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Mice with generalized influenza or tularemia of similar lethality were studied in an effort to compare biochemical responses of the myocardium during infections of viral and bacterial etiology. A progressive loss of body weight characterized the course of both infections. Accompanying this, the myocardial content of protein as well as the activities of lactate dehydrogenase, citrate synthase and cytochrome c oxidase all decreased. However, myocardial protein degradation appeared earlier and was more pronounced in influenza, and the
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Sequential metabolic alterations in the myocardium during influenza and tularemia in the mouse

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Short title: Infection and myocardial metabolism

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The USAMRIID facilities are fully accrediated by the American Association for Accrediation of Laboratory Animal Care.

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Infection and myocardial metabolism

Summary

Mice with generalized influenza or tularemia of similar lethality were studied in an effort to compare biochemical responses of the myocardium during infections of viral and bacterial etiology. A progressive loss of body weight characterized the course of both infections. Accompanying this, the myocardial content of protein as well as the activities of lactate dehydrogenase, citrate synthase and cytochrome c oxidase all decreased. However, myocardial protein degradation appeared earlier and was more pronouced in influenza, and the protein changes were accompanied by a rapid decline of myocardial RNA. Activation of acid hydrolases, such as cathepsin D and β-glucuronidase occurred in tularemia but not in influenza, whereas leakage of β-glucuronidase into the plasma occurred in both infections. Conversely, there was a considerably greater activation of myocardial catalase in influenza. These findings suggested that different control mechanisms or metabolic pathways were operative in the degradation of myocardial constituents in influenza as compared to tularemia. The absence of histologic signs of myocarditis in either infection appeared to exclude any direct local effects of an inflammatory process on myocardial cells. Since the infections were of comparable lethality (based upon the inoculated dose of organisms) the observed differences in pattern and extent of metabolic responses of the myocardium to these infections may be attributed to different pathophysiologic mechanisms evoked by the different microorganisms.

Infection and myocardial metabolism

Introduction

Myocarditis is a common complication in enterovirus infections[1,2] and has been reported at variable frequencies in other viral infections as well, including influenza.[3] Bacterial infections are less frequently associated with myocarditis.[4] Even in the absence of myocarditis, physical performance capacity of the host is often impaired following various acute infectious diseases.[5,6] Little is known, however, about the effects of generalized infections on myocardial metabolism. In the absence of a localized myocarditis, Newcastle disease virus infection in the chick causes a decrease of heart size and the myocardial content of protein, RNA and DNA.[7] Only recently was it demonstrated that a bacterial infection without myocarditis, i.e., one due to Francisella tularensis, could also cause myocardial protein degradation in the rat concomitant with a decreased physical performance capacity of skeletal muscles.[8] In tularemia, there was also evidence to suggest involvement of the lysosomal enzyme system of the rat myocardium.[8]

The purpose of the present study was to compare degradative processes and the metabolic consequences for the myocardium of a generalized viral infection with those of a bacterial infection of similar lethality. Histologic examination was performed in order to exclude the development of myocarditis and the possible confounding metabolic effects of a localized inflammatory reaction in the myocardium.

Infection and myocardial metabolism

Materials and methods

Animals. Male Swiss-Webster mice were used (Harlan, Sprague-Dawley Dawley Inc., Va.). Mice were maintained on a commercial diet (Wayne Lab Blox, Allied Mills, Chicago, Illinois) until the beginning of an experiment and were housed in rooms maintained at 23 $\pm$ 1°C. The initial mean body weights ($\pm$ S.D.) of the mice were for the influenza infection, 29.2 $\pm$ 1.5 g, and for the tularemia infection, 27.9 $\pm$ 1.0 g. Food and water were supplied ad libitum. Since inoculating doses were identified in preliminary studies to achieve median lethality after 7 days of influenza or after 6 days of tularemia, mice were preassigned at random to take part in the experiments for a total of 2, 4 or 7 days in the influenza infection and for 2, 4 or 6 days in the tularemia infection (Study 1).

Additional mice were randomized in similar groups in each infection, and after 6 days of influenza and after 4 days of tularemia, tissue samples were excised for histopathological examination (Study 2). Infected and noninfected control mice were studied simultaneously in each infection. Groups were sized to allow for losses due to lethality.

