Behaviour of tissue enzymes in the circulation

Citation for published version (APA):

van Dieijen-Visser, M. P. (1981). *Behaviour of tissue enzymes in the circulation*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg. <https://doi.org/10.26481/dis.19810116md>

Document status and date:

Published: 01/01/1981

DOI:

[10.26481/dis.19810116md](https://doi.org/10.26481/dis.19810116md)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Behaviour of tissue enzymes in the circulation

Marja P. van Dieijen-Visser

Behaviour of tissue enzymes in the circulation

Proefschrift

ter verkrijging van de graad van Doctor in de
Geneeskunde aan de Rijksuniversiteit Limburg te
Maastricht op gezag van de Rector Magnificus
Prof. Dr. W.H.F.W. Wijnen, hoogleraar in de
Faculteit der Geneeskunde, volgens het besluit
van het college van Dekanen in het openbaar
te verdedigen in de Aula van de Universiteit op
Vrijdag 16 januari 1981
des namiddags te 16.00 uur

door

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geboren te Amsterdam

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Het onderzoek beschreven in dit proefschrift is uitgevoerd onder leiding van
Dr. W.Th. Hermens

Typewerk : Mevr. R.M. Borgman-Hanssen
Drukkerij : Krips Repro Meppel

Aan mijn ouders
voor Gerbrand

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CHAPTER I

Introduction

In this thesis the behaviour of tissue enzymes in the circulation is studied, in order to validate and improve methods of estimation of tissue damage on the basis of enzyme levels in plasma. Tissue enzymes catalyze specific biological reactions in the cell. Normally only very small amounts of tissue enzymes are present in the circulation, but after tissue damage the levels of tissue enzymes in plasma increase due to leakage of enzymes from damaged cells. At the same time however the enzymes are cleared from the circulation and eventually enzyme levels in plasma return to normal.

In 1908 Wohlgemut introduced a sensitive assay for amylase and demonstrated the presence of this enzyme in serum and urine of patients with acute pancreatitis. New impetus to clinical enzymology was given by Bodansky, who demonstrated in 1933 that serum phosphatase activity is elevated in bone and liver diseases. The finding of elevated transaminase levels after acute myocardial infarction by La-Due *et al.* in 1954 and also during liver disease by Wroblewski *et al.* in 1955, marked the start of an explosive development in this field. Determination of serum or plasma enzyme activities has presently become an important tool in the diagnosis and confirmation of a variety of diseases. The specificity of enzymatic reactions makes it possible to measure enzyme activity in plasma or serum with high sensitivity. Each organ has its own specific enzyme pattern and tissue specific enzymes, isoenzymes and typical ratios of enzyme activity in plasma are determined routinely to verify the site of tissue injury and the clinical course of illness.

The level of tissue enzymes in the blood will depend on the quantity of cells damaged. In a number of studies a significant although not impressive correlation was found between peak levels of enzymes in plasma and histologically assessed infarct size. The latter method is based on specific staining techniques, marking either the damaged tissue or the undamaged tissue. After excision and weighing of the damaged tissue the amount of tissue injury can be expressed in terms of grams of injured tissue. The fact that correlations between peak levels, of enzymes in plasma and histologically assessed infarct size were insufficient to allow accurate determination of in-

infarct size in individual cases was attributed to such factors as insufficient sampling and individual differences in enzyme clearance rates.

The first study concerning quantitation of myocardial infarction, where clearance rates from the circulation and distribution of enzymes over different compartments were taken into account was performed by Witteveen *et al.* (1970, 1972). Since 1971 different models have been proposed for the determination of enzymatic infarct size and both good and poor correlations have been reported between enzymatic infarct size and histologically assessed infarct size. These contradicting results suggest that the events between cell death and enzyme release are not yet sufficiently understood and that existing methods to quantitate tissue damage from enzyme levels in plasma are inadequate. Models intended to describe the fate of proteins released into the circulation should account for the phenomena of (1) extravasation of protein (2) return of the protein from the extravascular pool to the circulation and (3) clearance of protein from the circulation.

Enzymes disappear from the plasma pool at rates, that are specific for the species as well as for the particular enzyme. In experimental animals enzyme clearance rates from the circulation can be estimated from plasma clearance curves after intravenous injection of purified or partially purified enzyme preparations. In man intravenous injection of such doses of enzyme to obtain enzyme clearance rates from the circulation is not feasible in view of immunological risks. The only way to measure enzyme clearance rates in man is from plasma enzyme curves after tissue injury like for instance myocardial infarction, acute hepatitis etc. In most studies the clearance rates of intracellular enzymes were estimated from the final part of the clearance curve after tissue injury. Such measurements can only give a rough approximation of enzyme clearance rates, because it is unknown when enzyme release from tissue has stopped, which may result in underestimation of the enzyme clearance rate and consequently in overestimation of tissue damage.

Some other factors which may affect enzymatic quantitation of tissue damage are: (1) incomplete release of enzyme from necrotic cells, (2) local inactivation of enzyme activity at the place of tissue injury and (3) extravascular inactivation of enzyme activity. Enzyme release from the cells after tissue injury is caused as a rule

by a relative or absolute lack of cellular energy, resulting in an increased permeability of the cell membrane. Hereafter enzymes diffuse directly through the capillary wall or reach the plasma indirectly by lymphatic transport. These factors are however rather difficult to investigate, because for clinical as well as experimental studies only the intravascular compartment is easily accessible for the investigator. Therefore to quantitate tissue injury on the basis of enzyme levels in plasma, enzymes have to be selected that show complete recovery in the plasma compartment, *i.e.* enzymes that are not inactivated locally at the site of injury or during transport through the interstitial fluid.

To obtain the weight of injured tissue in grams, the calculated total activity of enzyme released into the circulation has to be divided by the mean enzyme activity per gram of normal tissue. Selection of enzymes with small intra- and inter-individual variations in tissue enzyme content will therefore minimize errors in the quantitation of tissue damage, due to the use of a mean value for the enzyme content per gram of normal tissue.

Several aspects of enzymatic quantitation of tissue damage have been investigated in the present study. Dogs were used in all experiments. These animals are commonly used in experimental models to study enzymatic infarct quantitation.

Chapter II presents a review of the literature on protein turnover and on quantitation of tissue damage.

In chapter III intra- and inter-individual variations in enzyme content of dog heart and dog liver are investigated.

In chapter IV a method is described to estimate plasma volume by use of enzyme dilution. This parameter is essential for calculations of enzyme release in plasma.

In chapter V a model to estimate the plasma clearance and distribution of cardiac enzymes in the dog is presented. Intra- and inter-individual variations in model parameters are estimated.

In chapter VI a method is presented which allows estimation of individual clearance rates, even when there is still release of enzyme into the circulation. The method presented in this chapter is based on analysis of simultaneously infused enzymes with different clearance rates. The estimated clearance rate is used to quantitate the infusions. Intramuscular injections of enzyme activity were given to investigate whether all enzyme activity administered into the muscle

eventually reaches the circulation. Total recovery in plasma would indicate that there is no local inactivation of enzyme activity in the tissue, but also that enzyme breakdown occurs in the plasma pool or in immediate contact with that pool.

In chapter VII we describe a method to investigate the stability and homogeneity of enzyme preparations used for clearance studies. This method is based upon thermal inactivation of enzymes. Part of the contradictions in earlier work on the clearance of enzymes from the circulation were caused by use of different protein preparations. In chapter VIII a summary of the main conclusions of our work is presented.

Three appendices containing respectively the complete data of experiments presented in this thesis, a description of the mathematical models used and a thermodynamical description of thermal inactivation of enzymes complete this thesis.

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CHAPTER II

Review of the literature

II.1. Introduction

When this investigation was started in 1977 our aim was to study quantitation of tissue damage from increased plasma enzyme levels. In order to be able to quantitate the amount of enzyme released from necrotic tissue, the rate at which enzymes are cleared from the circulation and the distribution of enzymes throughout different compartments have to be known.

Determination of (1) enzyme clearance rates from the circulation, (2) the rate of enzyme exchange with the extravascular compartments and (3) the intra- and inter-individual variations in these parameters covers an important part of this thesis. Therefore a review will be given of the literature on the turnover of circulating proteins, with emphasis on circulating enzymes.

In the second part a literature review on enzymatic quantitation of tissue damage will be given. Literature on the more specific problems like: (1) the mechanism of cell death and subsequent enzyme release, (2) local inactivation of enzymes in the tissue and (3) inactivation of enzymes during transport from tissue to plasma will be discussed.

Throughout the literature the term protein turnover refers to different physiological processes like (1) turnover of circulating proteins, (2) hydrolysis of intracellular proteins to amino acids, (3) total protein turnover of a given organ etc. The present review will concern the turnover of enzymes in the circulation. The literature on the turnover of circulating albumin is so voluminous that it is impossible to summarize it in a few pages. Although there is no lack of data on the turnover and distribution of labeled plasma proteins and enzymes, much of the early work was carried out with inferior protein preparations. It is known that even slight damage of protein leads to a highly increased rate of catabolization as compared to the so-called native proteins. The mechanism responsible for this rapid elimination of protein from the circulation seems to be completely different from that involved in normal enzyme clearance from the circulation. Fallacious conclusions on the clearance and distribution of proteins have been drawn from such experiments.

Therefore in this review special attention will be paid to the clearance of damaged macromolecules.

II.2. Mathematical models to describe the catabolism and distribution of proteins that enter the circulation.

Models intended to describe the fate of proteins released into the circulation should account for the phenomena of: (1) extravasation of protein, (2) the return of protein from the extravascular pool to the circulation and (3) catabolism in the circulation.

It is now generally accepted that proteins escape from the circulation through the capillary membrane and that the principal mechanisms of transport from plasma to interstitium are filtration (solvent drag), diffusion and vesicular transport. The question which contribution should be attributed to each of these phenomena is not yet settled (Renkin *et al.*, 1977; Rippe *et al.*, 1979; Landis *et al.*, 1963; Lassen *et al.*, 1974 and Casley-Smith *et al.*, 1976). Approaches to this problem are indirect and it seems that laboratories using different techniques obtain different answers.

Generally one conceives that convective transport of solvent and macromolecules and diffusion of macromolecules is possible by the existence of canals (pores) in the capillary membrane. It is assumed that there exist a large number of small pores (diameter $< 50 \text{ \AA}$) and fewer large pores (diameter $> 200 \text{ \AA}$). The existence of pores of different sizes gives rise to a local paracapillary circulation. Through the large pores there is a bulk flow, consisting of solvent and proteins, from plasma to interstitium. This flow is mainly regulated by the hydrostatic pressure difference ΔP between capillary and interstitium. This flow through the larger pores is partially compensated by osmotic absorption of solvent through the small pores and across the endothelial cells. From this pathway macromolecules are excluded. This fast flow is mainly regulated by both the hydrostatic pressure difference ΔP and the osmotic pressure difference between capillary and interstitium.

Filtration

The net result of these flows is a net volume flow F_v of solvent and a net solute flow F_s , resulting from the solvent drag transfer of solute by the solvent flow F_{v1} through the pores, which can be passed

unhindered by macromolecules. Thus one obtains the following expression for the solute flow F_1 , due to convection:

$$F_1 = F_{v1} \cdot \bar{c} \quad (1)$$

F_1 - the amount of the specific solute extravasated per hour (mol/h)
 F_{v1} - the volume flow through the large pores per kg tissue (l/h)
 $\bar{c}$ - the concentration of solute in the large pores (mol/l); $\bar{c}$ has a value between the plasma concentration C_i and the interstitial concentration C_e .

In the literature (Renkin *et al.*, 1977; Rippe *et al.*, 1977 and Taylor *et al.*, 1977), a variety of different expressions are used to relate F_{v1} to the hydrostatic and osmotic pressure differences and the lymph flow. Because of this lacking agreement we do not specify this flow further. Note that F_{v1} increases with increasing hydrostatic and osmotic pressure differences between plasma and interstitium, and that F_{v1} decreases with increasing molecular size. The same disagreement exists about the relation between $\bar{c}$, C_i and C_e . Expressions used are all in the form: $\bar{c} = (1-\alpha)C_i + \alpha C_e$ but the α 's used vary widely. So we obtain instead of (1):

$$F_1 = F_{v1}((1-\alpha)C_i + \alpha C_e) \quad (2)$$

The diffusional and vesicular transport is assumed to be proportional to a permeability surface factor PS, which is the product of the area of the vascular wall, which is permeable to the macromolecules and a diffusion constant in case of diffusion or a rate constant of the transport rate in case of vesicular transport.

Assuming Fick's law for the diffusion or symmetry in case of vesicular transport we obtain the following expression for the solute flow, due to these mechanisms.

$$F_2 = PS \cdot C_i \quad (3)$$

F_2 - the amount of solute escaping from the vascular compartment per hour (mol/h)
 PS - permeability-surface area product (l/h)
 C_i - plasma concentration of the solute (mol/l).

$$F_{ex} = F_1 + F_2 \quad (4)$$

F_{ex} - the flow of protein to the extravascular space, the interstitium.

Return of protein

The return of protein from interstitium to plasma is formed by the back diffusion term $PS.C_e$ and the return by lymph flow. Hence one obtains the expression for the return F_R to plasma:

$$F_R = PS . C_e + F_L C_L \quad (5)$$

F_L - lymph flow per hour (l/h)

C_L - the concentration of solute in lymph (mol/l).

One generally agrees that catabolism of protein occurs in the vascular pool or in immediate contact with this pool. The evidence is indirect. Most information thus far is obtained from experiments with I-labeled proteins, especially albumin. It turns out that excretion of the iodine label and the decrease of whole-body content of the label runs closely in parallel to the plasma concentration. While certainly during the first 12 hours upon injection of the labeled albumin the extravascular concentration is still rising, and the plasma concentration decreasing. Most direct evidence on this point could be obtained by recording the quantitative recovery in plasma of extravascularly injected protein, see chapter VI of this thesis. However we do not know of reports comparing this return of extravascularly injected protein to the amount injected.

In case of rapidly catabolized protein, with a half-life in plasma of say less than 5 hours it follows from the mono-exponential decay curves of plasma activity and the fact that extravasation cannot account more than 5% per hour, that the major part of the protein is catabolized intravascularly and that the catabolic rate is proportional to the plasma concentration.

Thus the assumption is made that the protein is catabolized only from the intravascular pool and that the rate is proportional to the plasma concentration.

$$F_C = V_i k C_i \quad (6)$$

V_i - plasma volume (l), (intravascular volume)

k - rate constant of the first order reaction describing the catabolism (h^{-1})

C_i - plasma concentration of protein (mol/l)

F_C - the amount of protein or label catabolized per hour.

Collecting the different flows of protein, equations (2), (3), (4), (5) and (6) we can describe the dynamics in the time of the intravascular concentration C_i by:

$$V_i \frac{dC_i}{dt} = - F_c - F_{ex} + F_R + V_i f(t) \quad (7)$$

- F_c - the amount of protein or label catabolized per hour (mol/h)
 F_{ex} - the flow of protein to the extravascular space, the interstitium
 F_R - the return flow of protein from the extravascular space to plasma
 $f(t)$ - the input of enzyme.

Insertion of (2), (3), (4), (5), (6) in (7) results in:

$$\frac{dC_i}{dt} = - \frac{PS}{V_i} (C_i - C_e) - \frac{F_{v1}}{V_i} ((1-\alpha)C_i + \alpha C_e) + \frac{F_L C_L}{V_i} - kC_i + f(t) \quad (8)$$

In order to relate the interstitial concentration C_e to flows, we need the concept of the extravascular distribution space. Rutili *et al.* 1978 made a direct approach to the definition of this space by correlating the amount of extravascular macromolecules to the interstitial concentration.

It turned out that for increasing molecular size this distribution space V_d decreased, but for molecules larger than 50,000 Daltons this dependence of V_d on molecular size became rather faint. It is thought that the dependence of the distribution space on molecular size is due to exclusion of larger molecules from the gel phase in the interstitium (Wiederhielm *et al.*, 1979).

In this same study a direct comparison of lymph and interstitial fluid, sampled by a micropipette lead to the conclusion that lymph concentration and interstitial concentration are identical ($C_e = C_L$). Thus the change of the amount of protein or enzyme in the interstitium is described by:

$$\frac{dC_L}{dt} = + \frac{PS}{V_d} (C_i - C_L) + \frac{F_{v1}}{V_d} ((1-\alpha)C_i + \alpha C_L) - \frac{F_L C_L}{V_d} \quad (9)$$

Ordering of the terms in (8) and (9) results in:

$$\frac{dC_i}{dt} = - \left(\frac{PS}{V_i} + (1-\alpha) \frac{F_{v1}}{V_i} \right) C_i + \left(\frac{PS}{V_i} - \frac{\alpha F_{v1}}{V_i} + \frac{F_L}{V_i} \right) C_L - kC_i + f(t) \quad (10)$$

$$\frac{dC_L}{dt} = -\left(\frac{PS}{V_d} - \alpha \frac{F_{v1}}{V_d} + \frac{F_L}{V_d}\right) C_L + \left(\frac{PS}{V_d} + (1-\alpha) \frac{F_{v1}}{V_d}\right) C_P \quad (11)$$

Comparison of the equations (10) and (11) obtained by consideration of physiological processes with the more commonly used two-compartment model (Mathews *et al.*, 1967 and Hermens *et al.*, 1975) leads to the conclusion that both have identical form and are equivalent:

$$\frac{dC_i}{dt} = P/V_i (C_i - C_e) - kC_i + f(t) \quad (12)$$

$$\frac{dC_e}{dt} = -P/V_e (C_e - C_i) \quad (13)$$

The equations (12) and (13) are obtained from (10) and (11) by the transformations:

$$P/V_i = \frac{PS}{V_i} + (1-\alpha) \frac{F_{v1}}{V_i}$$

the quotient P/V_i is also known as transcapillary escape rate (TER).

$$P/V_e = \left(\frac{PS}{V_d} - \alpha \frac{F_{v1}}{V_d} + \frac{F_L}{V_d}\right)$$

$$k=k ; V_i=V_i$$

$$V_e = V_d \frac{PS + (1-\alpha) F_{v1}}{PS - \alpha F_{v1} + F_L}$$

$$C_e = \frac{PS - \alpha F_{v1} + F_L}{PS + (1-\alpha) F_{v1}} C_L$$

The main difference between these models is that model (10), (11) allows for the filtration of the protein in the capillary wall. In the steady state we find in this model the lymph concentration

$$C_L = \frac{PS + (1-\alpha) F_{v1}}{PS - \alpha F_{v1} + F_L} C_i$$

When F_L is larger than F_{v1} , C_L is smaller than C_i , which is in agreement with the experiments, but in contrast with the diffusional model, where it is assumed that $C_i = C_e$.

Note however that these models are a gross simplification by the fact that all capillary walls and all tissues are considered to be

equal and escape to and return from these tissues is lumped together in one term.

For the term representing the escape from plasma, this does not harm, as the summing up of several terms of this form leads to an expression of the same form. The problem however arises in the term representing the return from interstitium to plasma. The sum of this term over several tissues with a varying lymph flow and varying lymph concentration is not necessarily well represented by the same form.

Table I presents transcapillary escape rates (TER) and the ratio of the intra- and extravascular volumes (V_e/V_i) for different plasma proteins, as obtained from the literature

Table I

Plasma protein	MW	Reference	Species	Number of exponentials	TER (%/h)	V_e/V_i
Albumin	69000	Takeda 1963	man	3	3.9	1.2
Albumin	69000	Beeken 1962	man	3	4.2	1.3
Albumin	69000	Walker 1960	man	2	2.6	1.4
Albumin	69000	Reeve 1959	rabbit	2	4.3	1.44
γ -globulin	160000	Walker 1960	man	2	1.7	0.50
γ -globulin	160000	Andersen 1963	dog	2	1.8	0.72
Fibrinogen	341000	Tytgat 1971	man	2	2.5	0.41
α_1 -antitrypsin	56000	Jones 1978	man	2	3.3	1.07

TER = transcapillary escape rate (%/h)

V_e = extravascular volume (l)

V_i = intravascular volume (l)

MW = molecular weight.

