, and some other preparations (Link *et al.*, 2000). In conclusion, we suggest that future progress in toxicological studies of FA and development of effective therapy will depend on comprehensive consideration of these and other modern data, together with reevaluation of old and forgotten data.

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