Infection. On day 0, mice were inoculated intranasally (i.n.) with 0.1 ml of a 10-fold dilution of a mouse-virulent suspension of influenza virus, $10^{8.7}$ egg median infectious doses (EID_{50}) /ml), strain A/Aichi/2/68 (H3N2),[9] or intraperitoneally (i.p.) with 0.2 ml suspension of 3.15 x 10^4 colony forming units (CFU)/ml of nonwashed F. tularensis,

live vaccine strain, that had been grown on solid fortified glucose-cystein-blood agar.[10] The influenza virus suspension was delivered to the nose pad, encompassing both nares, to permit its inhalation during light anaesthesia with halothane. Control mice were administered similar volumes of heart infusion broth (HIB) in the influenza trial (i.n.) and sterile tryptose saline (Difco Laboratories, Detroit) in the tularemia trial (i.p.). Body temperatures were recorded by a rectally inserted thermocouple before sampling blood and tissue on each of days 2, 4 and 6 or 7.

Sampling. Study 1: On days 2, 4 and 7 in influenza and 2, 4 and 6 in tularemia, eight randomly preassigned mice from each group were anaesthetized using halothane. The thoracic cavity was opened, the caval vein severed, and blood was collected from the right pleural cavity using heparinized pipettes. The heart and spleen were removed and put into ice-cold homogenization buffer. The myocardium was rapidly opened, atria, vessels and blood removed and the entire remaining ventricular muscle was weighed and cut into small pieces with a pair of scissors. These pieces were homogenized in ice-cold homogenization medium (150 mM KCl, 50 mM $KHCO_3$ and 6 mM EDTA, pH 7,4) with all-glass Potter-Elvehjelm homogenizers operated manually. The entire procedure was performed at 0-4°C. The homogenates for acid hydrolase assays were made 0.1% with respect to Triton X-100 concentration. The spleen was cleaned of connective tissue, rinsed, blotted on filter paper and weighed.

Infection and myocardial metabolism

Study 2: On day 6 in influenza and on day 4 in tularemia, mice were killed by the same procedure as in Study 1 and the myocardium was similarly excised and placed in 10% formalin for routine histological processing and preparation of H & E slides.

Assays. Plasma. Study 1: Blood plasma was held in wet ice until used in individual analyses of β-glucuronidase activity (GUase: E.C. 3.2.1.31) (Sigma Analytical Kit).

Myocardium. Study 1: In all-heart homogenates, total protein (after incubating 0.1 ml of homogenate with 0.1 ml of 20% KOH at 80C for 60 min.),[11] RNA and DNA were measured.[12] Further, the activities of lactate dehydrogenase (LDH: E.C. 1.1.1.27),[13] citrate synthase (CS: E.C. 4.1.3.7),[14] cytochrome c oxidase (CYTOX: E.C. 1.9.3.1.),[15] cathepsin D (Cat. D.: E.C. 3.4.23.5),[16] β-glucuronidase (GUase: E.C. 3.2.1.31)[17] and catalase (E.C. 1.11.1.6) were determined. The activity of catalase, as expressed by oxygen production, was measured by an oxygen electrode, but otherwise essentially as previously described.[18]

All assays were performed immediately except those for plasma β-glucurondase and tissue protein, RNA and DNA. The latter tissue studies were performed on homogenates that had been frozen and thawed. All measured tissue variables were calculated and expressed as activity (or content) in the entire ventricular muscle and per gram of "wet" tissue.

Infection and myocardial metabolism

Study 2: Histopathologic examination of serial sections of the ventricular myocardium was performed in order to detect any myocardial necrosis, inflammation, or other lesions.

Statistics. The effect of each infection was calculated, for each variable, by means of a two-way analysis of variance by comparing results in infected groups with those of noninfected controls on each of days 2, 4, 6 and 7. Correlations were estimated and tested for significance by the method of least-squares.

Results

The disease intensity was similar in both infections in terms of lethality rates, the median lethality occurring at 7 days in influenza and at 6 days in tularemia.

There was progressively increased involvement of tissues throughout the course of the infections as reflected by alterations in the body, heart, and spleen weights (Fig. 1). The tissue weights and biochemical data of the ventricular myocardium in sham-inoculated control mice are given in Table 1.

Study 1: As a result of influenza or tularemia, heart and total body weights dropped. The general wasting of body tissues was more pronounced in influenza than in tularemia, but the relative effect on the myocardial weight was similar in the two infections. Spleen enlargement occurred in tularemia but not in influenza. Plasma β-glucuronidase activity increased in both infections and subnormal

rectal temperatures were recorded throughout the influenza infection and even late in tularemia (Fig. 1).