II.3. Clearance and distribution of circulating enzymes

Most of the research on circulating proteins has been performed with labeled plasma proteins like, albumin, immunoglobulin, fibrinogen etc. An extensive review on the turnover of plasma proteins in different species was given by Schultze and Heremans (1966). In the present review results obtained from studies on the clearance and distribution of circulating enzymes will be compared with results obtained for plasma proteins. The general principles to describe the clearance and distribution of circulating plasma proteins (II.2.) can be applied to circulating enzymes.

In turnover studies based on the measurement of enzyme activity some problems peculiar to enzymes may arise:

- Rapid elimination of partially denatured enzyme preparations. The higher the specific activity the less stable the enzyme becomes (Posen *et al.*, 1970 and Sodetz *et al.*, 1977).
- Enzyme activity may not parallel enzyme concentration when inhibitors or activators are present in plasma.
- The measurement of enzyme activity is dependent on small changes in pH and temperature.
- Hemolysis of plasma samples may increase the plasma levels of enzymes which are present in erythrocytes.

In a few studies radioactively labeled enzymes have been used (Roberts *et al.*, 1975; Schapira *et al.*, 1962; Masarat, 1968 and Qureshi *et al.*, 1976). These preparations are subject to the same errors as described for labeled plasma proteins (Schultze *et al.*, 1966; Luck, 1956 and II.6.).

In experimental animals enzyme clearance rates can be estimated from plasma curves obtained after intravenous injection of purified or partially purified enzyme preparations. In man the only way to measure enzyme clearance rates is from plasma enzyme curves obtained after tissue damage like for instance myocardial infarction (Witteveen, 1972), acute hepatitis etc. The clearance rates of intracellular enzymes are often estimated from the last part of the clearance curve until the activity returns to normal. Such measurements can however only give a rough approximation of enzyme clearance rates, because it is unknown when enzyme release from the tissue has stopped, which may result in an underestimation of the enzyme clearance rates.

One of the few studies on intravenous injection of intracellular tissue enzymes in man was performed by Dawson *et al.* (1969). In this study enzyme rich plasma of an alcoholic suffering from rhabdomyolysis was infused into the same patient after recovery (Table II). For human clotting factors VIII and IX plasma clearance and distribution could be measured after intravenous infusion of factor VIII and IX concentrates in patients suffering from bleeding disorders.

Table II shows the model parameters obtained after intravenous injection of various intracellular tissue enzymes in different species. Although the table is not complete it gives a general survey of the literature available on this subject. For most enzymes more than

one disappearance rate is recognizable from the plasma clearance curve. Plasma kinetics of intravenously injected proteins is based on the description of plasma curves as a sum of exponentials, each exponential corresponding to a separate compartment (II.2.). However until now many conflicting results have been reported on the clearance of tissue enzymes from the circulation and the distribution over different compartments because:

- purification and or labeling may cause molecular changes such that fractions of different *in vivo* stability may arise, resulting in a multi-exponential clearance pattern.
- isoenzymes or enzymes of different stability present in the preparations may cause the same problem.

These factors were not always recognized and as a consequence fallacious conclusions were drawn from such enzyme disappearance curves. As can be seen from table II even in the recent literature large discrepancies on the clearance and distribution of enzymes become apparent. For instance for AST and CK clearance in the dog both double-exponential (Dunn, 1958; Wakim *et al.*, 1963; Shell *et al.*, 1971 and Sobel *et al.*, 1977) and mono-exponential (Rapaport, 1975; Roberts *et al.*, 1975; Roberts *et al.*, 1977 and the present study) clearance has been reported.

Shell *et al.*, 1971 and Sobel *et al.*, 1977 measured extremely rapid diffusion of CK to the extravascular space. In both studies equilibration with the extravascular space occurred within two hours. The transcapillary escape rate (TER) as observed by Sobel *et al.* was 54% per hour. In sheep Boyd *et al.*, 1967 measured a TER of 73% per hour for LD-5. These values are in striking contrast with TER's as observed for plasma proteins of comparable molecular weight (see II.2.). For plasma proteins ranging in molecular weight from 69000 to 156000 Daltons TER's of less than 5% per hour were measured in different species.

As the blood-lymph barrier does not discriminate in the passage of molecules with molecular weights exceeding 40000 Daltons (Grotte, 1956; Pappenheimer, 1953 and Rutili, 1977) such large TER's as observed in some studies for CK, AST and LD (Table II) are not to be expected, because the respective molecular weights are 81000, 110000 and 140000 Daltons.

In dogs we were able to demonstrate that for CK and AST clearance rates as calculated from equilibrium conditions obtained after constant rate infusion of the enzyme equal the apparent clearance con-

Table II Clearance and distribution of intravenously injected enzymes.

Enzyme	Reference	MW	Source	Species ^x	Fast phase clearance			Slow phase clearance					TER %/h	V/V _e i
					T _{1/2} (b) (h)	SD %	C ₁ %	T _{1/2} (a) (h)	SD %	C ₂ %	SD %	k %/h		
ALD	Schapira '62	159000	rabbit muscle	rabbit (n = 4)	3.3			mono-exponential						
ALT	Fleisher '63	180000	dog liver dog heart	dog (n = 6)	16.3	22	58.3	14	61.7	16	37.4	13	2.0	1.0 0.42
ALT	Present study	180000	dog liver	dog (n = 5)	5.9	20	44.3	28	51.8	18	57.6	19	2.2	3.7 0.52
ALT	Mahy '67	180000	porcine heart	mice	10.0			mono-exponential						
ALT	Amelung '60	180000	rabbit liver	rabbit (n = 7)	1.32	17			5.1	27				
ALT	Amelung '60	180000	porcine heart	rabbit (n = 2)	9.7	5			-	-				
Amy- lase	Appert '68	45000	pancreatic juice dog	dog (n = 13)	3.13	31			mono-exponential					
AP	Clubb '65	100000	human placenta	human (n = 7)	19.3	24	30.2	17	139.0	26	58.8	8	0.7	0.8 0.33
AST	Amelung '60	110000	rabbit liver	rabbit (n = 7)	1.23	16			2.6	17				
AST	Amelung '60	110000	porcine heart	rabbit (n = 3)	0.92	3			2.9	34				
ASTC	Masarat '68	110000	porcine heart	pig (n = 6)			55.5		17.4	19	44.5			1.25
ASTC, 131I	Masarat '68	110000	porcine heart	pig (n = 6)			58.0		19.6	23	42.5			1.36
ASTC	Masarat '68	110000	porcine heart	rabbit (n = 1)					9.8		32.2			2.11

Enzyme	Reference	MW	Source	Species*	Fast phase clearance				Slow phase clearance				k %/h	TER %/h	V/V _{e1}
					T _{1/2} (h)	SD %	C ₁ %	SD %	T _{1/2} (a) (h)	SD %	C ₂ %	SD %			
ASTC, 131I	Masarat '68	110000	porcine heart	rabbit (n = 1)					10.4						
AST	Dawson '69	110000	human muscle	human (n = 1)	4.1				4.7						
ASTC	Wakim '63	110000	dog liver	dog (n = 8)	4.0	15	56.5	29	11.3	9	30.3	44	10.6	3.0	0.29
ASTC	Wakim '63	110000	dog muscle	dog (n = 4)	3.9	11	60.8	15	12.6	3	31.0	39	10.0	3.5	0.36
ASTC	Present study	110000	dog liver	dog (n = 5)	3.54	22			mono-exponential						
ASTC	Present study	110000	dog heart	dog (n = 10)	3.47	24			mono-exponential						
ASTC	Present study	110000	porcine heart	dog (n = 4)	3.26	33			mono-exponential						
CK-MM	Shell '71	81000	dog heart	dog (n = 11)					2.4	21					+ 2
CK-MM	Roberts '75	81000	dog heart	dog (n = 5)	2.3	24			mono-exponential						
CK-MM	Rapaport '75	81000	dog muscle	dog (n = 6)	1.77	28			mono-exponential						
CK-MB	Rapaport '75	81000	dog heart	dog (n = 6)	1.35	26			mono-exponential						
CK-BB	Rapaport '75	81000	dog brain	dog (n = 6)	0.69	37			mono-exponential						
CK-MM	Sobel '77	81000	dog heart	dog (n = 6)	0.34	48	52.0	23	2.2	51	48.5	23	53.7	59.7	0.48
CK-MM	Present study	81000	dog heart	dog (n = 10)	1.87	21			mono-exponential						

Table II Continued.

Enzyme	Reference	MW	Source	Species*	Fast phase clearance				Slow phase clearance				TER %/h	V/V _i e ⁻¹
					T _{1/2} (b) (h)	SD %	C ₁ %	SD %	T _{1/2} (a) (h)	SD %	C ₂ %	SD %	k %/h	
CK-MB	Yasmineh '76	81000	baboon heart	baboon (n = 6)	6.6	11			mono-exponential					
CK-MM	Dawson '69	81000	human muscle	human (n = 1)	2.3				4.5					
F VIII	Hermens '75	1100000	human plasma	human (n = 6)	4.0		37.3		14.0		62.7		6.7	2.8 0.22
F IX	Hermens '75	55000	human plasma	human (n = 20)	4.6		37.0		31.5		21.7		4.8	5.6 0.80
GPI	Present study	45000	dog heart	dog (n = 8)	1.45	26			mono-exponential					
GPI	Mahy '67	45000	yeast	mice	1.8				mono-exponential					
LD-1	Boyd '67	140000	sheep heart	sheep (n = 6)	2.3	108	48.3	36	57.2	43	51.7	31	2.2	12.7 0.80
LD-5	Boyd '67	140000	sheep muscle	sheep (n = 6)	0.33	322	40.8	50	5.9	41	59.2	35	19.3	74.0 0.57
LD-5	Qureshi '76	140000	rabbit muscle	rabbit (n = 5)	0.65	25	59.3	19	3.5	15	40.7	28	37.3	34.4 0.63
LD-5, 131I	Qureshi '76	140000	rabbit muscle	rabbit (n = 5)	0.97	38	21.1	65	12.4	19	47.5	49	8.2	25.6 0.39
Lb	Strandjord '59	140000	rabbit heart	dog (n = 1)	+2.5				mono-exponential					
LD-1,2	Present study	140000	dog heart	dog (n = 10)	1.58	26			mono-exponential					
lipase	Appert '68	45000	pancreatic juice	dog (n = 13)	2.9	34			mono-exponential					

Enzyme	Reference	MW	Source	Species*	Fast phase clearance				Slow phase clearance				TER %/h	V/V _e
					T _{1/2} (b) (h)	SD %	C ₁ %	SD %	T _{1/2} (a) (h)	SD %	C ₂ %	SD %	k %/h	
MD	Amelung '60	62000	rabbit muscle	rabbit (n = 6)	1.35	22			2.6	27				
MD	Dawson '69	62000	human muscle	human (n = 1)	2.1				12.0					
PK	Dawson '69	225000	human muscle	human (n = 8)	7.9				15.0					

Mode of expression of original data is modified to permit comparison.

T_{1/2}(b), (a) = half lives for the fast and slow phases of the clearance curves; T_{1/2}(b) = ln 2/b, T_{1/2}(a) = ln 2/a.

TER = transcapillary escape rate (%/h).

V_i, V_e = intravascular and extravascular volumes (l).

k = true clearance constant (%/h).

C₁, C₂, b, a = see chapter V.

* Species receiving the enzyme preparation.

Dawson: correction of the faster phase for the simultaneous effects of the slower phase was not done.

Strandjord: clearance rates are estimated from figure.

stants k_d as calculated from the clearance pattern after a loading dose of enzyme given 90 minutes before the infusion was started (chapter V). Constant rate infusion of enzyme permits direct calculation of enzyme clearance rates independent of the specific model used. It is concluded that for rapidly cleared enzymes like CK and AST the rate of transport to the extravascular compartment could be neglected in comparison to the plasma clearance rate and moreover in our study no initial rapid disappearance of enzyme activity occurred. This implies that double-exponential clearance, as presented throughout the literature for enzymes cleared as fast as CK or AST do not reflect distributional effects.

For ALT a slowly cleared enzyme ($T_{1/2} = 31$ h) a TER of 1.0% per hour was observed by Wakim *et al.* (1963). Comparable results were obtained in the present study (chapter VI). These values are comparable to TER's as observed for plasma proteins of about the same molecular weight (II.2., table II).

Enzymes like plasma proteins have characteristic biological half-lives depending on the (iso)enzyme, its source and the species involved. As can be seen in table II porcine AST disappears at about the same rate as observed for rabbit AST, while porcine ALT and rabbit ALT are cleared at completely different rates (Amelung *et al.*, 1960). Masarat (1968) also measured AST clearance in the rabbit resulting in a different value as obtained by Amelung. Injection of porcine AST in pigs resulted in a considerably smaller clearance rate as observed in the rabbit (Masarat, 1968). In the dog no significant differences in AST clearance rates were observed after injection of dog heart, dog liver and porcine heart AST.

For plasma proteins large inter-species variations in the biological half-lives have been observed. Allison *et al.* (1960) showed that the logarithm of the half-lives of albumin and γ G-immunoglobulin are directly proportional to the logarithm of the body weights. At this moment too few reliable data are present to examine such a relation for enzyme clearance rates.

II.4. Sites of catabolism of circulating enzymes

The mechanism of catabolism of proteins present in the circulation and the sites at which such catabolism takes place are still very much uncertain.

Exogenous Catabolism

Digestive and renal routes of loss of protein are described as exogenous catabolism.

The rôle of the kidneys

Conflicting results have been published on the rôle of the kidneys in the catabolism of circulating plasma proteins. Using unscreened ^{131}I -albumin Gitlin *et al.* (1958) demonstrated that in mice bilateral nephrectomy prolonged the half-life of ^{131}I -albumin from 0.7 to 1.4 days. In the rabbit Katz *et al.* (1960) and Rosenfeld *et al.* (1962) demonstrated that *in vivo* bilateral nephrectomy reduced unscreened albumin breakdown by about 20%, but had no effect on the breakdown of biologically screened iodoalbumin. To eliminate rapidly degrading fractions of damaged molecules, the biological filter method was developed (McFarlane, 1963). This involves intravenous injection and successive harvesting of the injected substance from plasma *i.e.* biologically screened preparations. The same results were observed in the perfused kidney. It was concluded that at most 10% of the turnover of native albumin in the body may occur in the kidneys. Normally intracellular tissue enzymes also are hardly excreted in the urine. Dunn *et al.* (1956) and Wakim *et al.* (1963) demonstrated respectively that no measurable amounts of AST and ALT were found in the urine after intravenous bolus injections or following myocardial infarction in the dog. In bilaterally nephrectomized dogs no significant effect on CK and AST clearance rates has been observed (Dunn *et al.*, 1956 and Roberts *et al.*, 1975), as compared to sham operated controls.

Appert *et al.* (1968) demonstrated that after injection of pancreatic juice in the dog, less than 1% of amylase and lipase activity was detected in the urine.

Blainey *et al.* (1967) found however that enzymes such as amylase, which have low molecular weights are readily filtrable by the glomerulus.

Kemp *et al.* (1960) demonstrated that in the rabbit and the cat aldolase (ALD), AST, ALT and LD are, - if at all excreted in very small amounts in normal urine.

Strandjord *et al.* (1959) obtained the same results for ICD and LD in dogs. Further studies are necessary to determine whether inactive enzyme is excreted in the urine, subsequent to their infusion into the

blood stream. For labeled plasma proteins it has been demonstrated that protein bound label is not excreted in the urine. This has not been examined for labeled enzymes.

The rôle of the gastrointestinal tract

An extensive review on the rôle of the gastrointestinal tract on the turnover of plasma proteins has been given by Schultze and Heremans (1966).

In the rat, splenectomy and combined or separate hepatectomy and evisceration depressed plasma albumin by 50% as compared to sham operated controls. It was demonstrated that the whole of the digestive tract may be responsible for about 50% of the fractional catabolism of albumin. If this assumption is correct it would consolidate the concept of an entero-hepatic cycle of protein nitrogen, since the amino acids liberated via the portal route reach the liver, which could straightforwardly use them for synthesis of plasma proteins. Rats given an intravenous injection of iodoalbumin excreted practically no protein bound label in their faeces, but if they received soybean trypsin inhibitor in their drinking water the trichloroacetic acid precipitable faecal activity corresponded to a daily excretion of 17-29 mg of protein (Tarver *et al.*, 1961). This represents 15-25% of the total mass of this protein degraded per day in this animal. Andersen (1963) demonstrated a significant correlation between intravascular, extravascular exchange rates of γ G-immunoglobulin and its intestinal degradation rate. This would imply that the leakage of plasma proteins into the gut is mainly a matter of capillary permeability. In the dog Andersen *et al.* (1961) demonstrated that intestinal clearance contributes substantially ($\pm$ 34%) to the metabolic clearance of γ G-immunoglobulin.

The observation that plasma proteins are able to cross the intestinal mucosa suggest that enzymes may do the same. In the dog intestinal clearance contributes for 18% to the metabolic clearance of ALT and only for 3% to that of cAST. For mAST the corresponding data were not calculated but it was assumed that its intestinal clearance (Wakim) contributes no more than tracers to its metabolic clearance. Masarat (1968) also observed no AST in the intestinal lumen of the dog after intravenous AST injections.

The small quantities of enzymes present in bile *e.g.* transaminases are out of proportion to the plasma turnover. After intravenous injections of LD and AST there is no increase of enzyme activity in the

bile (Dunn, 1958; Amelung, 1958).

Endogenous catabolism

Endogenous catabolism is the active uptake of proteins from plasma or interstitial fluid by tissue cells. Analogous to the behaviour of serum proteins, it may be assumed that the breakdown of serum enzymes takes place to the level of amino acids. Breakdown in the reticuloendothelial system (RES) has been demonstrated for a number of enzymes. Wakim and Fleisher (1963) demonstrated that in the dog zymosan, an insoluble polysaccharide from yeast cell walls has a marked inhibitory effect on the rate of disappearance from blood of injected cAST, mAST and LD, but not on the rate of clearance of ALT. This inhibitory effect was most noticeable during the first few hours after the administration of zymosan. Some time later the effect wore off and after 1-2 days there frequently were indications for enhanced enzyme clearance. Blockade of the reticuloendothelial system was without effect on the intestinal clearance rate of these enzymes. In 1975 Roberts *et al.* observed a significant effect of zymosan on CK clearance rate after bolus injections in the dog.

Injection of mice with RES blocking agents causes an increase in the levels of LD-5 and GPI, which enzymes are also raised after infection with Riley virus (Mahey *et al.*, 1967). For ALT and LD-1 no effect was observed on clearance rates after Riley virus infection and after injection of RES blocking agents. The effect of Riley virus infection is probably caused by inhibition of plasma enzyme clearance by the RES system.

The RES system can be seen as a diffusely distributed tissue with protein catabolizing properties. The major portion of this tissue is represented by the Kupffer cells. Furthermore the RES consists to a significant degree of fixed macrophages, localized along the vascular bed of the liver, spleen and bone marrow.

There is now plenty of evidence that Kupffer cells are the ones responsible for the swift clearance of particulate matter or denatured protein from the circulation. As described above, blockade of this system also influences the clearance rate of part of the intracellular enzymes. Blockade of the RES does not decrease the rate of breakdown of screened ^{131}I -albumin or biosynthetic labeled plasma proteins (Schultze *et al.*, 1966).

The liver, which may be regarded as a concentrated form of RES plays

only a minor rôle in the catabolism of native albumin. Cohen *et al.* (1958) estimated that the liver accounted to as much as 12-18% of the albumin catabolism *in vivo*. Cohen *et al.* (1958) demonstrated that the liver catabolized screened ^{131}I - γG -immunoglobulin much more actively than screened albumin.

In the case of enzymes Strandjord *et al.* (1959) demonstrated that ICD and LD clearance patterns were not altered in hepatectomized dogs, as compared to control animals. Injection of the enzymes in the portal vein resulted in similar clearance rates as found for intravenous injections.

Roberts *et al.* (1975) demonstrated that in the dog CK clearance rate decreased with about 10% after hepatic artery occlusion.

The influence of RES blocking agents on the rate of breakdown of biologically filtered enzyme preparations has not been examined.

For CK Sobel *et al.* (1977) demonstrated that the rate of clearance of biologically filtered CK diminishes with 44% as compared to purified CK. Wevers *et al.* (1978) demonstrated post-synthetic changes in creatine kinase isoenzymes. An *in vivo* more stable MM-band could be detected at latest 16-24 hours after myocardial infarction. This more stable MM-band was formed in the course of myocardial infarction and appeared to be induced by a thermolabile factor.

Sodetz *et al.* (1977) demonstrated that zymosan only inhibits the rate of breakdown of aggregated or denatured Factor VIII/von Willebrand molecules and not of native Factor VIII/von Willebrand Factor.

From the literature available until now it can be concluded that the RES plays a rôle in the clearance of part of the intracellular tissue enzymes. However caution is needed because an influence of RES blocking agents on enzyme clearance from the circulation, can wrongly be suggested when the enzyme preparations contain aggregated or denatured molecules (II.6.).

II.5. Mechanism of enzyme clearance from the circulation

Clinical and experimental studies have established that the disappearance rates of circulating enzymes are relatively constant in a given species (CV 20%), but very little is known on the mechanism. In the previous section the sites at which protein catabolism takes place have been discussed, but most of these results give little information on the clearance mechanism.

In the dog the clearance pattern of ^{35}S and ^{14}C is equal in doubly labeled lysine ^{14}C + cystine ^{35}S fibrinogen, albumin, γ -globulin and α -globulin (Goldsworthy, 1958). The authors interpreted these results as an indication for protein degradation after complete removal of the protein from the circulation.

In a few studies the disappearance of radioactive labeled enzymes was measured simultaneous with the disappearance of enzyme activity (Schapira *et al.*, 1962; Masarat *et al.*, 1965; Roberts *et al.*, 1975 and Qureshi *et al.*, 1976). Masarat concluded that enzyme activity and radioactivity disappeared at comparable rates after AST injections in the pig. However in the second part of the clearance curve, the disappearance rate of ^{131}I was 10% slower than the disappearance rate of AST activity. Schapira *et al.* (1962) measured aldolase (ALD) activity and labeled ALD clearance after bolus injections in the rabbit and concluded that ALD was not inactivated in plasma. In the dog Roberts *et al.* (1975) found that during the first several hours after injection, radioactivity and enzyme activity of CK decline in parallel suggesting again that the removal of enzyme molecules from the circulation accounted for the disappearance of activity. After about 3 hours radioactivity began to decline at a significantly slower rate. The same results were obtained by Qureshi *et al.* (1976) for LD-5 in the rabbit.

However Morin *et al.* (1979) demonstrated that in 6 out of 7 patients the clearance of CK-MB, released into the circulation after myocardial infarction, is due primarily to the loss of activity and not to the physiological removal of MB protein from the circulation. These results were obtained from immunological measurements to detect MB protein in the circulation. During the release phase after AMI the MB activity curve and the MB protein curve ran in parallel. These results suggest either intravascular breakdown resulting in disappearance of the enzyme activity, while the radioactive label remains in the circulation, or degradation of the enzyme after removal from the circulation followed by release of labeled fragments into the circulation.

Demonstration that enzyme and label disappear simultaneous does not necessarily conflict with the view that circulating enzymes are denatured by circulating proteases (Posen, 1970), if the time interval between denaturation and removal of the enzyme from the circulation is small (II.6.).

The view that enzymes may be destroyed by circulating proteases is supported by the work of Henley *et al.* (1960) who observed that the Michaelis constant K_m of malate dehydrogenase increased progressively in successive samples taken from a patient with acute myocardial infarction. Smith *et al.* demonstrated however that these results are introduced because after myocardial infarction the relative amounts of mitochondrial MD and cytoplasmic MD are changing, as is introduced by differences in clearance rates of these isoenzymes. Gradual loss of substrate affinity was not confirmed by Wakim *et al.* (1963) who were unable to detect any change in K_m value for purified cAST in successive samples after a bolus injection in the dog. From above mentioned results it remains therefore unanswered whether inactivation of enzymes causes immediate clearance from the circulation or whether clearance from the circulation causes subsequent breakdown of the enzymes.

In a number of studies a possible relation between molecular weight, susceptibility to proteolytic attack, thermal stability of enzymes and *in vivo* clearance rates has been examined. Dehlinger and Schimke (1970) found that the intracellular turnover rate is related to the logarithm of the subunit molecular weight of proteins. A higher intracellular turnover rate was found for the larger proteins. This observation led these workers to propose that a relation between size of a protein and its rate of degradation is based on the overall greater chance of a protein to be hit by a protease, producing an initial rate limiting peptide bond cleavage. Dice and Goldberg (1975) made the same observation for a larger group of intracellular proteins.

From our results it has become apparent that in the dog CK, LD, AST, ALT and GPI clearance rates from the circulation are not related to the logarithm of the respective subunit molecular weights (chapter VII). The same results had already been obtained for ICD, LD and AST clearance rates in the dog (Strandjord *et al.*, 1959). Moreover for isoenzymes of the same molecular weight completely different clearance rates from the circulation have been measured, which makes the theory of Dehlinger *et al.* (1970) rather improbable for the clearance of enzymes from the circulation.

Bond *et al.* (1971) demonstrated that in rat liver, enzymes with a short *in vivo* intracellular turnover rate were especially vulnerable to proteolytic attack as measured by the loss of activity *in vitro*,

in contrast to the long lived enzymes *in vivo*, which were more resistant to proteolytic attack *in vitro*.

In chapter VII we demonstrated that there is no relation between *in vivo* clearance rates from the circulation and *in vitro* thermal stability of the enzymes.

However from above mentioned relations no important indications on the mechanism of enzyme clearance from the circulation have become apparent.

Until now the only well documented clearance mechanism is the rapid elimination of asialoproteins from the circulation. Morell *et al.* (1971) and Rijk *et al.* (1970) demonstrated that upon injection into rats all of the desialylated plasma proteins (ceruloplasmin, haptoglobin, α_2 -macroglobulin etc.) with the exception of transferrin were promptly removed from the circulation and were recovered in the liver. The removal of sialic acid residues provides a means by which defective molecules can be recognized and quickly catabolized by the liver. Asialoproteins bind to specific receptors on the liver parenchym cells, from which they could be recovered apparently intact. Homogenated liver tissue binds desialylated proteins with great avidity, whereas rat tissue of the kidney, heart and brain exhibits no affinity to the modified proteins.

Morell also demonstrated that the uptake of asialoproteins by the liver was not inhibited by fully sialylated proteins or their glycopeptides. Denatured albumin a potent agent of hepatic Kupffer cell uptake was without effect on the clearance of asialoproteins. Sodetz *et al.* (1976) demonstrated that infusion of asialo- α_1 -glycoprotein, a protein known to bind to hepatic asialoglycoprotein receptors, dramatically extended the intravascular survival time of asialo ^{125}I -Factor VIII/von Willebrand Factor.

Although it has not been demonstrated, whether the removal of sialic acid residues is of physiological significance, the presence of receptors specific for a group of proteins with common characteristics is a very attractive mechanism as compared to a separate clearance mechanism for each protein present in the circulation. The presence of such receptors specific for a group of enzymes with common characteristics are to be examined.

However the exact sites and mechanisms of enzyme clearance not necessarily have to be known for the quantitation of tissue damage from increased enzyme levels in plasma. In those studies it is essential

to obtain the clearance rate and the total distribution volume of the enzymes.

From studies on albumin turnover (Schultze *et al.*, 1966, II.2.) and enzyme clearance from the circulation (II.3.), it has been concluded that protein clearance from the circulation is a first order rate process and that the clearance is strictly regulated by the protein levels in plasma, *i.e.* there is no extravascular breakdown.

From intramuscular injections of enzyme activity we conclude that at least 75-85% of the enzyme activity injected in the muscle is released in plasma, (chapter VI). Enzyme release from muscle continued to up to 40-50 hours. Even for AST an enzyme with a half-life of 3 hours as estimated from plasma curves after intravenous injection of the activity, release in plasma after intramuscular injection continues to up to 40-50 hours. These results indicate that the extravascular breakdown rate or local inactivation rate must be extremely small as compared to the clearance rate of AST activity from the circulation.

Moreover immediate blockade of enzyme clearance as observed for several enzymes after injection of zymosan (II.5.) also suggests that enzyme clearance mainly occurs at sites along the vascular bed and not in the extravascular space.

II.6. Catabolism of damaged macromolecules

Early turnover studies, carried out with labeled proteins or with purified enzyme preparations have been misleading because it was not realized that even the slightest damage to a protein molecule may cause a complete alteration of its metabolic fate. In the present section special attention is therefore paid to the clearance of damaged macromolecules.

In most studies on the turnover of plasma proteins isotopically labeled proteins are used. The most frequently used labels are: ^{131}I , ^{15}N , ^{14}C and ^{35}S . The values reported in the literature for the biological half-life of albumin cover a wide range, depending in the first place on the type of label used (Schultze *et al.*, 1966). For biosynthetic labels ^{15}N , ^{14}C and ^{35}S slow clearance was found as compared to clearance of ^{131}I -labeled plasma proteins, suggesting that biosynthetic labeling causes less molecular damage. However one of the main problems concerning biosynthetic labels is reutilization

of the label, resulting in an underestimation of the half-life. For ^{131}I -labeled plasma proteins used in turnover studies it has been demonstrated that *in vitro* labeling procedures may induce subtle alterations in the protein molecule. Freeman (1959) and Berson *et al.* (1953) demonstrated that most of their lots of ^{131}I -labeled albumin, contained variable quantities of rapidly degrading components due to self-irradiation damage. The major components in the best lots were degraded at a rate of about 4% per day or less, which agrees with turnover values for albumin with biosynthetic labels. As a rule the true half-life of a plasma protein was assumed to be approximated by the longest values obtained for radioiodine and by the shortest values for the biosynthetic methods of labeling. For ^{131}I -albumin Freeman (1959) demonstrated that iodination of albumin should be minimal, not exceeding the ratio of one atom of iodine per molecule. The damage due to self-irradiation can be minimized by diluting the preparation with unlabeled protein.

To eliminate rapidly degrading fractions of damaged molecules the biological filter method was developed (McFalan, 1963). This involves intravenous injection and successive harvesting of the injected substance from plasma. These so-called biologically screened preparations are used for turnover studies. This method can however not be used for experiments in humans. Until now no simple method is available to detect subtle alterations in the stability and homogeneity of protein preparations used for *in vivo* turnover studies.

Several authors have attempted to characterize the alterations in the protein molecules that give rise to rapid clearance. Berson *et al.* (1953) and Rossing *et al.* (1967) demonstrated the relative insensitivity of electrophoretic, chromatographic and ultracentrifugal methods in the detection of alterations in albumin molecules. Only heavily labeled albumin gave some evidence of heterogeneity upon electrophoretic or ultracentrifugal analysis (Berson *et al.*, 1953 and Fahey *et al.*, 1958). Rossing *et al.* (1967) compared two different albumin preparations, one prepared by alcohol fractionation (SS-albumin) and the other prepared by salt fractionation (BW-albumin). These preparations were studied both *in vivo* and *in vitro*. Paper-, immuno-, moving boundary electrophoresis gel filtration and ultracentrifugation revealed that both preparations contained at least 90% of monomeric albumin. Simultaneous injection of SS- ^{125}I -albumin and BW- ^{131}I -albumin or SS- ^{131}I -albumin and BW- ^{125}I -albumin resulted in a higher

metabolic clearance rate and distribution volume of the SS-albumin ($p < .001$). These results appeared therefore to be independent of the label used, but to depend on the method of isolation of the albumin preparation.

The rapid clearance of partially damaged proteins as mentioned above can be seen as a protection of the organism against damaged or otherwise changed molecules. Rapid clearance from the circulation was also observed in several studies on the clearance of enzymes after bolus injection (table II). For alkaline phosphatase (Clubb, 1965), AST (Wakim *et al.*, 1963), LD-5 (Qureshi *et al.*, 1976) and human clotting factor IX respectively 11%, 13%, 31% and 41% disappeared immediately after injection of the enzyme preparations. For CK we demonstrated that disappearance of 10% of the activity, immediately after injection in the dog was caused by an inhibition of enzyme activity in plasma. As has been described in II.3. in several studies (Shell *et al.*, 1971; Sobel *et al.*, 1977; Boyd *et al.*, 1967 and Qureshi *et al.*, 1976) this rapid elimination of enzyme activity from the circulation has been interpreted erroneously as a distribution of enzyme over different compartments.

In chapter VII we demonstrate that the thermal inactivation rate of enzymes at higher temperatures is very sensitive to inhomogeneities and instabilities of enzyme preparations. Intra-individual variation in AST clearance rates appears to be greatly reduced when homogeneous enzyme preparations of the same thermal stability are used.

Bernacerraf *et al.* (1957) showed that when denatured proteins and carbon particles are administered simultaneously, there is preferential uptake of the protein and the rate of removal of the carbon from the blood is temporarily decreased. Freeman (1958) demonstrated that both in the living animal and in the perfused liver, carbon injections resulted in large reduction of the rate of uptake and breakdown of thermally denatured proteins.

Proteins subjected to various forms of denaturation are known to be catabolized by the reticuloendothelial system and predominantly in the Kupffer cells. When fluorescein-conjugated proteins were used, the dye was detected in the Kupffer cells only (Freeman, 1958). Gordon *et al.* (1957) also found a much higher catabolism for denatured ^{131}I -albumin by the perfused rat liver *in vitro*, as was found for undenatured biologically screened albumin. He showed that the

liver plays an important rôle in the clearance of partially denatured material; 80-100% of 1 mg of heat denatured albumin is cleared in the liver, whereas the liver accounts to as much as 12-18% to the catabolism of biologically screened albumin (Cohen *et al.*, 1958). It was also shown that blockade of the RES with carbon particles does not decrease the rate of breakdown of biologically filtered albumin although it significantly reduces the rate of breakdown of heat denatured albumin.

II.7. Relation between cell death and enzyme release

Permeability of the cell membrane is a necessary condition for the release of enzymes into plasma. In case of exo-enzymes, enzymes may be excreted actively. Contrasted with this is a passive release of enzyme. We shall be concerned with the passive release of enzyme. Under physiological conditions the cell membrane retains intracellular enzymes at high concentrations, while extracellular enzyme activities are extraordinarily low.

Not much is known on the mechanism of retention of macromolecules in the cell. It is likely to be a function of the size of the membrane pores and the charge of the membrane components on the one hand and of the protein on the other.

Maintenance of a physiologically intact membrane requires energy on part of the cell. Disturbances of the permeability of the cell membrane may be the result of an insufficient oxygen supply to the cell membrane. Henley *et al.* (1969) found that heavy muscular activity resulted in considerable changes in the distribution pattern of serum enzymes. The pattern resembled that of the enzyme distribution pattern of skeletal muscle. It appears that physiological anaerobiosis of skeletal muscle leads to a change of membrane permeability. It is presently unknown whether this change in permeability is reversible. Löhr *et al.* (1959) demonstrated a critical ADP/ATP ratio, above which the continuity of the cellular membrane can no longer be maintained and hemolysis occurs. Zierler (1958) demonstrated aldolase efflux from peroneus longus muscle in young rats in glucose free medium and due to anoxia. Aldolase efflux was also greatly increased in muscles that had been stimulated. The amount of aldolase appearing in the medium could also be increased by agents that alter the muscle metabolism; iodoacetate, dinitrophenol or cyanide. Dawson (1966 a,b) measured the rate of exit of seven glycolytic enzymes from chicken biceps

into physiological saline. It was found that the enzymes left the muscle at approximately the same rate in spite of the wide variations in molecular weight and tissue concentration.

Pathological conditions may damage the intact cell membrane reversibly or irreversibly. Irreversible damage to the cell is identical with cell death.

After an infarction, when the myocardium is deprived of both oxygen, and glycolytic substrates like glucose and glycogen, intracellular energy depletion and increasing levels of waste products will soon lead to severe cellular injury. This has been studied in a variety of cardiac preparations perfused with either deoxygenated blood (Bornet *et al.*, 1977) or with oxygen-free perfusates (Weissler, 1968; Hearse *et al.*, 1975 a,b; Ganote *et al.*, 1975, 1976). It has been shown that when the period of anoxia is terminated by perfusing a preparation with an oxygenated substrate-containing perfusate a paradoxical acceleration of cell damage occurs, so-called reperfusion damage.

For the quantitation of tissue damage from increased plasma enzyme levels it is important to know whether (1) there may be leakage of enzyme from cells which are not dying and (2) whether incomplete enzyme release from necrotic tissue or local inactivation, *i.e.* inactivation before the blood stream is reached occurs.

Van der Laarse (1979) investigated whether enzyme release reflects impairment of the cellular structure and function in rat heart cells in monolayer cultures. The authors concluded that the activity of HBD released from the culture is closely related with both functional damage (assessed by measuring depression of post anoxic frequency of steady-state beating) and structural damage. It was concluded that a transient period of anoxia lasting 3 hours or less causes necrosis of a number of cardiac cells in culture (irreversible injury), leaving the other cells still viable (reversible injury). After 16-20 hours cells had become totally depleted of enzyme.

Nordbeck *et al.* (1977) demonstrated that myocardial lymph under normal conditions shows a distribution of the LD and CK isoenzymes specific for heart muscle. Furthermore the concentrations of all determined enzymes in lymph are higher than in plasma, while the concentration of total protein, not only of globulin, but also of albumin in lymph is lower than in plasma. It was concluded that enzyme release is a normal physiological process. Hearse *et al.* (1979) concluded however that for the heart the onset of myocardial enzyme re-

lease indicates the onset of irreversible damage.
The above mentioned controversy is still unsolved.

II.8. Quantitation of myocardial tissue damage from increased plasma enzyme levels

In 1954 LaDue *et al.* reported that after myocardial infarction AST activity in human serum is increased. This observation has been confirmed and accepted by many authors. Further investigations showed that apart from the increase in AST activity other enzymes such as CK, HBD, LD, GPI and ALD also show increased levels in serum. This elevation of plasma (serum) enzyme activity can be ascribed to a release of enzyme from necrotic cells. A number of authors have demonstrated loss of enzyme from infarcted areas of cardiac muscle after coronary occlusion with simultaneous increase of enzyme levels in serum (Nydyck *et al.*, 1955; Strandjord *et al.*, 1959 and Kjekshus *et al.*, 1971). Several authors investigated the relation between enzyme release and infarct size. In these studies aspartate aminotransferase (AST) and lactate dehydrogenase (LD) were measured in dogs during the acute phase of experimentally induced myocardial infarction and the results were related to infarct size as assessed morphologically post-mortem (Agress *et al.*, 1955; Nydyck *et al.*, 1955 and Reugsegger *et al.*, 1959). In these studies a significant although not impressive correlation was found between peak levels of the enzymes in plasma and morphologically assessed infarct size. Comparable results were obtained in human studies (Kibe *et al.*, 1967; Meurman *et al.*, 1963 and Sjögren, 1970).

With the exception of one investigation (Meurman *et al.*, 1963) where clearance rates from the circulation were already taken into account, these studies also used only maximal serum activities and the fact that correlations were insufficient to allow accurate determination of infarct size in individual cases was attributed to such factors as insufficient sampling, differences in clearance rates and non cardiac sources of enzyme release.

Since the observation by Dreyfus *et al.* in 1960, that CK is elevated in serum of patients with acute myocardial infarction, attention in infarct diagnosis is mainly focussed on this enzyme, because it is predominantly found in myocardial and skeletal muscle and only negligible amounts are found in liver, kidney, lung and intestine. CK plays a rôle in the metabolism of high energy phosphate compounds in-

timately related to the process of muscle contraction. The specific isoenzyme CK-MB has become very important in the diagnosis of myocardial infarction. In man about 14% of total CK activity in heart tissue consists of this heart specific isoenzyme, whereas in dogs and baboons, animals frequently used in experimental studies on myocardial infarction, respectively 2% (chapter III) and 18% (Yasmineh *et al.*, 1975, 1976) of total CK consists of CK-MB.