The myocardial content of protein and RNA and the total myocardial activity of lactate dehydrogenase, citrate synthase and cytochrome c oxidase were reduced in the course of each infection (Figs. 2-3). However, a clear difference was evident in that the degradation appeared earlier, developed faster and became more severe in influenza (Figs. 2-3). RNA increased early in tularemia, a trend that was later reversed, whereas in influenza, RNA dropped progressively (Fig. 2). The DNA content was unaffected in either infection (Fig. 2). In tularemia, the activities of the mitochondria-associated oxidative enzymes citrate synthase and cytochrome c oxidase decreased more than the activity of cytoplasmic lactate dehydrogenase, but in influenza no such difference was evident; these enzymes decreased their activities in parallel (Fig. 3).

The lysosomal enzymes β-glucuronidase and cathepsin D were activated only in tularemia; a consistent decrease of β-glucuronidase activity was found in influenza (Fig. 4). Conversely, the myocardial activity of catalase showed a major increase only in influenza (Fig. 4). The catalase increase correlated negatively with the activity of cytochrome c oxidase ($r = -0.720$, $p<0.01$, infected mice on days 4 and 7 combined). No similar correlation was observed in noninfected or tularemia infected mice. When expressed as activity per mg of myocardium, cathepsin D showed an increase even in influenza ($p<0.001$

on day 7), whereas the decrease of β-glucuronidase remained significant ($p < 0.01$ on day 4 and $p < 0.001$ on day 7).

Study 2: No histologic signs of necrosis of myocytes or infiltration of inflammatory cells could be found in the myocardium in any of the influenza or F. tularensis infected or sham-inoculated mice.

Discussion

The present study, in which one viral (influenza) and one bacterial (tularemia) infection without myocarditis were compared, showed that influenza caused a more pronounced tissue wasting including the ventricular myocardium, than did tularemia of similar lethality. In influenza, alterations occurred earlier and progressed further than in tularemia. The myocardial changes included a decrease in total protein and glycolytic and oxidative enzyme activities in both infections. In influenza, the protein synthetic capacity as assessed by RNA content was depressed, but in tularemia, RNA was initially stimulated. Different mechanisms or pathways for the degradation of myocardial constituents in viral as compared to bacterial infections are suggested by the different patterns of activations of myocardial tissue degrading enzymes observed in these two infections.

The intensities of the two infections were comparable since similar lethality rates were recorded. In tularemia in the rat, spleen weight is useful for estimating the progression of the infection,[19] and, not unexpectedly, a progressive increase of spleen weight was recorded in the present mouse tularemia model, whereas in influenza there was a

significant decrease in spleen weight. Similarly, plasma β-glucuronidase activity is known to increase in tularemia, the enzyme probably originating in the liver.[20] We observed an increased β-glucuronidase activity even in influenza. The plasma activity of this enzyme does not seem to have been studied in viral infections before.

Cardiac changes known to be associated with some viral infections, such as coxsackie virus infections, include histologic signs of myocarditis; i.e., myofiber necrosis, infiltration of inflammatory cells,[2] acute increase of heart weight,[1,21] and subsequent fibrosis.[22] Multiplication of virus particles within the myocardium is considered to initiate this process.[2,22] Such an ingress of inflammatory cells would alter the myocardial content of DNA, RNA, and protein, and make it difficult to identify any metabolic changes in myocardial cells per se. Since no histologic signs of myocarditis were observed in the present infections, the recorded biochemical alterations should be considered to be part of the general catabolic response of the host to these infections. Skeletal muscle protein degradation occurs in both viral and bacterial infections[23] and the heart muscle seems not to be spared in this process (Figs. 1 and 2).[7,8]

The altered myocardial RNA content may have reflected an initially increased protein synthetic capacity in tularemia that was later normalized. Conversely, RNA showed a progressive decrease below baseline values in influenza, probably indicating a deceleration of the

rate of protein synthesis.[24] Indirect evidence has previously been presented of a decreased protein synthetic capacity of somatic proteins in Salmonella. typhimurium infection.[25,26]

The control mechanisms that initiate altered myocardial cell metabolism during generalized infections have yet to be identified. Infections are accompanied by multiple hormonal responses, and by the secretion by phagocytic cells of endogenous mediators of fever and other physiologic responses. One of these, leukocytic endogenous mediator (LEM), is released by activated phagocytic leukocytes early in bacterial infections and to a lesser extent in viral infections.[27] LEM stimulates RNA replication and subsequent protein formation in the liver[28] but its effects on skeletal muscle protein may be catabolic.[27] Additional studies will be required to determine if an interplay of hormonal and mediator effects can explain the proteolytic and enzymic changes in myocardial tissues during generalized bacterial and viral infections. Similarly, the myocardial changes could be influenced to some degree by the sizable acute nutritional losses of body mass observed during each of these mouse infections.