Kjekshus *et al.* (1971) showed that myocardial CK activity is reduced in whole left ventricular homogenates of the rabbit heart within 24 hours after experimental coronary occlusion. Although depression of CK activity is linearly related to the proportion of infarcted tissue a considerable scatter was demonstrated. This may well reflect the difficulty in excising infarcted tissue, as judged by morphological changes. These results partly explain the rough correlations observed between enzymatically estimated infarct size and morphologically assessed infarct size (Roe *et al.*, 1975, 1977 and Jarmakani *et al.*, 1976).

Serum myosin light chains (Trahern *et al.*, 1978) and myoglobin (Sylvén *et al.*, 1978) have also been advocated as sensitive markers in the diagnosis of infarction.

The first study concerning quantitation of myocardial infarct size, where different compartments were taken into account was performed by Witteveen *et al.* (1970, 1972). The authors used a two-compartment model in which the change in enzyme activity is described in terms of release of enzyme from the infarcted area, intravascular and extravascular volumes, the diffusion constant governing the passage of enzymes between intravascular and extravascular space and the disappearance constant expressing the rate at which the enzyme is broken down (table IV). The model is based upon turnover studies in man. The total release of enzyme was expressed in grams of myocardial tissue and was calculated for CK, HBD and AST. Correlation between the amounts of enzyme are: $HBD/AST = 0.88$; $HBD/CK = 0.63$ and $AST:CK = 0.64$ (Witteveen *et al.*, 1972).

In experimental studies both poor (Jarmakani *et al.*, 1976 and Roe *et al.*, 1977) and good correlations (Bleifeld *et al.*, 1977) between the morphological assessment of infarct size and the calculated total release of CK in the circulation have been observed.

Estimation of infarct size strongly depend upon the specific model used and each parameter influences the final estimate of infarct

size. In the study by Roe *et al.* (1977) enzymatic infarct size (EIS) as calculated according to the model parameters by Shell *et al.* (1971), Norris *et al.* (1975) and Roberts *et al.* (1975) was related to histologically assessed infarct size (HIS) (see table III).

Table III Enzymatic estimation of infarct size, based on a one-compartment model.

Enzymatic Infarct size =

$$\frac{\int_0^t f(t)dt \cdot K_W \cdot BW}{CK_D \cdot K_R}$$

, $f(t) = \frac{dE}{dt} + k_d E$

recommended parameter values					
	k_d (h ⁻¹)	K_W	CK_D (U/gr)	K_R	species
Shell <i>et al.</i> , 1971	0.27	0.114	800	0.30	dog
Norris <i>et al.</i> , 1975	from curve	0.114	800	0.30	man
Roberts <i>et al.</i> , 1975	from curve	0.044	800	0.15	dog
Bleifeld <i>et al.</i> , 1977	0.06	0.05	1105	0.15	man

K_R : the ratio of the amount of enzyme entering the serum, compared to the amount of enzyme depleted from the myocardium.

K_W : the designation for the percentage of body weight (BW) in which enzymes are distributed. Therefore $\int_0^t f(t)dt \cdot K_W \cdot BW$ represents the total amount of enzyme in the distribution space ($K_W \cdot BW$).

k_d : apparent clearance constant (h⁻¹).

CK_D : the estimated amount of enzyme (U) depleted per gram of infarcted myocardium (U/gr).

E : enzyme activity (U/l).

Especially for the larger infarcts poor correlations were observed between EIS and HIS. It has however been demonstrated that CK shows accelerated denaturation in lymph (Robison *et al.*, 1975 and Clark *et al.*, 1978). Crains *et al.* (1978) found an inverse relation between the ratio of the amount of enzyme entering the plasma pool to the amount of enzyme depleted from the myocardium (K_R) and EIS. It was postulated that prolonged contact of CK with cardiac lymph during slow transport from the center of a large zone of homogeneous infarction might lead to extensive local inactivation of enzyme activity. Above mentioned results may clarify why in the study by Roe *et al.* (1977) better correlations between HIS and EIS were found for the smaller infarcts. This explanation is however in contradiction with the observation by Spieckermann *et al.* (1979) that only 15% of the enzyme is transported by lymph.

As for the other model parameters presented in table II, the model by Shell *et al.* (1971) is based upon bolus injections of CK preparations in dogs and consists of a one-compartment model with a total distribution volume ($K_W \cdot BW$), which is almost three times plasma volume. In the model by Norris *et al.* (1975) individualized clearance rates (k_d) were obtained from the final part of the CK clearance curve after myocardial infarction. For the distribution volume the same value as observed by Shell *et al.* (1971) was used.

The model by Roberts *et al.* (1975) was a one-compartment model including plasma volume only and individual clearance rates were obtained as described by Norris *et al.* (1975).

As stressed by Slutsky (1978), individually estimated values for k_d contain errors, because when CK appearance in the circulation is tailing, plasma CK activity *versus* time may still be mono-exponential. Determination of apparent CK clearance rates from this part of the curve will therefore result in an underestimation of CK clearance rate.

Willems *et al.* (1979a) demonstrated that release of enzyme from the infarcted area continues even to up to 96 hours and estimation of individual clearance rates of CK from the final part of the plasma curve results into large underestimations. The obtained value for CK clearance (table IV) was about 3 times higher than the clearance rate reported by Norris *et al.* and Bleifeld *et al.* (see table III).

Table IV Enzymatic estimation of infarct size, based on a two-compartment model.

	recommended parameter values			
	V_e/V_i	TER (%/h)	k_{CK} (h^{-1})	k_{HBD} (h^{-1})
Witteveen <i>et al.</i> , 1972	0.30	6.0	0.07 ± 0.0177	$0.015 \pm 0.0042^*$
Willems <i>et al.</i> , 1979	$0.80 \pm 0.5^*$	$1.7 \pm 1.1^*$	$0.20 \pm 0.12^*$	$0.015 \pm 0.066^*$

* mean $\pm$ SD

TER : transcapillary escape rate (%/h), also indicated as P/V_i .

V_e : extravascular volume.

V_i : intravascular volume.

k : clearance constant.

The method used by Willems *et al.* (1979a) was based upon a two-compartment model with a mean transcapillary escape rate, as estimated from the individual plasma curves of 1.7% per hour, which is a factor

3 lower than the value used by Witteveen *et al.* (1972). Willems *et al.* (1979b) also concluded that using HBD as a reference enzyme individual clearance constants of CK, AST and GPI can be obtained from the first 36 hours after infarction, when there is still release of enzyme into the circulation. The ratio of the released quantities of different enzymes agreed with heart tissue content. The released quantities of CK showed considerable variation in proportion to the other enzymes, as is in agreement with the bad results for CK in other studies (Roe *et al.*, 1977 and Craig *et al.*, 1978). The method of Willems *et al.* has thus far only been applied to patient curves after acute myocardial infarction. From the experiments presented in chapter VI; quantitation of continuous infusions, we validated that this method can indeed be used to obtain individual clearance rates resulting in an improvement of the quantitations.

Individual variations in enzyme content of normal myocardium.

In order to obtain the weight of injured tissue in grams, the calculated total activity of enzyme released into the circulation has to be divided by the mean enzyme activity per gram of normal tissue. Selection of enzymes with small intra- and inter-individual variations in tissue enzyme content will therefore minimize errors in the quantitation of tissue damage, due to the use of a mean value for the enzyme content per gram of normal tissue.

In chapter III we examine the distribution of several enzymes in heart and liver tissue of the dog and conclude that CK, LD and GPI can be used to quantitate heart tissue damage and ALT can be used to quantitate liver tissue damage. Variations for samples taken from different sites of the same organ are less than 5-7%. Inter-individual variations in enzyme activity are less than 10% for these enzymes. Comparable results have been obtained from other studies, although in these studies only a limited amount of data was used (Shell *et al.*, 1971; Witteveen 1972; Witteveen *et al.*, and Hermens *et al.*, 1979). Further details on this subject will be given in chapter III.

Factors that prevent enzymes from reaching the blood stream.

Incomplete recovery of enzymes from necrotic tissue in blood can be caused by incomplete cellular release or by local denaturation,

i.e. inactivation before the blood stream is reached. Localization in organelles or adsorption to local structures can retard release of enzymes and enhance the possibility of local denaturation. For instance it has been shown in rat myocardium that mitochondrial CK was not released into the circulation after hypoxic damage (Baba *et al.*, 1976) and the same phenomenon was demonstrated for mitochondrial AST after infarction in dogs (Malmberg, 1973). Even cytoplasmic enzymes undergo a time delay in their release into the blood, caused by impairment of the local circulation and this increases the possibility of local denaturation (Clark *et al.*, 1978).

From studies on cultures of beating rat heart cells (Van der Laarse *et al.*, 1975 and 1978) it has been concluded that enzyme release from myoblasts in these cultures started about 3 hours after anoxia and lasted for 16-20 hours, after which time the cells had become totally depleted of enzyme. Release of CK and HBD after anoxia in rat heart cell cultures runs completely parallel ($r=0.996$). However CK was inactivated much more quickly than was HBD.

Studies on experimental infarction in dogs have shown that although cardiac lymph obtained directly after experimental infarction showed the same enzyme composition as cytoplasmic heart extracts (Malmberg, 1973), CK shows accelerated denaturation upon *in vitro* incubation of dog lymph in contrast to HBD (Robinson, 1975), while interruption of cardiac lymph flow resulted into significantly less CK appearing in plasma (Clark *et al.*, 1978). These data are supported by a study on *in vitro* enzyme release from human myocardium (Hermens *et al.*, 1975), showing that AST, CK and HBD are released in quantities representing 35%, 43% and 82% respectively of the amounts originally present. Only for HBD could the remaining 18% be recovered in the residual tissue. For AST and CK only 33% and 16% could be recovered in the tissue, implying that 32% and 42% were respectively inactivated locally. This shows that HBD is remarkably stable compared to AST and CK.

Bleifeld *et al.* (1977) demonstrated that 15% of CK depleted from the myocardium reaches the circulation. Taking however parameter values as estimated by Willems *et al.* (1979a), 50% of the quantity of both CK and HBD is recovered in the plasma of patients (Table IV).

Rapid washout of enzymes, as is characteristic for reperfusion, leaves no time for local denaturation. This rapid washout should therefore cause a marked increase in the total amount of CK released. Such an increase should become apparent by comparing the total amount of CK released to the total release of HBD, which is not subject to

local denaturation. This was tested by comparing patients with acute myocardial infarction with patients undergoing heart surgery. As skeletal muscle damage is present in the surgery group the specific isoenzyme CK-MB was compared to HBD. Determination of the linear regression equations between calculated amounts of CK-MB and HBD gave the following ratios: Infarction group 0.27 ± 0.08 (n=17) and in the surgery group 0.23 ± 0.03 (n=32), indicating that no significant inactivation of CK-MB occurs (Hermens *et al.*, 1979).

From *in vivo* experiments in dogs (Roberts *et al.*, 1975) it was calculated that 24 hours after myocardial infarction 11% of CK activity originally present in the infarcted tissue had reached the blood stream, while 26% remained in the myocardium. This implies that 63% of the original CK activity was not recovered. Using a different model to study enzyme release in patients with acute myocardial infarction (Witteveen, 1972) it was calculated that AST, CK and HBD are released in quantities representing 25%, 45% and 80% respectively of the amounts originally present in the damaged area. Considering the discrepancies between *in vitro* and *in vivo* recovery of CK, one has to keep in mind that *in vivo* results strongly depend upon the parameter values of the circulatory model used, such as the magnitude of the extravascular pool and the rate of exchange of enzyme between the vascular and extravascular space (Hermens, 1975).

From the results presented by Hermens *et al.* (1979) and Willems *et al.* (1979) and from the experiments presented in chapter VI it can be concluded that in order to minimize errors in calculations of enzymatic infarct size, one should use enzymes that are slowly metabolized and show a complete washout from the infarcted tissue. In both respects HBD is to be preferred to CK for quantitation of myocardial infarction in man.

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CHAPTER III

Enzyme distribution in dog heart and liver

Summary

In studies on the quantitation of tissue damage from increased plasma enzyme levels it has been assumed that enzymes are homogeneously distributed throughout the tissue and that the inter-individual^{*} variation in enzyme content of the tissue is small. In this study we checked the validity of these assumptions by examining the distribution of several enzymes throughout heart and liver tissue of the dog. This species is commonly used in experimental models to study quantitation of tissue damage by means of increased plasma enzyme levels.

In liver tissue alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glucosephosphate isomerase (GPI) activities, expressed in units per gram wet tissue, were determined in samples taken from different liver lobes. Intra-assay variations^{*} in enzyme measurements are within 5%. The enzymes are almost homogeneously distributed throughout the liver and the intra-individual^{*} variations were less than 6% for all three enzymes. For AST a 5% higher activity was observed in the left liver lobes, as compared to the activity in the right liver lobes ($p < .01$).

In heart tissue creatine kinase (CK), lactate dehydrogenase (LD), AST and GPI activities have been determined in samples taken from various sites of the left ventricle, right ventricle and septum. In the left ventricle and septum transmural samples were taken. We found a significant transmural CK gradient in the left ventricle, with a 10% higher CK activity at the epicardial side of the left ventricle wall as compared to the activity at the endocardial side ($p < .0001$).

^{*}Coefficient of variation (CV): standard deviation expressed as a percentage of the mean.

Intra-assay variation	: coefficient of variation for enzyme measurements on the same day.
Inter-assay variation	: coefficient of variation for enzyme measurements on different days.
Intra-individual variation	: coefficient of variation for samples taken from different sites of the same organ.
Inter-individual variation	: coefficient of variation for samples taken from different organs.

In the septum a 5% higher LD activity ($p < .01$) and in the left ventricle a 5% higher AST activity ($p < .02$) was found, as compared to the activity in the right ventricle.

Inter-individual variations of 7.0%, 7.6%, 12.6% and 5.7% were respectively found for CK, LD, AST and GPI activities of the dog heart.

We conclude that CK, LD and GPI are suitable for the quantitation of heart tissue damage and ALT can be used to quantitate liver tissue damage. The use of these enzymes for quantitation of tissue damage will introduce errors of less than 10%.

Introduction

Normally only very small amounts of intracellular tissue enzymes are present in plasma. This normal steady state plasma enzyme concentration is assumed to be related to the normal tissue breakdown. After tissue injury the intracellular enzymes are released into the circulation and a qualitative indication of tissue damage can be obtained from tissue specific enzyme or isoenzyme elevations in plasma or from typical enzyme ratios (Schmidt *et al.*, 1970 and Wilkinson *et al.*, 1970).

A quantitative assessment of tissue damage is based on the plasma enzyme pattern after acute tissue injuries like for instance myocardial infarction (Shell *et al.*, 1971; Witteveen *et al.*, 1975 and Hermens *et al.*, 1979). In these studies the total activity of enzyme released from necrotic tissue into the circulation was calculated. In order to obtain the weight of injured tissue the calculated total activity of enzyme released was divided by the mean enzyme activity per gram of normal tissue. In addition the enzyme activity remaining in the tissue and the locally inactivated activity should be known. The amount of enzyme activity remaining in the tissue has been estimated from experimental studies in the rabbit and the dog (Kjekshus *et al.*, 1970 and Shell *et al.*, 1971). In such computation of tissue damage, using the mean enzyme activity for a specific organ, it is assumed that inter- and intra-individual variations in enzyme activity of the tissue are small.

In human and porcine hearts it has been demonstrated that variations in CK, HBD (α -hydroxybutyrate dehydrogenase), AST and GPI activities, in samples taken from the same heart are small. Inter-individual variations of 7-13% were observed for these enzymes (Witteveen,

1972; Van der Laarse *et al.*, 1980 and Hermens, 1979).

In studies on the local metabolism of the ventricles and the septum of the heart significant transmural gradients in enzyme activity have been observed for 4 out of 8 glycolytic enzymes (Lundsgaard-Hansen *et al.*, 1967), phosphorylase (Jedeikin *et al.*, 1964), succinate cytochrome c reductase (Tota *et al.*, 1973) and citrate synthase (Dowell *et al.*, 1978).

The aim of the present study is to analyze the homogeneity in enzyme activity throughout dog heart and dog liver and to estimate inter-individual variations in enzyme activity of dog heart and liver tissue.

These questions are relevant to the selection of enzymes suitable for the quantitation of acute heart or liver damage from increased plasma enzyme levels. Dog heart and dog liver were taken, because dogs are commonly used in experimental models to study the quantitation of myocardial infarction by means of plasma enzyme levels (Shell *et al.*, 1971; Roberts *et al.*, 1975; Roe *et al.*, 1977 and Sobel *et al.*, 1977).

Materials and methods

Mongrel dogs of either sex, unknown age and ranging in weight from 15 to 35 kg were used in all experiments. Dogs were killed and

Fig. I

RL = right lobe
LL = left lobe
CL = caudate lobe
QL = quadrate lobe.

heart and liver were rapidly excised and hereafter rinsed in saline. Samples ranging in weight from 200-400 mg, were taken from different regions of the liver; 4 samples from the right lobe (lobus dexter), two samples from the left lobe (lobus sinister), two samples from the quadrate lobe (lobus quadratus) and two samples from the caudate lobe (lobus caudatus) (fig. I).

For the heart, three left ventricle samples, taken from apex to base were cut transmurally and each sample was divided in an epicardial, a middle and an endocardial sample. Three transmural samples were also taken from the septum and divided in an endocardial sample at the left ventricle side, a sample from the middle and an endocardial sample at the right ventricle side. For the right ventricle three adjacent transmural samples were taken at three different regions of the right ventricle. The right ventricle wall was mostly too thin to be accurately divided in an epicardial, middle and an endocardial sample (Fig. II).

Fig. II

- LV1 = left ventricle epi
- LV2 = left ventricle middle
- LV3 = left ventricle endo
- SE1 = septum left
- SE2 = septum middle
- SE3 = septum right
- RV = right ventricle

All samples were homogenized in 15 ml pasteurized plasma protein solution with a colloid osmotic pressure equivalent to that of plasma (albumin 85 gr/l, glucose 30 gr/l, Na^+ 1.3-1.6 gr/l and K^+ < 2 mmol/l). Homogenization occurred in 8 bursts of 4 seconds and hereafter the

homogenized sample was sonicated in 8 bursts of 15 seconds. Sonication was performed using a MSE Mark II 150-Watt disintegrator set at 12 microns peak to peak amplitude. During homogenization and sonication the tubes were kept in ice. The supernatant fraction was immediately frozen at -70°C until determination.

All enzyme determinations were carried out in duplicate in an Eppendorf spectrophotometer and enzyme activities are expressed in units per litre (U/l). One unit converts one micromole of substrate per minute under the given conditions. For CK and AST the Technicon Auto-analyzer continuous flow system was also used.

With the Eppendorf spectrophotometer the change in extinction caused by the appearance or disappearance of the reduced form of either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) was measured over a fixed period of time at a wavelength of 334 nm. NADH or NADPH disappeared or was formed in the reaction catalyzed by the enzyme, which was measured as such or in a reaction coupled to it. The change in extinction was measured at a constant temperature ($25.0 \pm 0.05^{\circ}\text{C}$) in a Sarstedt disposable cuvette with a light path of one cm and the enzyme activity in U/l was calculated using the following formula:

$$\text{U/l} = \frac{\Delta E/\text{min} \times V \times 10^3}{\epsilon \times v \times d} = \Delta E/\text{min} \times F$$

$\Delta E/\text{min}$ = change in extinction in one minute

V = total test volume (ml)

ϵ = extinction coefficient of NADH or NADPH at 334
millimicron : $6.18 \times 10^6 \text{ cm}^2/\text{mole}$

v = volume of sample in cuvette (ml)

d = light path of cuvette (cm)

Enzyme activities in tissue homogenates are expressed in units per gram wet tissue.

The following enzymes were determined:

1. LACTATE DEHYDROGENASE (LD) (EC 1.1.1.27)

This enzyme catalyses the reaction:

The rate of disappearance of NADH, measured as a diminution of extinction is proportional to the LD activity of the reaction mixture ($F=5097$). Boehringer test kits (124893) based on the method of Wroblewski and LaDue (1955) were used.

2. CREATINE KINASE (CK) (EC 2.7.3.2)

This enzyme catalyses the reversible transfer of a phosphate group:

The reaction is carried out at 37°, using the Technicon Autoanalyzer continuous flow system, according to the method of Siegel *et al.* (1967). It is a colorimetric method dependent upon the color complex formed by creatine with diacetyl-orcinol. The colored solution is pumped through the flow cell colorimeter fitted with 520 nm filters. This method, based on direct assay of creatine is not generally used, therefore the first two hearts were assayed both on the Eppendorf and Autoanalyzer.

For a spectrophotometric determination the first reaction is coupled to an indicator reaction catalyzed by hexokinase (HK) (EC 2.7.1.1):

which is then coupled to a reaction catalyzed by glucose 6-phosphate dehydrogenase (G6P-D) (EC 1.1.1.49)

Baker test kits (3107 and 3108), based on the method of Oliver and modified by Rosalki (1967) were used (F = 3733).

The conversion factor for CK activity measured on the Eppendorf to CK activity measured on the Autoanalyzer is:

$\text{CK (Autoanalyzer, 37}^\circ\text{C)} / \text{CK (Eppendorf, 25}^\circ\text{C)} = 3.13 \pm 0.08$ (mean $\pm$ SD, $n = 54$).

This conversion factor is obtained by measuring the first two hearts both on Eppendorf and Autoanalyzer.

3. GLUCOSEPHOSPHATE ISOMERASE (GPI) (EC 5.3.1.9)

The following reaction is catalyzed:

and is coupled to a reaction catalyzed by glucose 6-phosphate dehydrogenase

Sigma test kits (355A,B) based on the method of Bueding *et al.* (1955) were used ($F = 5016$).

4. ALANINE AMINOTRANSFERASE (ALT) (EC 2.6.1.2)

This enzyme catalyses the reaction:

and is coupled to a reaction catalyzed by LD:

Boehringer test kits (124559) based on the reaction conditions of Wroblewski *et al.* (1955) were used ($F = 1197$).

5. ASPARTATE AMINOTRANSFERASE (AST) (EC 2.6.1.1)

The reaction catalyzed is:

The activity of AST is measured by coupling it to the following reaction catalyzed by malate dehydrogenase (MD) (EC 1.1.1.37)

On the Eppendorf spectrophotometer Boehringer test kits based on the reaction conditions of Karmen were used (1955) ($F = 1197$).

In chapter V AST determinations have been performed on the Auto-analyzer using a procedure modified by Kessler *et al.* (1971). In this procedure the same reactions as described for the spectrophotometric method were used and the change in absorbance at 340 nm, caused by the disappearance of NADH is measured at 37°.

The conversion factor for AST activity measured on the Eppendorf to AST activity measured on the Autoanalyzer is:

$\text{AST (Autoanalyzer, 37}^\circ\text{C) / AST (Eppendorf, 25}^\circ\text{C)} = 2.25 \pm 0.12$ (mean $\pm$ SD, $n = 25$).

Isoenzyme separation of CK and LD was performed with a column chromatographic method based on stepwise elution of isoenzymes from the column (Mercer, 1975). For CK $98 \pm 0.4\%$ (mean $\pm$ SD, $n = 8$) of the total activity in dog heart consisted of the muscle type isoenzyme CK-MM. The analytical recovery of CK from the column was: $99.3 \pm 3.0\%$ (mean $\pm$ SD, $n = 8$).

For LD $5.8 \pm 0.4\%$ (mean $\pm$ SD, $n = 8$) consisted to the isoenzymes LD-3,4,5. The analytical recovery of LD activity from the column was: $98 \pm 2.1\%$ (mean $\pm$ SD, $n = 8$).

For separation of AST isoenzymes, i.e. cytoplasmic AST (cAST) and

mitochondrial AST (mAST), a column chromatographic method of Sampson (1978) was used. The analytical recovery of AST activity from the column was: $97.4 \pm 7.6\%$ (mean $\pm$ SD, $n = 32$). In dog liver and dog heart resp. $54.2 \pm 4.5\%$ ($n = 5$) and $54 \pm 3\%$ (mean $\pm$ SD, $n = 3$) of the activity consisted of mAST.

In order to standardize our enzyme determinations, a standard was prepared by homogenizing about 1 gram of dog left ventricle in 2 litre pasteurized plasma protein solution. After centrifugation in a JA-20 rotor at 30,000 g for one hour, the supernatant fraction was divided in small aliquots of 1 ml and frozen at -70°C .

For ALT the standard was obtained from dog liver on a similar way as described for the standard obtained from dog heart.

Intra-assay variations of the enzyme measurements were obtained by measuring 5 aliquots of this standard on the same day, under the same conditions. This procedure was repeated on 5 different days. For CK, LD, AST, GPI and ALT mean intra-assay variations of resp. 3.9%, 2.8%, 2.2%, 2.4% and 2.4% were found.

Inter-assay variations of the enzyme measurements were obtained by measuring 2 aliquots of the standard on 5 different days during one month.

Inter-assay variations of CK, LD, AST, GPI and ALT are respectively 8.1%, 3.7%, 2.9%, 6.6% and 4.1%.

Each day at least two different aliquots of the standard were measured together with all other samples in order to correct all our measurements for inter-assay variations. This implies that the inter-individual variations, calculated from mean enzyme activities of all samples of the same organ do not contain inter-assay variations. For each enzyme, activities from one and the same heart were determined on the same day.

All enzyme activity determinations of dog heart and liver were performed within 6 months. During this time no significant decline of the enzyme activity of the standard was observed.

Statistical significance was judged from a paired student t test.

Results

Distribution of enzymes in dog liver

The mean AST, ALT and GPI activities of 10 different dog livers are given in table I,1. For complete data see appendix I, table I.

Table I.1 Mean values of enzyme activities of samples taken from 10 different dog livers.

In each liver samples were taken from: right lobes, n = 4 (RL),
left lobes, n = 2 (LL),
caudate lobe, n = 2 (CL) and
quadrate lobe, n = 2 (QL).

Sample site	ALT activity		AST activity		GPI activity	
	mean U/gr	CV %	mean U/gr	CV %	mean U/gr	CV %
RL	88.2	10.4	110.2	11.7	113.2	17.4
LL	91.0	12.0	115.2	13.3	115.2	19.6
QL	87.2	10.8	110.5	13.6	111.3	16.4
CL	86.7	12.0	109.9	11.4	112.6	15.8
Mean*	88.3	10.4*	111.1	12.5	113.1	17.5

* Inter-individual variation of the mean enzyme activity of different livers.

The only significant difference found is a 5% higher AST activity in the left liver lobes ($p < .01$). For sample sites as used in our study the intra-individual variations for AST, ALT and GPI activities were respectively 4.7%, 5.7% and 5.7%. These values were determined by averaging the intra-individual variations of all livers.

Inter-individual variations of respectively 12.5%, 10.4% and 17.3% were found for the mean AST, ALT and GPI activities of different livers.

Table I.2 presents intra- and inter-assay variations of measured enzyme activities and intra- and inter-individual variations for enzyme activities of liver tissue.

Table I.2 Intra- and inter-assay variations of measured activities and intra- and inter-individual variations of enzyme activities of dog liver.

Enzyme	intra-assay variation (%)	inter-assay variation (%)	intra-individual variation (%)	inter-individual variation (%)
ALT	2.4	4.1	5.7	10.4
AST	2.2	2.9	4.7	12.0
GPI	2.4	6.6	5.7	17.3

Variation coefficients for enzyme ratios in samples taken from the same liver and inter-individual variations in mean enzyme ratios are presented in table II.

Table II Variations in enzyme content ratios of dog liver.

Enzyme ratio	mean	intra-individual variation	inter-individual variation
AST/ALT	1.28	4.1	15.8
AST/GPI	1.01	5.4	23.7
ALT/GPI	0.80	5.4	19.8

Intra-individual variations were obtained by averaging intra-individual variations of all livers.

Inter-individual variations were obtained from mean enzyme ratios of different livers.

Distribution of enzyme activity in dog heart

The mean enzyme activities of 10 different dog hearts are presented in table III.1. For completa data see appendix I; tables II, III, IV, V.

Table III.1 Mean values of enzyme activities of samples taken from 10 different dog hearts.

In each heart samples were taken from: left ventricle, n = 9 (LV), septum, n = 9 (SE) and right ventricle, n = 9 (RV).

Sample site	LD activity		CK activity		AST activity		GPI activity	
	mean U/gr	CV %	mean U/gr	CV %	mean U/gr	CV %	mean U/gr	CV %
LV-epi	311	8.5	3733	5.4	177	15.2	160	4.4
LV-mid	315	6.6	3476	7.6	175	15.2	159	7.9
LV-endo	312	7.0	3407	7.3	179	13.6	161	8.2
SE-left	328	7.9	3548	8.5	179	13.0	170	8.9
SE-mid	321	10.8	3472	8.5	177	14.0	166	7.3
SE-right	318	10.1	3551	8.5	180	12.3	165	5.4
RV	306	7.9	3689	9.0	187	10.4	167	6.6
Mean ^x	313	7.6 ^x	3583	7.0	181	12.6	165	5.7

^x Inter-individual variation of the mean enzyme activity of different livers.

A significant transmural CK gradient has been observed in the left ventricle with a 10% higher CK activity at the epicardial side ($p < .0001$), as compared to CK activity at the endocardial side. For LD a 5% higher activity in the septum and for AST a 5% higher activity in the left ventricle, as compared to their activities in the

right ventricle have been observed.

Intra-individual variations of 6.8%, 5.2%, 6.8% and 6.2% were respectively found for CK, LD, AST and GPI activities of the dog heart. These values were also obtained by averaging the intra-individual variations of all hearts. It should be noted that intra-individual variations in this case depend on the specific sample scheme.

Inter-individual variations for the mean CK, LD, AST and GPI activities of different dog hearts were respectively 7.0%, 7.6%, 12.6% and 5.7%.

Table III.2 presents intra- and inter-assay variations for the determination of enzyme activity and intra- and inter-individual variations for the enzyme activity of dog heart.

Table III.2 Intra- and inter-assay variations of measured activities and intra- and inter-individual variations of enzyme activities of dog heart.

Enzyme	intra-assay variation (%)	inter-assay variation (%)	intra-individual variation (%)	inter-individual variation (%)
LD	3.9	8.1	5.2	7.0
CK	2.8	2.9	6.8	7.6
AST	2.2	3.7	6.8	12.6
GPI	2.4	6.6	6.2	5.7

Variation coefficients for the enzyme ratios in samples taken from the same heart and inter-individual variations in enzyme ratios are presented in table IV.

Table IV Variations in enzyme content ratios of dog heart.

Enzyme ratio	mean	intra-individual variation	inter-individual variation
CK/LD	11.3	4.9	9.7
CK/AST	19.8	3.1	12.3
CK/GPI	21.7	3.9	8.6
LD/AST	1.76	2.9	18.2
LD/GPI	1.93	2.4	12.9
GPI/AST	0.92	2.4	11.0

Intra-individual variations were obtained by averaging intra-individual variations of all organs.

Inter-individual variations were obtained from mean enzyme ratios of different organs.

Discussion

In order to estimate heart or liver tissue damage in grams of tissue, from increased plasma enzyme levels, the mean enzyme activity expressed in units per gram of normal tissue should be known. To make a reliable estimation possible intra- and inter-individual variations in enzyme activity per gram of normal tissue should be small. When these requirements are fulfilled the mean enzyme activity, as estimated from different hearts or from different livers can be used to quantitate tissue damage.

In human left and right ventricles (Van der Laarse *et al.*, 1980) and in porcine left ventricle (Hermens, 1979) the intra-individual variations in CK, HBD (α -hydroxybutyrate dehydrogenase) and AST activities per gram wet tissue are small.

In the dog we conclude that the intra-individual variations in CK, LD, AST and GPI activities in 27 samples taken from various sites of the same heart are less than 6.8% for all four enzymes.

In the left ventricle and septum the existence of transmural gradients in enzyme activity was investigated. A significant transmural gradient was only observed for CK. CK activity is 10% higher at the epicardial side as compared to the activity at the endocardial side of the left ventricle wall, whereas in the septum no transmural gradient in CK activity was observed. After myocardial infarction tissue necrosis seldomly remains confined to the epicardial side of the left ventricle wall. The intra-individual variation in CK activity estimated from 27 samples taken throughout the dog heart was only 6.8% and therefore this transmural gradient in CK activity as observed in the left ventricle wall will not disturb the quantitation of the myocardial tissue damage as described above.

The epicardial side of the left ventricle wall has the potential to generate ATP at a 10% higher rate than the endocardial side. It remains to be seen whether the observed gradient in left ventricle CK content is of physiological significance, *i.e.* that the CK content of normal heart muscle is rate limiting in the generation of ATP during exercise.

The myocardial CK content of 3600 U/l implies a maximal rate of ATP turnover of 3.6 mmol/min/gr. Assuming that ATP transport from the mitochondria to the myofibrils occurs only by creatine phosphate, in steady state conditions CK catalyses the forward reaction at the same

rate as the backward reaction, so net consumption of ATP could be maximally 1.8 mmol/min/gr. In rest ATP consumption as computed from O_2 consumption is 0.015 mmol ATP/min/gr (Personal communication G. v.d. Vusse).

During exercise myocardial O_2 -consumption increases by a factor of about 5 (Restorff *et al.*, 1977). Therefore about 20 times as much ATP as is consumed can be supplied by CK during exercise.

At first sight the extra potential of the epicardium to generate ATP seems contradictory to the supposed higher energy demand of the endocardium.

However, from model studies it has been inferred that both epi- and endocardial side of the heart perform the same amount of work (T. Arts, 1978).

Lundsgaard-Hanssen *et al.*, 1967 measured transmural gradients for the glycolytic enzymes in the left ventricle of the dog heart. A significant transmural gradient was observed for 4 out of 8 glycolytic enzymes. The authors reported extremely large variations in enzyme activities of the dog heart. The variations were caused, for a considerable part by the inherent scatter of the assay methods available at that time. Their data are in striking contrast with all more recent studies on enzyme activity determinations per gram wet tissue. For the glycolytic enzymes we studied LD and GPI no transmural gradients were found in the dog left ventricle and septum. Dowell *et al.*, 1978 found no transmural gradient in LD activity in samples taken from the same heart, whereas for citrate dehydrogenase a 10% higher activity was observed at the epicardial side of the left ventricle wall.

For CK, LD and GPI both intra- and inter-individual variations, corrected for intra-assay variations are small and allow quantitation of heart tissue damage using mean values for enzyme content per gram heart tissue.

Variations in enzyme ratios are in accordance with the assumption of uncorrelated variation in the enzyme content of heart tissue. A higher activity of one of the enzymes not necessarily implies a higher activity for the other enzymes.

For the enzymes AST, CK, HBD and GPI Witteveen (1972) reported variation coefficients of 14%, 14%, 13% and 6% between rat hearts and Hermens (1979) reported variation coefficients of respectively 7.4%, 12.5% and 10.2% for AST, CK and HBD activity between porcine left

ventricles. In samples obtained from 15 patients at thoracic surgery coefficients of variation of 19%, 10%, 11% and 10% were found for AST, CK, HBD and GPI activities (Witteveen 1972). Large variations as found for AST activity were caused by insufficient homogenization, resulting in incomplete release of AST from the mitochondria. In our study total AST activity was determined after homogenization, sonication and freezing, in order to release all cytoplasmic (cAST) and mitochondrial AST (mAST) present in the cell. Hereafter a coefficient of variation of 6.2% was observed as is in agreement with variation coefficients of cytoplasmic enzymes, like CK, LD and GPI.

After acute myocardial infarction very few mitochondrial AST is released into the circulation. Therefore to quantitate tissue damage from the total AST activity released into the circulation, the amount of cytoplasmic AST per gram tissue should be known. Cytoplasmic AST content in the dog heart is: $46 \pm 5\%$ (mean $\pm$ SD, $n = 4$) of total AST activity. In human heart tissue Van der Laarse *et al.* (1980) demonstrated that differences in enzyme and isoenzyme activity in different regions of the same heart are minimal and inter-individual variations in enzyme and isoenzyme content per gram wet tissue vary from 10-20%. Variations in isoenzyme activity were somewhat larger, because of the inherent larger experimental errors of column chromatographic and electrophoretic separation techniques as compared to the simple spectrophotometric assay of total enzyme activity. In our study isoenzyme contents have not been studied in all samples. For CK 98% of total activity in the dog heart is of muscle type (CK-MM). For LD 5.8% of total activity is of heat labile type LD-3,4,5. LD-1 and LD-2 have not been separated in this study.

ALT, AST and GPI contents in samples taken from different regions of the same liver were rather constant, coefficients of variation were less than 5.7%. Comparable results have been obtained for AST and ALT in samples from the human liver (Schmidt *et al.*, 1970b). Liver tissue contains completely different cell types, hepatocytes and Kupffer cells. The small variations in enzyme activity throughout liver tissue indicate that these different cell types are homogeneously distributed in liver tissue.

We observed that in the dog inter-individual variations in enzyme activities of liver tissue are somewhat larger than inter-individual variations in enzyme activities of different hearts. Especially for GPI activity in liver tissue large variations have been observed

(17%). Therefore this enzyme is not suitable for quantitation purposes.

Until now increases in plasma of intracellular liver enzymes are commonly used as a qualitative indication of liver damage (Schmidt *et al.*, 1970). We conclude that ALT can be used for a quantitative assessment of liver tissue damage, using a mean value for the ALT content per gram wet tissue.

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CHAPTER IV

Plasma volume determination by use of enzyme dilution

Summary

In this study the use of enzymes for plasma volume measurements has been examined. In dogs plasma volume was determined from dilution of intravenously injected enzymes. A comparative study with high molecular weight fluorescein isothiocyanate labeled dextrans (FITC-dextrans) was used as an independent check on the validity of these plasma volume measurements. For simultaneous plasma volume measurements with aspartate aminotransferase (AST) and alanine aminotransferase (ALT), obtained from liver tissue, no significant differences were found. For ALT a single sample point, taken within ten minutes after intravenous injection of the enzyme preparation, is sufficient for an accurate plasma volume determination. Simultaneous plasma volume measurements with creatine kinase (CK), lactate dehydrogenase (LD) and AST resulted in a significantly higher plasma volume for both CK and LD as compared to AST. This overestimation in plasma volume determinations, as obtained from CK and LD dilution can be explained by inhibition of enzyme activity in plasma. Thus CK and LD can only be used when the amount of injected activity is determined from *in vitro* dilution of the enzyme preparation in plasma of the same dog.

It is concluded that plasma volume can be determined with less than 0.045 mg enzyme per kg of body weight. This method offers an attractive alternative to methods presently used, especially because no radioactive isotopes are needed.

Introduction

All current methods used for plasma volume determination are based on the dilution principle. Dilution of Evans Blue dye (T-1824) and especially of ^{131}I -labeled albumin have become widely accepted as measures for plasma volume determinations. Both methods use tagging of albumin and plasma volume is estimated from the dilution immediately after injection. Comparative studies have however lead to conflicting results. Several authors suggested that T-1824 dilution results in an overestimation of the plasma volume, because part of the injected dye escapes from the vascular system before binding to albu-

min has occurred (Krieger *et al.*, 1948; Storaasli *et al.*, 1950 and Aust *et al.*, 1951). Especially in the rabbit and the rat T-1824 is less firmly bound to plasma proteins (Allen *et al.*, 1953 and Zizza *et al.*, 1958). Other authors, however, did not find significant differences between the two methods (Freinkel *et al.*, 1953 and Schultz *et al.*, 1953). It has also been shown that both hemolysis and turbidity of the plasma samples interfere with the dye method (Gregerson *et al.*, 1958).

The use of labeled albumin has also been criticized by several authors, who demonstrated the presence of labile fractions in their preparations, caused by self-irradiation damage (Yalow *et al.*, 1957; Bloom *et al.*, 1958 and Freeman, 1959). As a result several authors advocated the use of high molecular weight dextrans for plasma volume determinations (Wasserman *et al.*, 1955; Semple, 1956; Craig *et al.*, 1957 and Jaenike *et al.*, 1957). Indirect estimation of the plasma volume from the labeled red cell volume together with the hematocrit has also been criticized. This method does not take into account local differences in hematocrit values (Berson *et al.*, 1952 and Belcher *et al.*, 1957).