In tularemia the loss of mitochrondial enzyme activity, i.e., citrate synthase and cytochrome c oxidase, preceded the decrease in lactate dehydrogenase located in the cytoplasm, whereas in influenza all these enzyme activities decreased early (Fig. 3). Thus, mitochondrial involvement occurred early in both infections. In previous studies of mitochrondrial and cytoplasmic enzymes in skeletal muscle in viral,

mycoplasma and bacterial infections in humans[29,30] and rats,[31] the membrane-attached enzyme cytochrome c oxidase, was considerably more resistant to the catabolic stimulus than cytoplasmic enzymes, such as lactate dehydrogenase. It is noteworthy that in skeletal muscle, isoenzyme 1 of lactate dehydrogenase, which predominates in myocardium,[32] is less affected than the other LDH isoenzymes by similar infections.[33] Thus, even minor biochemical differences in enzyme structure and intracellular environment seems to influence the sensitivity of an enzyme to stresses such as those of an infection.

In tularemia the lysosomal enzyme system was activated in the myocardium as indicated by the increase in total activity of acid hydrolases, such as cathepsin D and β-glucuronidase. Similarly, these and other lysosomal enzymes showed increased activity in rat myocardium and skeletal muscle in tularemia[8,31] and in S. typhimurium myocarditis.[34] Further, increased activity of these enzymes may also be recorded in skeletal muscle undergoing necrosis as a result of overloading.[35] However, histologic signs of myocarditis were absent in tularemia- or influenza-infected mice. Thus, the lysosomal enzyme system was involved in the degradative turnover of various tissue components in normal and pathological states.[7,36] It may have mediated myocardial proteolysis in tularemia.

In influenza, on the other hand, the total activity of cathepsin D was unchanged and that of β-glucuronidase even decreased. In

contrast, when expressed as tissue concentration, the activity of cathepsin D, but not that of β-glucuronidase, was significantly elevated. In degenerative processes such as those following vitamin E deficiency or irradiation, decreased β-glucuronidase activity has been recorded.[37] The mechanisms of protein degradation in influenza are not clear, but reduced protein synthesizing capacity as reflected by the abrupt decrease of RNA at a normal or even slightly increased protein degradation rate, seems to fit our data best. However, alternative or additional factors cannot be excluded, such as damage of mitochondrial membranes and/or leakage of macromolecules through altered myocyte membranes. Macromolecules of m.w. 88,000 have previously been demonstrated to leak from muscle into serum in patients suffering from influenza without cardiac complications.[38] Leakage of β-glucuronidase into the plasma was evident in both of the present infections, although the myocardial content of this enzyme decreased only in influenza.

Lipid peroxidation and hydrogen peroxide formation is the basic mechanism of destruction of unsaturated fatty acid chains in biological membranes. If the mechanism of regulation is unbalanced, detrimental effects, such as increasd membrane permeability, enzyme solubilization or inactivation may occur.[39] This process is associated with hydrogen peroxide formation which may exert cytotoxic effects, but hydrogen peroxide is degraded by catalase.[18] Increased catalase activity has been proposed to be a valid indicator of ongoing skeletal muscle

wasting.[40] One intracelular location of catalase in myocardium has been shown to be the mitochondrial matrix.[41] Thus, the present finding of a negative correlation between catalase and cytochrome c oxidase activity in influenza, suggests a role for catalase in the more pronounced and rapid wasting of myocardial tissues and cellular constituents in this infection than in tularemia.

Thus, the present results suggest different pathways for myocardial degradation in influenza and tularemia. As both infections were of the same lethality, the different metabolic responses to these infections may be explained by a different pathogenic mechanism associated with these specific microorganisms. It remains to be established whether or not these differences are characteristic of viral versus bacterial infections in general.

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Table I. Tissue weights and normal enzyme activities and concentrations of protein, RNA and DNA in mouse myocardium of all (n = 48) sham-inoculated control mice (study 1).