It is clear that most methods do not fulfil the exact standards required for accuracy, ease of preparation or stability of the injected preparation. Because of the high specificity and sensitivity of the current routine enzyme determinations, dilution curves of enzymes could form an attractive alternative to the methods presently used. We investigated whether a number of enzymes, which are normally present in plasma, can be used to this end. Such a method could possibly replace the use of radioactivity with its inherent precautions and limitations.

Materials and methods

Mongrel dogs of both sexes and 15-35 kg of body weight were used in all experiments. The enzyme preparations were obtained from dog heart or dog liver. Pieces of about 1 gram of freshly cut tissue were suspended in dog plasma (2 ml/gram). Nitrogen was led through the medium in order to maintain hypoxic conditions during 15-20 hours at 4°C. Under these conditions enzymes are released from the tissue into the plasma. Hereafter the preparation was centrifuged first in a Beckman JA-20 rotor at 30,000 g for 30 minutes and thereafter in a

TI-45 rotor at 100,000 g for 24 hours.

Enzyme activities of creatine kinase (CK) (EC 2.7.3.2), lactate dehydrogenase (LD) (EC 1.1.1.27), aspartate aminotransferase (AST) (EC 2.6.1.1) and alanine aminotransferase (ALT) (EC 2.6.1.2) were assayed spectrophotometrically at 25°C, using a Boehringer test kit for LD, AST and ALT, and a Baker test kit for CK determinations. Enzyme activities were determined in duplicate and expressed in units per litre plasma (U/l) (see chapter III).

Isoenzyme separation of CK, LD and AST was performed as described in chapter III. For CK $98 \pm 0.4\%$ (mean $\pm$ SD) of total activity was of the MM-type while for AST $90 \pm 2.6\%$ of total activity consisted of the cytoplasmic isoenzyme. For LD $5.8 \pm 0.4\%$ of total activity consisted of the "heat labile" isoenzymes LD-3,4,5.

Fluorescence measurements were performed with an Aminco Bowman spectrophotofluorometer. The excitation wavelength of FITC-dextran is 490 nm and the emission peak occurs at 510-515 nm. A bandwidth of 5 nm was taken for both excitation and emission (Rutili *et al.*, 1970). A 5% solution of FITC-dextran, in isotonic buffered saline (0.14 M NaCl, 0.01 M Tris-HCl), containing 1% of dog plasma was used to obtain reference curves (Fig. I). Each point in this reference curve

Fig. I
A reference curve for
FITC-dextran.

was obtained from five different dilutions (SE indicated in Fig. I). Determination of fluorescence in undiluted plasma introduced errors. Therefore, plasma samples after FITC-dextran injections were diluted in buffered saline and all samples were measured in triplo. For the reference curves dilutions were performed in buffered saline containing plasma of the same dog. In order to prevent light scattering all

buffers were passed through a cathivex disposable filter unit (0.22 μm). Protein determinations were performed with a commercially available Biorad protein assay, based on the binding of Coomassie Brilliant Blue G-250 to protein, resulting in a shift of the absorption maximum of the dye from 465 to 595 (Bradford *et al.*, 1976).

Enzymes and fluorescein isothiocyanate labeled dextrans (FITC-dextran, pharmacia fine chemicals AB Upsala Sweden), mean molecular weight 150,000 D were injected through a venous catheter (Braunüle B. Braun Melsungen A.G.) in the foreleg vein (vena cephalis). A simultaneous bolus injection of enzymes and FITC-dextran was not possible, because FITC-dextran interferes with our enzyme determinations. For a bolus injection $\pm$ 0.20 ml of the enzyme preparation was injected per kg of body weight. The preparations contained about 10 mg tissue protein per ml (spec. act. AST: 3 U/mg; CK 36 U/mg and LD 11 U/mg). Enzyme preparations used in this study were not purified. Purification and or labeling may cause rapidly cleared fractions, which could result in an overestimation of plasma volume (Sodetz *et al.*, 1977 and Hermens, 1975). These preparations can, however, only be used for laboratory animals and therefore plasma volume determinations with purified AST from porcine heart were also performed. In these experiments 0.045 mg of AST from porcine heart (Sigma G2751: spec. act. 90 U/mg at 25°C) was injected per kg of body weight. For FITC-dextran dilution 20 mg per kg body weight of the same 5% solution as used for the reference curve was injected 2 hours after injection of the enzyme preparation (Fig. II). Injected quantities were determined from weighing and reweighing of syringes (density plasma: 1.025 gr/cm^3 ; dextran: 1.020 gr/cm^3). The syringe was cleared by repeated drawing and expelling of about 5 ml blood, hereafter 5 ml of saline was injected twice in order to clear the catheter.

Blood samples of about 4 ml were collected from a venous catheter in the other foreleg vein. The first 0.5-1.0 ml were always discarded, as well as hemolytic samples. Clotting was prevented with 5 U of heparin per ml blood. After a bolus injection of enzymes a first sample was taken after approximately 10 minutes and thereafter blood samples were collected at regular intervals during 90 minutes. All samples were centrifuged immediately (1000 g, 20 minutes) and plasma was separated. For enzyme determinations platelet free plasma was obtained by centrifuging at 30,000 g for 30 minutes and plasma samples

were stored at -70°C until determination.

After FITC-dextran injection 4-5 samples were taken during the first 20-30 minutes. Fluorescence measurements were performed immediately after centrifugation of plasma samples at 30,000 g for 60 minutes. Normal steady state plasma enzyme activities were determined from three successive plasma samples taken at 5 minute intervals before the bolus injection was given.

The influence of ten different dog plasma's on the enzyme activity was measured by diluting the enzyme preparation both in plasma and saline. Six different dilutions were made. The activity in each plasma was expressed as a percentage of the activity in saline. Hematocrit values were determined routinely in all blood samples with the microcapillary centrifuging technique by centrifuging at 15,000 g for 5 minutes. During successive plasma volume determinations with FITC-dextran and enzymes, hematocrit values remained constant within 2-3%.

In our experiments the dogs were not fed at least eight hours before the experiments were started, because an effect of feeding on the plasma volume has been reported (Reece *et al.*, 1970).

Analysis of data

Plasma enzyme activity and FITC-dextran concentration immediately after injection (zero time) were estimated from the best fit to a straight line (least square procedure) in the logarithmic plot,

$$\ln (C(t)-C_n) = kt + \ln C_0$$

where $C(t)$ is the plasma activity at time t , C_n is the steady state activity before injection, k is the clearance constant and C_0 was taken as the plasma activity extrapolated to zero time. A standard linear regression computer program was used. 95% confidence limits of C_0 were calculated and expressed as a percentage of obtained values. Because the error in enzyme determinations is mainly percentual, a linear fit to the logarithmic plot should be preferred to direct exponential fitting of obtained data. The latter procedure would result into exaggerated influence of data points with the highest enzyme activities. Plasma volume was calculated by dividing the total injected amount of activity by C_0 . Tests of statistical significance were performed using a paired student t test.

Results

Table I shows results of simultaneous plasma volume measurements by enzyme dilution of CK, LD and AST in 26 experiments, using enzyme

Table I Plasma volume determinations from simultaneous dilution of CK, LD and AST.

Experiment number	CK (ml/kg)	95% conf. limit (%)	LD (ml/kg)	95% conf. limit (%)	AST (ml/kg)	95% conf. limit (%)	AST (ml/kg)
1	55.3	2.1	55.5	3.3	54.4	3.0	55.6
2	60.0	5.2	60.2	3.3	56.4	1.9	56.5
3	59.4	5.8	58.2	2.3	58.2	3.2	58.0
4	74.8	6.1	67.8	7.7	55.2	3.7	54.7
5	52.4	6.4	50.6	3.8	53.3	2.4	51.5
6	51.1	3.3	50.4	5.7	49.8	4.7	48.5
7	45.0	5.5	45.8	6.2	48.8	5.1	49.2
8	56.2	5.0	55.8	6.3	53.8	1.8	52.5
9	42.0	3.7	40.5	5.7	39.9	5.5	39.4
10	46.9	3.3	48.3	3.6	43.7	2.4	44.6
11	47.4	1.7	47.3	3.0	46.1	1.4	48.2
12	49.8	2.3	57.5	4.4	42.9	4.0	42.8
13	44.4	2.7	45.4	5.4	42.7	4.5	41.5
14	50.8	3.6	50.1	3.9	47.3	3.2	45.1
15	55.2	4.6	52.0	4.2	50.1	1.5	49.6
16	46.4	2.1	45.3	4.0	42.2	2.5	42.4
17	53.8	4.2	42.1	7.6	44.8	4.2	45.1
18 ^x	49.1	3.0	47.8	3.1	48.1	1.3	47.4
19 ^x	46.8	2.9	46.4	5.6	42.0	2.2	42.0
20 ^x	51.2	2.7	53.3	3.6	46.9	2.3	45.8
21 ^x	49.2	3.6	49.4	6.1	43.5	1.9	43.1
22 ^x	41.9	0.6	41.0	3.0	34.7	3.1	34.3
23 ^x	50.7	2.4	44.9	5.9	45.6	4.6	44.5
24 ^x	50.7	9.1	46.0	11.3	49.0	8.0	46.5
25 ^x	52.9	6.3	48.5	5.7	48.6	2.3	49.8
26 ^x	43.0	5.1	43.2	4.3	43.5	4.5	42.0
Mean	51.0	4.0	49.7	5.0	47.4	3.3	47.0
SE	1.35	0.37	1.26	0.98	1.10	0.31	1.10

- For experiments indicated by (x) a subsequent plasma volume determination with FITC-dextran is performed (see Table II).

- The last column represents plasma volume, by AST dilution, as estimated from sample points taken during the first 20 minutes after injection.

preparations obtained from heart tissue. The difference in plasma volumes estimated from CK and AST dilution after bolus injection is statistically highly significant ($p < .02$). So overestimations of about 8% and 5% were respectively found for plasma volume determinations obtained from CK and LD dilution.

In the last 9 experiments indicated by *, subsequent plasma volume determinations with FITC-dextran were performed. The results of these experiments are indicated in Table II. No significant difference is

Table II Comparison of plasma volume determination by dilution of AST and FITC-dextran.

Experiment number	AST (ml/kg)	95% conf. limit (%)	FITC-dx (ml/kg)	95% conf. limit (%)
18	48.1	1.3	44.6	0.7
19	42.0	2.2	45.3	6.9
20	46.9	2.3	51.7	2.4
21	43.5	1.9	41.1	6.5
22	34.7	3.1	33.5	1.9
23	45.6	4.6	46.3	0.8
24	50.0	8.0	43.6	2.3
25	48.6	2.3	48.2	7.2
26	43.5	4.5	43.5	1.9
27 [□]	57.2	4.9	57.7	3.3
28 [□]	53.5	3.9	58.4	5.5
29 [□]	53.0	3.4	57.2	6.3
30 [□]	47.3	3.5	43.5	1.3
31 [□]	51.1	5.1	49.3	5.6
Mean	47.5	3.6	47.4	3.8
SE	1.50	0.46	1.87	0.65

- In experiments indicated by (□) purified AST from porcine heart was used.

obtained for plasma volumes determined from AST and FITC-dextran dilution. The last column of Table I shows plasma volumes obtained from the first 20 minutes of decay curves of AST.

Enzymes have different clearance constants and depending on the requirements a fast or a slow cleared enzyme can be chosen (clearance constants; CK $0.37 \pm 0.024 \text{ h}^{-1}$; AST $0.20 \pm 0.015 \text{ h}^{-1}$; LD $0.44 \pm 0.037 \text{ h}^{-1}$, mean $\pm$ SE, $n=10$ and ALT: $0.023 \pm 0.034 \text{ h}^{-1}$, mean $\pm$ SD, $n=3$).

Fig. II shows clearance curves of CK, LD, AST and FITC-dextran. From Table II it can be concluded that purified AST from porcine heart is

Fig. II

Plasma enzyme and FITC-dextran clearance curves after successive intravenous injections.

- = CK
- = LD
- ▲ = AST
- = FITC-dextran.

also suitable for plasma volume determination.

Table III shows that there is no significant difference between plasma volumes estimated from AST or ALT dilution, using enzyme preparations obtained from liver tissue. The last column presents plasma volumes calculated from only the first sample point of ALT.

Table IV shows the influence of twelve different dog plasma's on CK, LD and AST activity of an enzyme preparation obtained from dog

Table III Plasma volume determination from simultaneous dilution of AST and ALT.

Experiment number	AST (ml/kg)	95% conf. limit (%)	ALT (ml/kg)	95% conf. limit (%)	ALT ml/kg from first sample point
32	47.5	8.6	46.3	5.3	46.7
33	49.2	7.3	47.9	4.8	49.2
34	45.2	3.1	45.8	2.1	46.6
35	53.2	2.2	52.3	2.9	54.0
36	45.1	4.2	47.3	2.0	46.8
37	43.7	6.6	47.2	4.1	48.4
38	49.6	2.8	50.3	1.3	50.5
39	43.7	1.4	43.6	1.7	43.9
40	49.7	1.6	47.0	1.8	48.1
41	47.9	4.1	46.5	1.6	47.3
Mean	47.5	4.2	47.4	2.8	48.2
SE	0.97	0.79	0.76	0.46	0.86

heart. For CK significant inhibition of enzyme activity occurs ($p < .02$). For LD this trend does not become significant.

Table V shows the influence of ten different dog plasma's on AST and ALT activity of an enzyme preparation obtained from liver tissue. For these enzymes no important influence of plasma on the measured activity has been observed.

Table IV Influence of 12 different dog plasma's on CK, LD and AST from dog heart.

Plasma number	AST	CK	LD
1	101	100	103
2	101	99	103
3	105	100	104
4	100	86	94
5	99	108	103
6	103	94	94
7	95	96	99
8	99	93	88
9	100	90	95
10	101	93	85
11	98	84	100
12	97	83	103
Mean	100	94	98
SE (%)	0.8	2.2	1.8

Table V Influence of 10 different dog plasma's on AST and ALT from dog liver.

Plasma number	AST	ALT
1	103	101
2	111	110
3	101	101
4	98	98
5	93	104
6	98	108
7	96	99
8	100	98
9	102	91
10	100	101
Mean	100	101
SE (%)	1.5	1.7

Activities are expressed as a percentage of activity in saline.

Discussion

A first requirement for plasma and blood volume determinations is that no unknown fraction of the injected substance is lost from the circulation before mixing is completed. This would result in an overestimation of plasma volume. Although dilution of ^{131}I -labeled albumin and T-1824 are widely used for plasma volume determinations methodological problems, as mentioned above have been reported.

Jaenike *et al.* (1957) found a significant difference between the distribution volume of high molecular weight dextran and ^{131}I -labeled albumin. An overestimation of $6 \pm 0.3\%$ was found for albumin dilution as compared to dextran dilution. The ^{131}I -labeled albumin preparation

used in this study contained 50-140 microcuries per mg albumin and 50-100 microcuries have to be injected for a plasma volume determination in man. In several studies it was demonstrated that even slightly overiodinated albumin preparations contain fractions of denatured albumin, which are rapidly cleared, resulting in overestimation of plasma volume (Yalow *et al.*, 1957; Bloom *et al.*, 1958 and Freeman 1959). In 1957 Bloom *et al.* demonstrated that the effects of self-irradiation become significant when the specific activity exceeds 100 microcuries ^{131}I per mg of albumin. Moreover, a comparative study of ^{131}I -labeled albumin and T-1824 might be disputable because both methods are based on the dilution of tagged albumin.

These results indicate that an independent check on the validity of plasma volume measurements is necessary. In our study fluorescein labeled dextrans were used. Schröder *et al.* (1967) showed that FITC-dextrans have the same *in vivo* and *in vitro* lability as found for unlabeled dextrans.

Our results show that overestimation of plasma volume can also be found using dilution of enzymes. Enzyme determinations are assays of activity, not of concentration of a protein in plasma, accordingly the apparent activity may be influenced by the presence of inhibitors, activators or cofactors. In our case especially for CK inhibition of enzyme activity in plasma was found, resulting in an overestimation of plasma volume. This effect of inhibition on CK activity has been described (Wong *et al.*, 1976 and Morin *et al.*, 1977). Some examples of inhibitors of CK activity are maleate, succinate and anions (I^- , SO_4^{2-} , NO_3^- etc.). For LD inhibition is less pronounced although significant in some of the cases. Therefore the amount of injected CK and LD has to be estimated from *in vitro* dilution of the enzyme preparation in plasma of the same dog.

Overestimation of plasma volume as estimated from LD dilution might also be introduced, because preparations obtained from heart tissue contain $(5.8 \pm 0.43)\%$ (mean $\pm$ SD, $n=4$) of "heat-labile" LD (isoenzymes LD-3,4,5). Although our AST preparations contain $\pm 10\%$ of the mitochondrial isoenzyme (mAST) no overestimations of AST plasma volumes are observed. For CK, the enzyme preparations we used contain $(98 \pm 1.0)\%$ of muscle type isoenzyme CK-MM. For AST and ALT no significant inhibition or activation is observed, which makes these enzymes especially suitable for plasma volume determinations.

Moreover we demonstrated that there is no significant difference between plasma volume determinations with purified AST from porcine

heart and plasma volume determinations with FITC-dextran. From these results it is concluded that purified AST preparations do not contain quickly metabolized fractions, resulting in an overestimation of plasma volume.

From the 95% confidence limits in table I and table II it follows that plasma volume determinations with AST and ALT are accurate to approximately 3%. Moreover it follows from table II that determination of ALT plasma volume from the first sample point only does not result into decreased accuracy. For AST, estimation from a single sample point introduces a systematic overestimation of plasma volume, because the half-life time for AST clearance is less than two hours in some dogs. Estimation from the first 20 minutes of AST clearance curves, *i.e.* from at least two sample points gave however satisfying results (*cf.* Table I). In man the half-life for AST clearance is approximately 9 hours and a single sample point for AST would probably be sufficient (Willems *et al.*, 1980).

The enzymes we used in our study normally occur in the circulation and by choosing an enzyme with a short half-life, the presence in the plasma can be of short duration and the risk for immunological effects is minimized. Several dogs received 15 repeated injections of the same enzyme preparation, obtained from heart or liver tissue, during ten weeks and no fever or immunological complications were observed. By contrast FITC-dextran is not suitable for routine plasma volume determinations, because hypersensitivity was observed after two injections. Also the method is very laborious.

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CHAPTER V

Selection of a suitable model for the plasma clearance and distribution of cardiac enzymes in the dog

Summary

This study concerns the existing controversy about the validity of a one- vs. a two-compartment model for the distribution of intravenously injected enzymes in the dog. Enzyme preparations were obtained by leakage of enzyme from heart tissue under hypoxic conditions.

It is shown that infusion of the enzyme preparation at a constant rate permits direct calculation of the enzyme clearance constants, independently of the specific model used.

The plasma clearance constants k of creatine kinase (CK) and aspartate aminotransferase (AST) are respectively $0.36 \pm 0.030 \text{ h}^{-1}$ and $0.21 \pm 0.014 \text{ h}^{-1}$ (mean $\pm$ SE, $n=8$). These values were compared with clearance rates as estimated from a bolus injection of enzyme given 90 minutes before the infusion was started. For both enzymes the value of k , as estimated from the infusion equals the apparent disappearance constant k_d , as obtained from the preceding bolus injection.

From these experiments it is concluded that plasma clearance of CK and AST is adequately described by a one-compartment model.

In 10 experiments plasma CK and AST clearance after bolus injection was sampled during 8 hours. Disappearance of AST was strictly mono-exponential, while for CK a slight biphasic effect was observed in 5 out of 10 cases.

It is concluded that this effect is not due to exchange of enzyme with an extravascular pool but is caused by a fraction of enzyme with a slightly higher elimination rate. This phenomenon is illustrated by showing that AST also shows biphasic disappearance upon injection of an enzyme preparation obtained by mechanical disruption of cells.

Individual and day to day variations in k values obtained for CK and AST do not show any correlation. This suggests an independent clearance mechanism for both enzymes.