Component		Unit	Mean ± S.D.
Body weight		g	28.1 ± 1.7
Spleen weight		mg	108 ± 21
Heart weight		mg	110 ± 6
Plasma β-glucuronidase		units/ml	4.92 ± 2.20
Heart tissue variables (units per g "wet" muscle)			
Protein		mg	166 ± 13
RNA		mg	3.25 ± 0.49
DNA		mg	1.82 ± 0.21
Glycolytic enzyme			
Lactate dehydrogenase	(LDH)	μmoles x min^{-1}	210 ± 20
Oxidative enzymes			
Citrate synthase	(CS)	μmoles x min^{-1}	178 ± 32
Cytochrome c oxidase	(CYTOX)	μmoles O_2 x min^{-1}	5.69 ± 0.81
Lysosomal acid hydrolase enzymes			
β-Glucuronidase	(GUase)	nmoles x min^{-1}	11.9 ± 3.8
Cathepsin D	(Cat.D)	μg albumin x min^{-1}	1124 ± 157
Peroxisomal enzyme			
Catalase		μmoles O_2 x min^{-1}	35.7 ± 7.4

LEGENDS TO FIGURES

Figure 1. Alterations in rectal temperature(■), β-glucuronidase activity in plasma (△), body- (○) heart- (●) and spleen weight (□) in influenza and F. tularensis infected mice at different times after inoculation expressed in per cent deviation from corresponding values in sham-inoculated control mice (unit of variables in table 1). Means ± S.E. are given. Asterisks denote statistically significant differences (*P<0.05, **P<0.01, ***P<0.001) between infected and non-infected mice.

Figure 2. Total myocardial contents of protein, RNA and DNA in influenza (○) and F. tularensis (● infected mice at different times after inoculation expressed in per cent deviation from corresponding values in sham-inoculated control mice. Unit of variable contents in table 1. Means ± S.E. are given. Asterisks denote statistically significant differences (*P<0.05, **P<0.01, ***P<0.001) between infected and non-infected mice ▣.

Figure 3. Total myocardial enzyme activity of lactate dehydrogenase, citrate synthase and cytochrome c oxidase in influenza (○) and F. tularensis (●) infected mice at different times after inoculation expressed in per cent deviation from corresponding values in sham-inoculated control mice. Unit of enzyme

activities in table 1. Means ± S.E. are given. Asterisks denote statistically significant differences (*P<0.05, **P<0.01, ***P<0.001) between infected and non-infected mice.

Figure 4. Total myocardial enzyme activity of β-glucuronidase , cathepsin D and catalase in influenza (O) and F. tularensis (●) infected mice at different times after inoculation expressed in per cent deviation from corresponding values in sham-inoculated control mice. Unit of enzyme activities in table 1. Means ± S.E. are given. Asterisks denote statistically significant differences (*P<0.05, **P<0.01, ***P<0.001) between infected and non-infected mice.

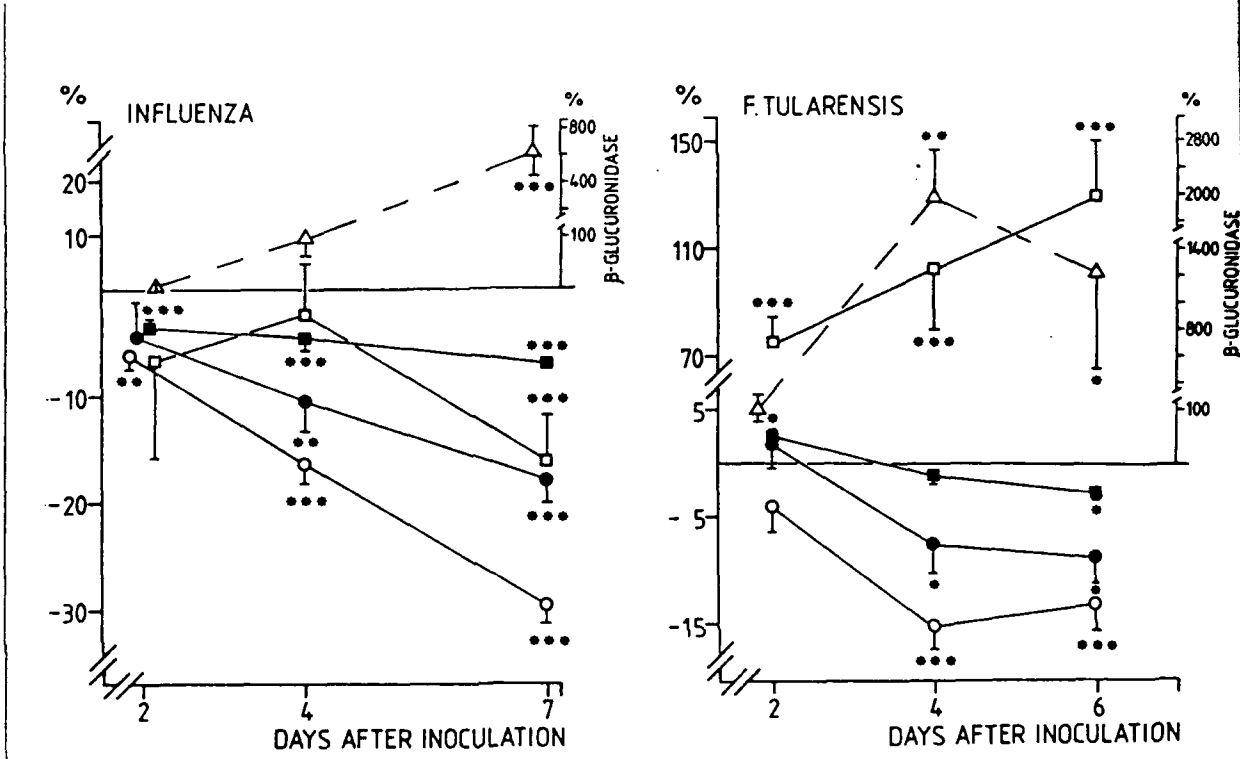
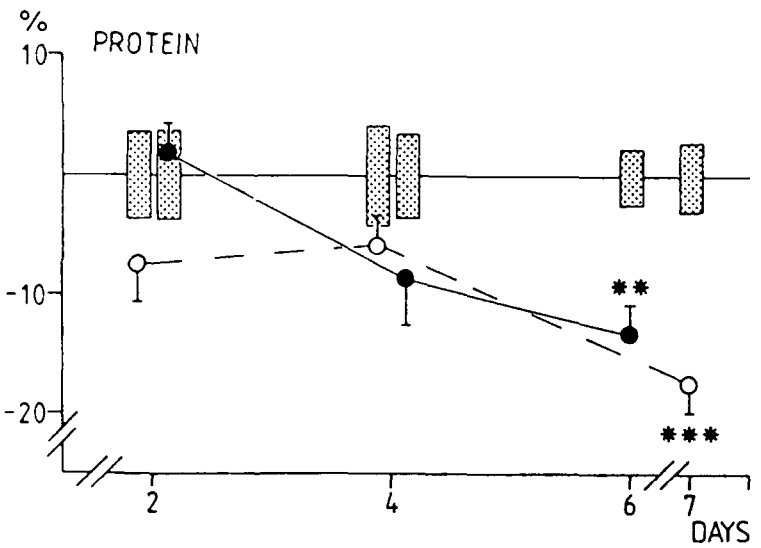
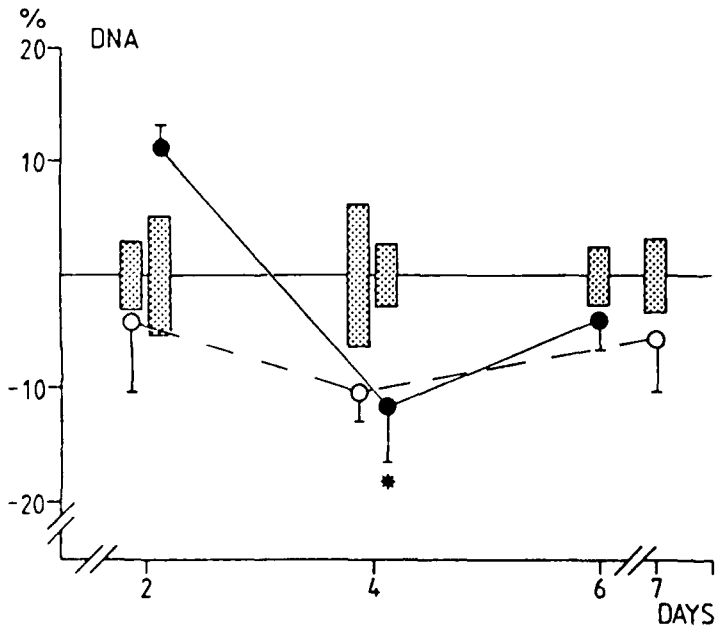