Introduction

Following the appreciation of the clinical importance of serum

enzyme determinations in the diagnosis of acute myocardial infarction, several authors investigated the relation between enzyme release and infarct size. In the dog a rough correlation was demonstrated between peak serum levels of AST and histologically assessed infarct size (Agress *et al.*, 1955; Nydick *et al.*, 1955; Ruegsegger *et al.*, 1959). In 1970 myocardial CK depletion was demonstrated after experimental infarction (Kjekshus *et al.*, 1970) and ever since most studies on enzymatic infarct quantitation are based on CK clearance curves. However, a model which adequately describes the plasma clearance of CK in the dog is still at issue. Shell *et al.* (1971) introduced a two-compartment model with a total distribution space of about 3 times the plasma volume. This model was based upon observed double-exponential clearance curves of CK. Rapaport (1975) reported mono-exponential clearance for different CK isoenzymes. Roberts *et al.* (1975, 1977) also found mono-exponential clearance and rejected the two-compartment model used by Shell *et al.* (1971). They suggested that the formerly proposed double-exponential function was an artefact introduced by a partially denatured enzyme preparation. However, these authors thereafter reconsidered their data and proposed a two-compartment model with an extravascular space of about 48% of the plasma volume (Sobel *et al.*, 1977). All cited studies were based upon bolus injections of enzyme preparations in the dog.

In the present study this controversy is investigated by successive determination of the enzyme clearance rate on the same day in the same dog, by the traditional bolus injection technique and by an infusion technique. The latter technique permits direct calculation of clearance constants from infusion equilibrium conditions, independently of the specific model used.

Materials and methods

Mongrel dogs of either sex, unknown age and ranging in weight from 15 to 35 kg were used in all experiments. Enzyme preparations were obtained from dog heart as described in chapter IV.

CK and AST activities were assayed in a Technicon Autoanalyzer continuous flow system at 37 °C (chapter III). Enzyme activities were determined in duplicate and expressed in units per litre plasma (U/l). Methods used for the separation of isoenzymes have been described in chapter III. For CK $98 \pm 0.4\%$ (mean $\pm$ SD) of total activity was of

the MM-type, while for AST $90 \pm 2.6\%$ of the total activity consisted of the cytoplasmic isoenzyme (cAST).

One of the enzyme preparations was obtained by homogenizing 2 grams of dog heart in 2 ml of dog plasma at 0°C by using an ultra-turrax (Janke & Kunkel type 18-10) in 8 bursts of 4 seconds. Hereafter the enzyme preparation was sonicated in 8 bursts of 15 seconds and centrifuged as described for the other enzyme preparations (chapter IV). Sonication was performed using a MSE Mark II 150-Watt disintegrator set at 12 μ peak to peak amplitude.

This preparation contained $98 \pm 1.0\%$ of CK-MM and $54 \pm 1.0\%$ (mean $\pm$ SD) of mitochondrial AST (mAST). Before administration the preparations were sterilized by filtration through a Millipore disposable filter unit (0.22 μm).

Blood samples were collected as described in chapter IV. For the continuous infusion experiments the following protocol was used. First a bolus injection of enzyme was given. The apparent disappearance constant k_d and the plasma volume was calculated from a mono-exponential fit on sample values taken 3 minutes after injection and at 10 minute intervals during 90 minutes. This first bolus of enzyme has the effect of a loading dose and considerably shortens the period of time needed to attain equilibrium after subsequent constant rate infusion. After this 90 minute period the same enzyme preparation as used for the bolus injection was diluted with physiological saline and infused at a constant rate of approximately 6 ml/hour, using a Harvard pump (model 903). During infusion samples were taken every 30 minutes for another 6 to 7 hours.

In experiments involving only bolus injections a first sample was taken 3 minutes after injection and hereafter every 20-30 minutes for 8 hours.

During continuous infusion dogs were premedicated at the start of the experiment with Vetranquil[®] 1 mg/kg (azepromazin base), symoron 1 mg/kg and atropine 0.1 mg/kg. A possible effect of premedication on enzyme clearance rates was verified by control experiments in which premedication was given 2 to 3 hours after the bolus injection. The normal steady state plasma enzyme activities were determined from three successive samples taken at 5 minutes intervals at the start of each experiment.

Analysis of continuous infusions

Fig. 1 shows the one- and two-compartment models as proposed in the literature. In both models first-order clearance is assumed *i.e.* a clearance rate proportional to the plasma activity with a proportionality constant k .

When an enzyme preparation is infused at a constant rate and for a sufficient length of time an equilibrium state will be established for the one- as well as the two-compartment model. This saturation by means of continuous infusion has been used in pharmacological studies (Wagner, 1975). During equilibrium the rate of enzyme entry into the plasma equals the rate of enzyme clearance, thus:

$$f = k(C_{eq} - C_n) \quad (1)$$

where C_{eq} is the attained equilibrium activity (U/l), C_n is the normal steady state plasma activity (U/l), k is the clearance constant (h^{-1}) and f is the rate of input of enzyme (U/(l.h)). Expression (1) allows direct calculation of k from C_{eq} , C_n and a known rate of input of enzyme f .

Analysis of bolus injections

For the one-compartment model, the time-activity curve after an intravenous bolus injection of enzyme is a mono-exponential function (Hermens, 1975):

$$C(t) = C_0 \exp(-kt) + C_n \quad (2)$$

where $C(t)$ is the plasma activity at time t , C_0 is the amount of enzyme injected at t is zero and C_n is again the normal steady state plasma activity.

For the two-compartment model the combined effect of plasma clearance and exchange with the extravascular pool results into a bi-phasic disappearance and the time-activity curve after an intravenous bolus injection of enzyme becomes a double-exponential function (Hermens, 1975):

$$C(t) = C_1 \exp(-bt) + C_2 \exp(-at) + C_n \quad (0 < a < b) \quad (3)$$

where a and b are respectively the slow and the fast disappearance constants. The parameters k , V_e/V_i and P/V_i (cf. Fig. I) can be computed from C_1 , C_2 , a and b (Hermens, 1975):

$$k = \frac{(C_1 + C_2)ab}{C_1a + C_2b} ; \quad \frac{V_e}{V_i} = \frac{C_1C_2(b-a)^2}{(C_1a + C_2b)^2} ; \quad \frac{P}{V_i} = \frac{C_1C_2(b-a)^2}{(C_1 + C_2)(C_1a + C_2b)} \quad (4)$$

Fig. I

- One- and two-compartment models.
 C_i , C_e : intra- and extravascular enzyme activities (U/l)
 V_i , V_e : intra- and extravascular volumes (l)
 f : rate of input of enzyme per litre of plasma (U/(l.h))
 P : permeability constant (l/h)
 k : clearance constant (h^{-1}).

To fit mono- and double-exponential functions a standard least squares procedure (Dell *et al.*, 1973) was used which minimizes the mean deviation of all datapoints to the fit functions (2) and (3). As the error in enzyme determinations is mainly percentual, the mean deviation expressed as a percentage of observed activities was minimized. This implies a weighted fit with the reciprocal activity as a weight function. The quality of obtained fits is expressed in the residue, *i.e.* the residual mean percentual deviation per observation. The significance of improvement of the fits was tested by a Fisher test for the ratio of residues (Beck *et al.*, 1977). For all parameters 95% confidence limits were computed.

Results

Fig. II shows plasma enzyme activities as obtained after constant rate infusion following an initial loading dose. Table I presents the results of 8 such experiments. No significant differences were observed between clearance constants k as calculated from equilibrium

* The quotient P/V_i is also known as transcapillary escape rate (TER).

Fig. II

Plasma activities of CK (●) and AST (▲) after an initial loading dose and subsequent constant rate infusion.

Experiment nr. 5 of table I is shown.

Table I Constant rate infusions of AST and CK.

Exp.	Dog	Enzyme prep.	AST				CK			
			f (U/l.h)	$C_{eq} - C_n$ (U/l)	k (h^{-1})	k_d (h^{-1})	f (U/l.h)	$C_{eq} - C_n$ (U/l)	k (h^{-1})	k_d (h^{-1})
1	D2	P3	49.3	215	0.23	0.22	636	1356	0.47	0.36
2	D3	P3	54.4	193	0.28	0.26	702	1996	0.35	0.28
3	D4	P6	31.3	196	0.16	0.17	467	1700	0.27	0.31
4	D5	P3	30.2	165	0.18	0.19	390	1053	0.37	0.33
5	D6	P4	44.2	246	0.18	0.16	659	2442	0.27	0.24
6	D6	P3	41.4	221	0.19	0.18	534	1963	0.27	0.26
7	D7	P4	41.6	178	0.23	0.24	620	1339	0.46	0.52
8	D7	P5	24.7	125	0.20	0.17	356	822	0.43	0.40
Mean			38.9	188	0.21	0.20	545	1584	0.36	0.34
SE			3.5	11	0.014	0.014	46	191	0.030	0.032

f : infusion rate of enzyme (U/(l.h))

C_{eq} : plasma activity during equilibrium (U/l)

C_n : normal steady state plasma activity (U/l)

k : clearance constant calculated from equilibrium conditions (h^{-1})

k_d : apparent disappearance constant calculated from the first 90 minutes after an initial loading dose (h^{-1}).

Complete data see appendix I; data of chapter V, table I.

conditions and apparent disappearance constants k_d as calculated from the first 90 minutes after the initial loading dose. This implies that the rate of transport to the extravascular space can be neglected in comparison to the plasma clearance rate and no initial rapid disappearance occurs.

Fig. III and table II present results of mono-exponential fits after intravenous bolus injections. Reasonable accurate values of k_d were obtained as shown by 95% confidence limits. Comparison of values for k_d from tables I and II shows no significant difference between values calculated over 90 minutes and 7-8 hours respectively.

Fig. III

Clearance curves for CK (●) and AST (▲) after a single bolus injection. Experiment nr. 6 of table II is shown.

As shown by the small residue of 3.1%, AST time-activity curves conformed very closely to a mono-exponential function and convergence to a double-exponential function was never obtained.

For CK however, 5 out of 10 experiments (*cf.* table II) showed significantly improved double-exponential fits. The results of these 5 fits to equation (3) and the parameter values calculated from these results by use of equation (4) are presented in table III. As is apparent from the large 95% confidence limits, the double-exponential fit is ill-conditioned in these cases and extremely sensitive to experimental error. This fact also became apparent from computer simulations. Although the random error added to the simulated CK curves was 7.5% for mono-exponential fits against only 3.5% for double-exponential fits (*cf.* tables II and III), the mean value for dk_d as calcu-

Table II Mono-exponential fits after bolus injections of AST and CK.

Exp.	Dog	Enzyme prep.	AST			CK		
			k_d (h ⁻¹)	dk_d (h ⁻¹)	residue (%)	k_d (h ⁻¹)	dk_d (h ⁻¹)	residue (%)
1	D1	P1	0.15	3.7	3.0	× 0.35	3.6	7.5
2	D2	P1	0.27	4.5	4.0	× 0.41	6.3	12.6
3	D2	P2	0.23	6.4	4.6	0.41	3.3	7.2
4	D3	P2	0.27	3.9	4.0	0.52	4.6	9.2
5	D3	P2	0.23	1.9	1.8	0.43	1.9	3.8
6	D4	P3	0.19	3.5	2.8	× 0.33	2.9	4.1
7	D4	P3	0.22	1.9	1.7	0.36	4.3	4.8
8	D4	P3	0.17	7.0	5.7	× 0.26	12.4	10.9
9	D5	P3	0.15	3.5	2.4	0.29	5.7	7.3
10	D5	P3	0.14	1.9	1.4	× 0.31	4.8	7.8
Mean			0.20	3.8	3.1	0.37	5.0	7.5
SE			0.015	0.56	0.44	0.024	0.92	0.90

k_d : apparent disappearance constant calculated from samples taken during 7-8 hours.

dk_d : 95% confidence limits of k_d expressed as a percentage of k_d .

residue : quality of fit expressed as the mean residual deviation per datapoint expressed as a percentage of measured activity.

Curves indicated with × in the CK-column showed improved double-exponential fits (table III). Complete data see appendix I; data of chapter V, table II.

Table III Double-exponential fits after bolus injections of CK.

Exp.	C_1 (U/l)	dC_1 (%)	C_2 (U/l)	dC_2 (%)	C_n (U/l)	b (h ⁻¹)	db (%)	a (h ⁻¹)	da (%)	res. (%)	k (h ⁻¹)	V_e/V_i	P/V_i (h ⁻¹)
1	3003	24	541	140	40	0.50	21	0.14	95	2.3	0.36	0.43	0.08
2	3595	15	408	164	42	0.63	25	0.12	153	4.7	0.44	0.76	0.13
6	718	67	1569	33	99	0.77	56	0.29	14	2.1	0.36	0.13	0.08
8	2468	39	697	157	77	0.55	54	0.53	370	4.6	0.18	1.60	0.26
10	1421	52	559	145	43	0.53	43	0.18	75	3.8	0.35	0.31	0.09
Mean	2241	39	755	128	60	0.60	40	0.25	141	3.5	0.34	0.65	0.13
SE	522	9	209	24	12	0.05	7	0.08	137	0.6	0.10	0.58	0.08

Experiment numbers correspond to table II.

C_1 , C_2 , C_n , a , b : obtained best-fit values for the double-exponential function (see text).

k , V_e/V_i , P/V_i : calculated circulatory parameters (see text).

dC_1 , dC_2 , da , db : 95% confidence limits expressed as a percentage of obtained parameter values.

residue : cf. table II.

lated for simulated curves was only 6.5% while the mean values of dC_1 , dC_2 , da and db were 66%, 216%, 146% and 45% respectively. So the biphasic behaviour of CK is insufficiently pronounced to allow accurate discrimination between fast and slow phases. This is also shown by the fact that k_d as determined from the first 90 minutes (table I) does not differ significantly from k_d as determined from 7-8 hours (table II) or from k as calculated from the double-exponential fit (table III).

Fig. IV shows that premedication, as used during infusions, does not interfere with clearance rates of CK and AST.

Fig. IV

Administration of premedication during the clearance phase after a single bolus of CK (●) and AST (▲). Time of administration is indicated.

Fig. V shows clearance as observed after injection of a preparation obtained by mechanical disruption of cells. Four bolus injections of the latter preparation resulted into biphasic disappearance for AST with a faster disappearing fraction of $37 \pm 8\%$ (mean $\pm$ SE, $n = 4$) cleared at a rate of $b = 1.42 \pm 0.44 \text{ h}^{-1}$ and a remaining fraction cleared at a rate of $a = 0.17 \pm 0.03 \text{ h}^{-1}$. The mean residue improved from 6.5% for the mono-exponential to 2.3% for the double-exponential fit. Improvement of the residue was significant at $p < 0.001$ for all 4 cases. For CK a value of $k_d = 0.32 \pm 0.02 \text{ h}^{-1}$ was found in these 4 cases while a significant improvement of the residue for a double-exponential fit occurred in 2 cases.

Fig. V

Clearance of CK (●) and AST (▲) using an enzyme preparation obtained from mechanical disruption of cells.

From table II a considerable day to day variation in clearance rates is apparent, even for the same preparation in the same dog. Fig. VI shows 8 experiments in dog D7. Large day to day variations in clearance constants of AST and CK, which are apparently uncorrelated, are observed. However, from the small residue for AST (*cf.* table II) it follows that variations in clearance constant during the experimental period of 8 hours must remain small.

Fig. VI

Variation in apparent disappearance constants k_d for CK and AST.

Discussion

Since Sterling's work on radioactive labeled albumin (Sterling, 1951) many clinical and experimental studies on plasma kinetics of intravenously injected proteins have been based upon description of plasma curves as a sum of exponentials, each exponential corresponding to a separate compartment (Jacquez, 1972). However, many conflicting results were reported and it gradually became apparent that purification and/or labeling of proteins may cause molecular changes such that fractions of different *in vivo* stability may arise. For instance it was shown that the clearance rate of ^{131}I -labeled albumin depends upon the degree of labeling (Freeman, 1959). A well known clinical example is clotting factor IX which, after purification and infusion into patients with hemophilia B, shows a rapid elimination of about 50% of infused activity (Hermens, 1975). The presence of fractions with different *in vivo* lability will result into multi-exponential clearance curves even if only a single distribution compartment is present. In this respect it should be noted that although anatomically extravascular compartments are obviously present in most animals, one-compartment behaviour occurs if the rate of exchange of protein between compartments is much slower than the rate of plasma clearance.

Data on clearance of infused AST and CK preparations in the dog have also been conflicting. Double-exponential clearance (Dunn *et al.*, 1958; Wakim *et al.*, 1963; Shell *et al.*, 1971; Sobel *et al.*, 1977) as well as mono-exponential clearance (Rapaport, 1975; Roberts *et al.*, 1975; Roberts *et al.*, 1977) has been reported. As these results were interpreted by compartmental analysis, different circulatory models were proposed as mentioned in the introduction. Model independent determination of clearance constants, as presented in this study, offers the possibility of checking the results from the literature. From the fact that clearance constants as determined from infusions equal the mono-exponential disappearance constants it is concluded that a one-compartment model allows adequate description of plasma kinetics of CK and AST in the dog. This implies that double-exponentials as presented in the literature do not reflect distributional effects. CK and AST are of comparable size. The molecular weights are 81,000 Daltons and 90,000 Daltons respectively (Noda *et al.*, 1954, Martinex-Carrion *et al.*, 1967). As the blood-lymph barrier does not discriminate in the passage of molecules with molecular weights ex-

ceeding 40,000 Daltons (Grotte, 1956; Pappenheimer, 1953; Rutili, 1977) differences in distribution space for CK and AST are not to be expected.

The conclusion that biphasic clearance does not reflect distributional effects is further strengthened by showing that AST may show mono- or double-exponential clearance depending upon the specific enzyme preparation used. Although in this specific case one is tempted to explain the biphasic clearance of AST by the fact that a mixture of mitochondrial and cytoplasmic enzymes was injected, this hypothesis is weakened by the fact that the preparation contained $54 \pm 1\%$ of mitochondrial AST while the faster disappearing fraction formed only $37 \pm 8\%$ of total activity. So only part of the mitochondrial pool could be of a more labile form. Different explanations such as differences in exposure to lysosomal proteolytic activity are equally well tenable.

The same holds true for the biphasic effect in the clearance of CK as found in 50% of the cases. The percentage of mitochondrial CK in dog heart is still a matter of debate and pH-dependent binding of mitochondrial CK to membrane structures has been described (Vial *et al.*, 1979). Moreover, it follows from table II that the biphasic clearance was not consistently found even if the same preparation was used in the same dog.

Several authors have demonstrated that within a few hours after intravenous injection of enzyme in the dog, enzyme levels of lymph have reached plateau values (Wakim *et al.*, 1963; Dunn *et al.*, 1958). At first sight this finding may seem to contradict our conclusion that diffusion of AST and CK to the extravascular space can be neglected when compared to clearance. However, although locally measured enzyme activities in lymph may soon reach steady state values, total transport of enzyme through the lymphatics is limited and complete equilibration between intra- en extravascular pools may need a considerable period of time.

It would be of interest to know if use of different batches of enzyme preparation could explain part of the variation in clearance constants. However, from table II we conclude that large variations are found even if the same preparation is used. Variations within the same dog are somewhat less although still considerable. This is in contrast to a report in the literature (Roberts *et al.*, 1977). Even if we conclude that variations in clearance constants are mainly

animal dependent, it follows from fig. VI that dogs having a relatively high clearance constant for CK may have a relatively low clearance constant for AST and *vice versa*. So enzyme clearance is not determined by some overall property of the dog and clearance mechanisms for CK and AST seem to be independent.

In conclusion our results show that for enzymes which are cleared as fast as CK and AST, or faster, diffusion to the extravascular space can be neglected and the one-compartment model allows adequate description of plasma time-activity curves in the dog.

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CHAPTER VI

Quantitation of intravenously and intramuscularly administered doses of enzyme

Summary

This study regards the validation of methods presently used for the quantitation of tissue necrosis. Intravenously and intramuscularly administered doses of liver enzymes were quantitated from the enzyme activity in serial plasma samples. The calculated release in plasma was compared to the dose administered.

For quantitation of the total activity infused in plasma a method was used, which allows estimation of the individual clearance constants of rapidly cleared enzymes (AST and GPI), using a simultaneously measured slowly cleared enzyme (ALT) as a reference (Willems *et al.*, 1979b).