FIGURE 1 Note to publisher -- use glossy prints in this format





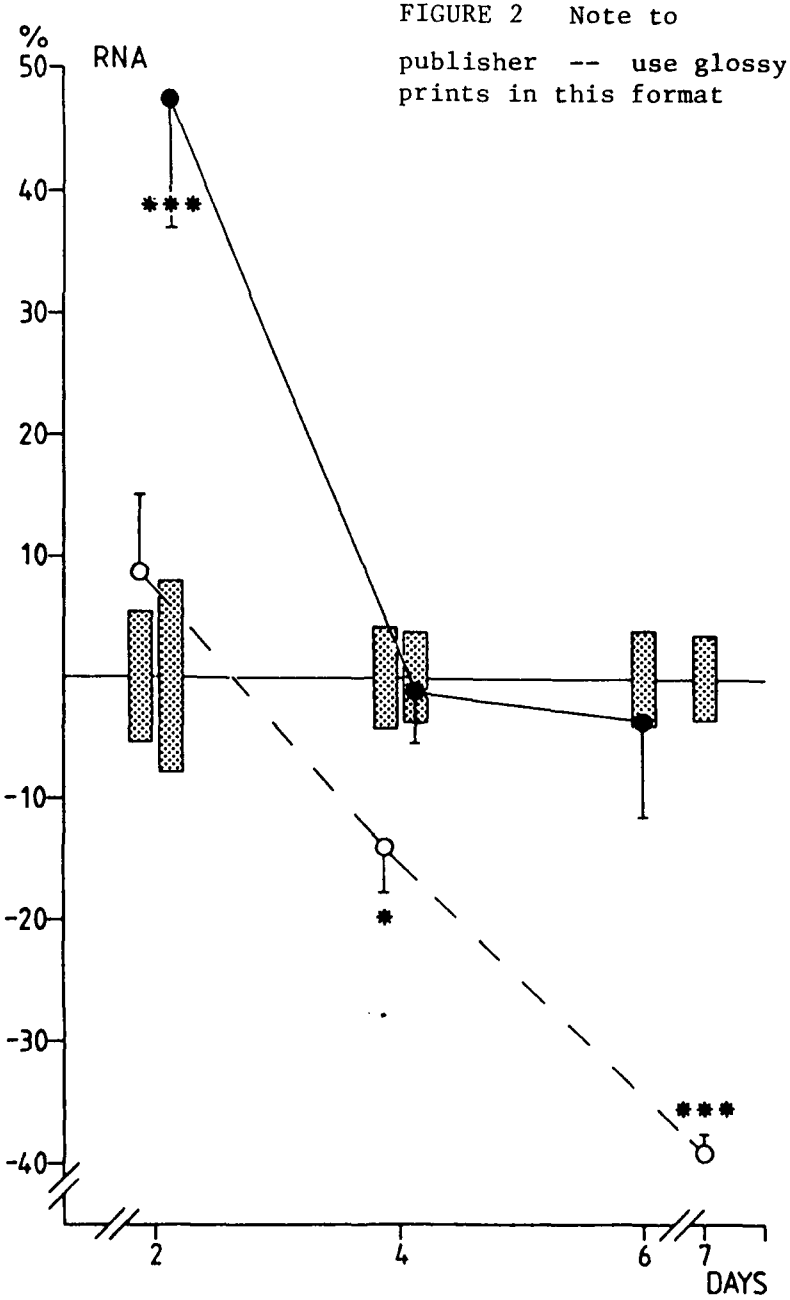
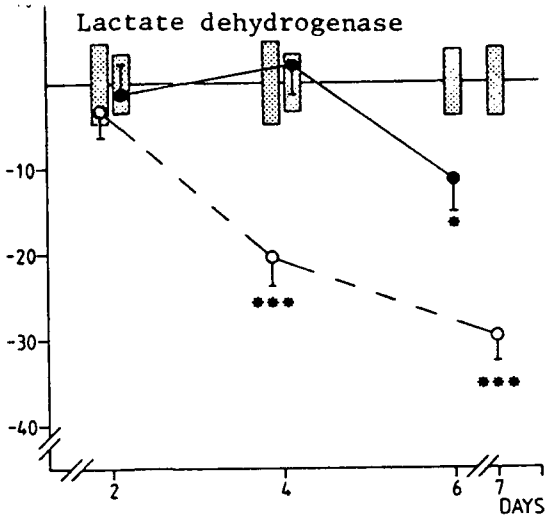
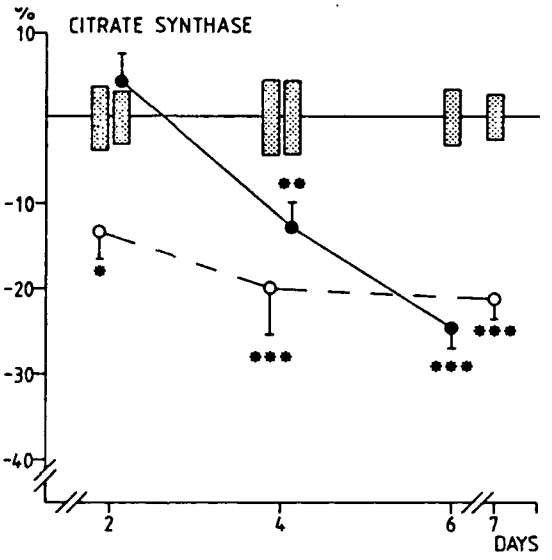


FIGURE 2 Note to publisher -- use glossy prints in this format





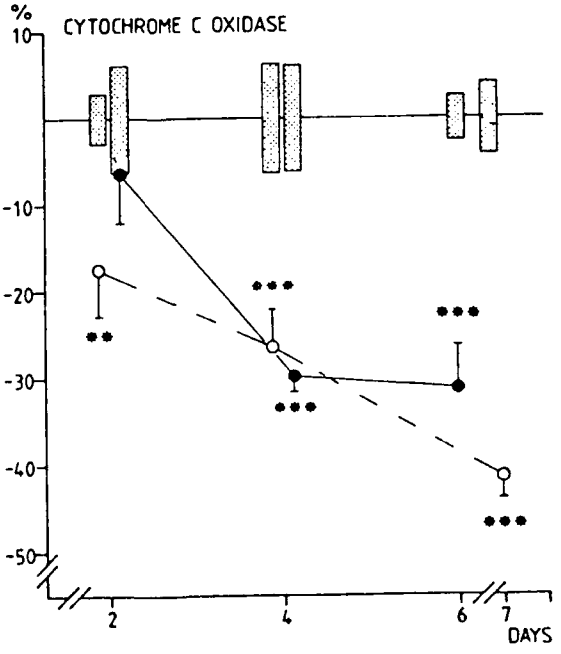


FIGURE #3

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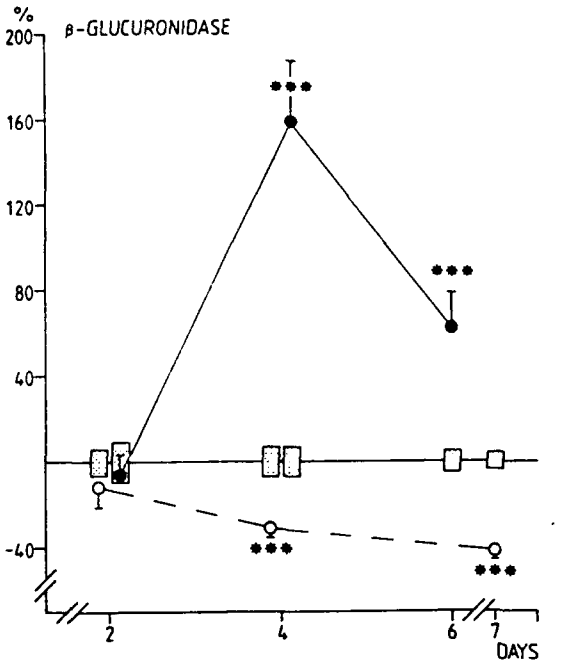

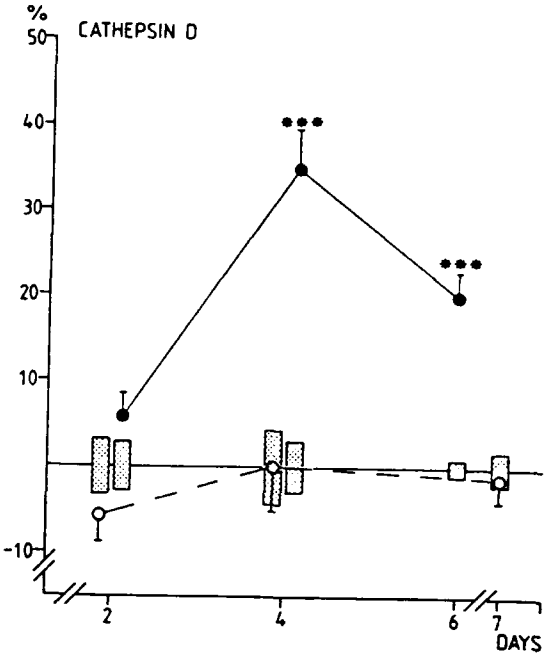

FIGURE 4 Note to publisher use glossy prints in this format