It was demonstrated that using ALT as a reference enzyme, simultaneously measured AST infusions could be quantitated with a mean recovery of $95.8 \pm 12.2\%$ (mean $\pm$ SD, $n = 12$), whereas simultaneously measured GPI infusions were quantitated with a mean recovery of $98 \pm 17.7\%$ (mean $\pm$ SD, $n = 12$).

Using the mean plasma volume value and the mean value for the clearance rate of ALT, as estimated from separate bolus injections, the infused amount of this slowly metabolized enzyme could be quantitated with a mean recovery of $100 \pm 7.7\%$ (mean $\pm$ SD, $n = 12$). The proportionality constants ρ , *i.e.* the ratios of the enzyme activities ρ (ALT/AST), ρ (ALT/GPI) and ρ (AST/GPI), as obtained from the fit corresponded very closely to the ratios of enzyme activities in the enzyme preparations used for the experiments.

We conclude that for rapidly metabolized enzymes AST and GPI, the use of individualized clearance constants as obtained from the fit, gives an improvement of the results, as compared to results obtained with fixed mean values of the clearance constants. The best results for quantitations of enzyme levels in plasma were however obtained with a slowly metabolized enzyme ALT, using the mean value of the clearance constant in all experiments.

After intramuscular injections at least 75% of the ALT activity

administered was recovered in plasma, whereas for AST 86% of the activity injected was released into the plasma compartment. These results indicate that for AST there is very few local inactivation of enzyme activity at the site of injection, or during transport through the interstitial fluid. Release of enzyme from the muscle continued up to 40-50 hours.

We conclude that in order to minimize errors in calculations of tissue damage from enzyme levels in plasma, one should select enzymes that are slowly metabolized, and show complete washout from the tissue.

Introduction

Since 1970 several authors have investigated the quantitative relation between total enzyme release in plasma and myocardial infarct size. Estimation of enzymatic infarct size strongly depends on the specific circulatory model used and each model parameter influences the final estimate of infarct size.

In experimental studies in dogs poor correlations between the morphological assessment of infarct size and the calculated total release of creatine kinase have been observed (Jarmakani *et al.*, 1976 and Roe *et al.*, 1977).

In the study by Roe *et al.* (1977) enzymatic infarct size (EIS) has been calculated according to model parameters by Shell *et al.* (1971), Norris *et al.* (1975) and Roberts *et al.* (1975).

The model by Shell *et al.* is based upon bolus injections of CK preparations in dogs and consists of a one-compartment model with a total distribution volume, which is almost 3 times plasma volume; a fixed value of the clearance rate was used in all experiments. In chapter V we demonstrated however that CK clearance after bolus injection is best described by a one-compartment model including plasma volume only.

In the model by Norris *et al.* (1975) individualized clearance rates were obtained from the final part of the CK clearance curve after myocardial infarction. For the distribution volume the same value as observed by Shell *et al.* (1971) was used.

The model by Roberts *et al.* (1975) was a one-compartment model including plasma volume only and individual clearance rates were obtained as described by Norris *et al.* (1975).

Willems *et al.* (1979a) demonstrated that release of enzymes from the

infarcted tissue continues even up to 96 hours and therefore estimation of individual clearance rates of CK from the plasma curve after for instance 36-48 hours, as advocated by Norris *et al.* results in a large underestimation of CK clearance rate. The observed CK clearance rate by Willems *et al.* (1979a) was about three times higher than the value measured by Norris. In this study the transcapillary escape rate between V_i and V_e was about 1.7% per hour and is a factor three lower as observed by Witteveen *et al.* (1975). The extravascular volume was about 80% of the plasma volume.

Errors in the model parameters will result in errors in EIS, but also errors in histologically assessed infarct size (HIS) may explain the lack of a correlation between EIS and HIS after coronary occlusion. This possibility is suggested by a number of studies in dogs (Shell *et al.*, 1971) and baboons (Yasmineh *et al.*, 1976) demonstrating a good correlation between time-integrated CK activities and myocardial CK depletion.

Willems *et al.* concluded (1979b) that using HBD as a reference enzyme individual clearance constants of AST, CK and GPI can be obtained from the first 36 hours after infarction, when there is still release of enzyme in the circulation. The ratio of the released quantities of different enzymes agreed with heart tissue content. The released quantities of CK showed considerable variance in proportion to the other enzymes.

The aim of the present study is validation of the method by Willems *et al.*, which thus far has only been applied to patients with acute myocardial infarction. In the present study different types of infusions have been quantitated from plasma enzyme levels. Plasma enzyme patterns comparable to those obtained after acute myocardial infarction, were obtained by varying the rate of infusion.

Also in the present study intramuscular injections of AST and ALT activity were quantitated in order to estimate the total recovery of enzyme activity in plasma.

Materials and methods

Mongrel dogs of either sex and ranging in weight from 15-35 kg, were used in all experiments. D1, D2, D3, D10 and D11 were labradors. Enzyme preparations were obtained from dog liver as described in chapter IV. AST, ALT and GPI activities were assayed spectrophotomet-

rically at 25°C, as described in chapter III. Enzyme activities were expressed in units per litre plasma (U/l). Methods used for the separation of isoenzymes have been described in chapter III. For AST 90.5 $\pm$ 0.4% (mean $\pm$ SD) of the total activity consisted of the cytoplasmic isoenzyme (cAST).

Infused and injected enzyme preparations were used as a standard to correct for inter-assay variations (*cf.* chapter III).

Administration of enzyme preparations and collection of blood samples was performed as described in chapter IV. After bolus injections samples were taken at regular intervals during 5 days (see fig. I). No premedication (*cf.* below) was administered during these experiments.

Enzyme preparations as used for infusions were diluted 4-5 times in physiological saline. A Harvard pump (type 903) was used to infuse the enzyme preparations. During infusion of the enzyme preparations, samples were taken every 15 minutes for 3 to 4 hours. In the first 5 experiments the enzyme preparations were infused at a constant rate of 6.108 ml/hour. In the last 7 experiments the enzyme preparations were infused at variable rates (3-10 ml/hour).

The infusion pump was stopped after 3-4 hours and hereafter samples were taken every 15 minutes, during 1-2 hours, in order to obtain an independent indication of the apparent enzyme clearance rate from a mono-exponential fit on the latter part of the curve (k_d).

The total number of units infused was read from the syringe. Calibration of the syringe was checked by weighing and reweighing; the number of ml infused could be read within 2-3% accurately. The total number of units infused could be estimated within 4-6% accurately.

For each dog the individual value of the plasma volume was obtained from ALT dilution after bolus injection, using the same enzyme preparations as used for the infusion experiments.

During continuous infusion experiments the dogs were premedicated with vetranquil[®] 1 mg/kg (azepromazin base), symoron 1 mg/kg and atropine 0.1 mg/kg, given at the start of the experiment. A possible effect of premedication on enzyme clearance rates was verified by control experiments, in which premedication was given 2 to 3 hours after the bolus injection (*cf.* chapter V); no effects were observed.

Hematocrit values were determined in most of the experiments (see complete data, appendix I), using the microcapillary centrifug-

ing technique, by centrifuging at 15,000 g for 5 minutes.

Intramuscular injections were given deep into the gluteus muscle. Enzyme preparations were not diluted and about 7 ml of the preparation was injected at each side. No premedication was administered during these experiments. CK activity was measured in all samples in order to estimate muscle damage, caused by the intramuscular injections. In all experiments CK activity remained below 25 U/l (see complete data, appendix I). Dog muscle contains about 30 times as much CK activity as AST activity per gram wet tissue. Therefore the amount of AST released because of muscle damage was less than 1 unit per litre.

Normal steady state plasma enzyme activities were determined from 3 successive samples taken at 5 minute intervals before the enzyme preparations were administered.

Analysis of bolus injections

Mono- and double-exponential fits on the plasma curves were performed as described in chapter V.

Analysis of infusions

For rapidly cleared enzymes, like AST and GPI, the quantity of enzyme diffused to the extravascular space can be neglected, if compared to the amount of enzyme cleared. This implies that a one-compartment model can be used in good approximation. As the infusions were given during 3-6 hours a fixed mean value can be used for the clearance constant of the slowly metabolized reference enzyme ALT. We used the value of $k_d = 0.034 \text{ h}^{-1}$ as estimated from bolus injections of ALT, sampled during the same period as taken for the infusion experiments (*cf.* results).

The amount of enzyme released into plasma at any time t is:

$$Q(t) = C(t) + k \int_0^t C(s) ds \quad (1)$$

If the release of enzymes into plasma runs in parallel:

$$Q_{E1} = \rho \cdot Q_{E2} \quad (k_{E1} \ll k_{E2})$$

$$C_{E1}(t) + k_d E1 \int_0^t C_{E1}(s) ds = \rho C_{E2} + k\rho \int_0^t C_{E2}(s) ds \quad (2)$$

Using E1 (ALT) as a reference enzyme, individual values of enzyme clearance constants of E2 (AST or GPI) can be determined. Hereafter the amount of enzyme released in plasma can be computed from (1).

Analysis of intramuscular injections

Enzyme release in plasma after intramuscular injection of enzyme activity is stretched over a considerably longer period, i.e. 40-50 hours. Therefore a one-compartment model approximation is no longer sufficient and a two-compartment model, taking into consideration the diffusion to V_e , should be applied to these data. The fitting procedure as described above was not yet available for the two-compartment model. Therefore the intramuscular injections were quantitated with mean values of AST and ALT clearance constants as obtained from bolus injections: $k_{AST} = 0.19 \text{ h}^{-1}$ and $k_{ALT} = 0.022 \text{ h}^{-1}$. For AST, enzyme activity in plasma after intramuscular injections had returned to normal values and total release in plasma could be quantitated from (1). For ALT the slowly cleared enzyme in most experiments activity had not returned to normal values and the total release in plasma was calculated from:

$$Q(t) = C(t) + k \int_0^t C(s) ds + P/V_1 \int_0^t e^{-P/V_e(t-s)} C(s) ds \quad (3)$$

When extravascular breakdown or local inactivation of enzyme activity occurs, the total recovery in plasma as computed from the above mentioned equations will be less than 100%.

Computer simulations

Computer simulations were used to evaluate the variation in the estimated parameter values. Simulated curves were computed from equations (1) and (2) by substituting the mean values of the parameters indicated in tabel II. The sample scheme of the simulated curves was comparable to that of the experimental curves. A random error equal to the mean percentage of deviation as found for the experimental curves was added to the data points of the simulated curves, which thereupon were used as if they were obtained from dogs. The mean pa-

parameter values and the 95% confidence limits were computed from 25 simulations.

Results

Fig. I.1. shows ALT, AST and GPI activities in plasma samples taken during 100 hours after an intravenous bolus injection. In fig. I.2. the first 10 hours are represented separately.

Fig. I.1.,2. Clearance curves of ALT, AST and GPI after a single bolus injection. Fig. I.2.: The first 10 hours of the experiment (Experiment 3, table I).

Complete equilibration between intra- and extravascular ALT pools occurred after about 20 hours. During this period AST and GPI were already cleared from the circulation. Therefore equilibrium between intra- and extravascular concentrations was not attained for these fast cleared enzymes and AST and GPI clearance curves were best described by a mono-exponential function.

Table I shows results of double-exponential fits on ALT clearance curves. Large inter-individual variations in model parameters were observed, whereas repeated bolus injections in the same dog resulted in very much similar results.

Fig. II.1. and fig. II.2. show respectively plasma enzyme patterns during and after (1) constant rate infusions and (2) infu-

Table I Double-exponential fits after intravenous bolus injections of ALT.

Dog	C ₁ (U/l)	dC ₁ (%)	C ₂ (U/l)	dC ₂ (%)	b (h ⁻¹)	db (%)	a (h ⁻¹)	da (%)	C _n (U/l)	res. (%)	k (h ⁻¹)	v _e /v _i	P/v _i (h ⁻¹)
D1	184.5	9	181.2	8.8	0.12	23	0.015	8	22.1	3.5	0.027	0.62	0.042
D1	244.2	10	275.8	5.4	0.15	18	0.015	6	16.3	3.1	0.026	0.61	0.052
D3	125.7	20	284.8	8.4	0.11	40	0.014	9	7.8	3.7	0.019	0.30	0.024
D2	233.4	7	282.7	6.6	0.10	15	0.010	11	42.0	1.1	0.016	0.56	0.031
D3	181.0	14	298.6	9.1	0.09	27	0.014	8	11.9	2.5	0.021	0.35	0.021
Mean	193.8	12	265.0	7.7	0.11	25	0.014	9	20.0	2.8	0.022	0.49	0.034
CV	24.5	42	17.9	21	20.2	40	15.0	22	66.9	39.0	21.4	31.1	38.0

C₁, C₂, C_n, a, b : obtained best-fit values for the double-exponential function.
See analysis of bolus injections chapter V.

k, v_e/v_i, P/v_i : calculated circulatory parameters (chapter V).

P/v_i (TER) : transcapillary escape rate (h⁻¹)

dC₁, dC₂, da, db : 95% confidence limits, expressed as a percentage of obtained values.

res. : residue quality of fit expressed as the mean residual deviation per datapoint expressed as a percentage of the measured activity.

Complete data see appendix I; complete data chapter VI, table II.

Fig. II.1. Constant rate infusion

Q : total number of units infused per litre plasma, as estimated from plasma enzyme pattern during and after infusion.

Fig. II.2. Infusion at varying rates

Q : total number of units infused per litre plasma, as estimated from plasma enzyme pattern during and after infusion.

sions at varying rates.

The computed enzyme release functions are also depicted (Q). The fitting procedure based upon equations (1) and (2) was applied to AST, GPI and ALT activities determined simultaneously in 12 different experiments. Table II.1. presents results of quantitation of AST infusions using ALT as a reference enzyme. The reference k_d was obtained from a mono-exponential fit on ALT clearance curves after bolus injections, sampled during the same period as taken for the infusion experiments. The apparent clearance constant k_d was measured in the same dogs as used for the infusion experiments ($k_d = 0.034 \pm 12\%$, mean $\pm$ SD, $n = 5$).

Using the mean value for the apparent disappearance constant k_d of ALT and the mean value for the plasma volume (47.4 ml/kg), ALT infusions were quantitated with a mean recovery of $100 \pm 7.7\%$ (mean $\pm$ SD, $n = 12$). Quantitation of AST infusions using ALT as a reference enzyme resulted in a mean recovery of $95.8 \pm 12.2\%$ (mean $\pm$ SD, $n = 12$) (cf. Rec₂ column table II.1). The ratio of the two enzymes in the enzyme preparation ρ (ALT/AST) was estimated within 13% accurately. Table II.2. presents results of quantitation of GPI infusions, using ALT as a reference enzyme. GPI infusions were quantitated with a mean recovery of $98 \pm 17.7\%$ (mean $\pm$ SD, $n = 12$) (cf. Rec₂ column table II.2.). Using individual plasma volume values, as obtained from enzyme dilution after bolus injection (chapter IV), ALT, AST and GPI infusions were quantitated with mean recoveries of respectively $100 \pm$

Table II.1. Quantitation of AST infusions using ALT as a reference enzyme.

Dog nr.	Pr. nr.	PV (ml/kg)	BW kg	k _d (h ⁻¹)	AST (h ⁻¹)	dk _d (%)	res (%)	ρ (ALT/ AST)	dk (h ⁻¹)	k (h ⁻¹)	AST (%)	Q _{AST} (U/l)	rec ₁ AST (%)	rec ₂ AST (%)	rec ₃ AST (%)	Q _{ALT} (U/l)	rec ₄ ALT (%)	res (%)
D4	P1	49.6	23	0.18	22	2.5	1.54	5	0.23	7	207	115	110	95	320	104	99	2.7
D5	P1	50.0	32	0.17	15	1.9	1.60	6	0.19	8	174	111	105	110	276	103	97	3.2
D8	P1	43.6	33.5	0.29	7	1.0	1.90	3	0.25	4	154	89	97	68	295	100	108	4.8
D6	P1	43.6	22	0.18	10	1.5	2.04	17	0.17	8	218	82	89	92	443	98	106	4.6
D8	P1	43.6	32	0.23	8	2.1	1.67	5	0.24	4	175	103	113	82	307	106	116	3.4
D5	P1	50.0	33	0.19	13	1.4	1.71	8	0.19	17	116	99	94	99	202	101	96	3.2
D5	P1	50.0	33	0.18	14	1.7	2.15	6	0.14	16	102	76	72	103	242	106	106	4.5
D4	P1	49.6	24	0.13	32	2.9	1.54	10	0.17	21	148	111	106	123	236	104	99	4.7
D9	P2	50.1	27	0.19	21	2.1	2.00	6	0.15	16	155	97	91	122	308	96	90	4.5
D8	P2	43.6	32	0.19	26	3.0	2.24	5	0.20	9	146	85	92	80	326	94	102	4.9
D8	P1	43.6	31	0.26	16	2.3	1.86	10	0.17	27	219	90	98	101	393	95	103	6.4
D9	P2	50.1	26	0.16	17	2.2	2.11	2	0.14	6	122	87	83	118	260	93	88	4.5
Mean		47.4		0.20	17	2.1	1.86	7	0.19	12		95.4	95.8	99.4		100	100	4.2
CV(%)		7.3		22.5	44	29	13.3	59	20.0	62		13.2	12.2	17		4.7	7.7	25.

PV = plasma volume, as estimated from ALT dilution after bolus injection. BW = body weight.

k AST = clearance rate of AST, as obtained from the fit (equation 2). Pr. nr. = preparation number.

 ρ (ALT/AST) = ratio of ALT and AST activities obtained from the fit. True values of ρ as estimated from the enzyme preparations are $\rho_{P1} = 1.71$ and $\rho_{P2} = 2.01$.rec₃ AST = recovery of AST computed with fixed k for AST: k AST = 0.19 h⁻¹ and mean PV value. See table II.2.rec₄ = recovery, i.e. calculated amount of ALT as a percentage of the true amount of ALT infused, computed with a fixed k for ALT: k ALT = 0.034 h⁻¹ and individual PV values.

Q AST = calculated enzyme release in plasma, as computed with equation (1) and individual values of AST clearance rates, as obtained from (2).

k_d AST = apparent disappearance constant of AST obtained from mono-exponential fit on plasma curve after the infusion pump was set off. ρ , res, dk_d, dk, ρ , rec₁, rec₂, see table II.2.

Complete data see Appendix I; data of chapter VI, table I.

Table II.2. Quantitation of GPI infusions using ALT as a reference enzyme.

Exp. nr.	k_d GPI (h^{-1})	dk_d (%)	ρ (ALT/GPI)	$d\rho$ (%)	k GPI (h^{-1})	dk (%)	Q_{GPI} (U/l)	rec_1 GPI (%)	rec_2 GPI (%)	rec_3 GPI (%)	res (%)
1	0.39	7	0.606	5	0.38	5	514	142	136	136	2.7
2	0.37	15	0.662	15	0.37	7	410	130	124	126	3.2
3	0.74	8	0.884	8	0.56	4	320	93	101	68	4.8
4	0.32	8	1.05	7	0.26	9	411	79	86	125	4.6
5	0.37	8	0.957	5	0.34	4	290	86	93	104	3.4
6	0.39	16	0.845	7	0.34	8	235	100	95	106	3.2
7	0.38	16	1.174	7	0.38	7	193	72	68	68	4.5
8	0.20	18	0.929	7	0.28	7	259	97	93	126	4.7
9	0.42	20	0.785	6	0.31	7	379	103	97	119	4.5
10	0.59	14	0.886	8	0.40	7	368	92	100	95	4.9
11	0.42	10	0.973	9	0.32	12	396	82	89	105	6.4
12	0.69	2	0.802	1	0.59	4	329	102	97	62	4.5
Mean	0.44	12	0.879	7	0.38	7		98	98	103	4.3
CV(%)	35.3	46	17.9	45	26.9	35		20.6	17.7	24.5	2.4

Experiment numbers correspond to experiments in table II.1.

ρ (ALT/GPI) ratio of ALT and GPI activities obtained from the fit. True values of ρ as estimated from the enzyme preparations: $\rho P_1 = 0.855$ and $\rho P_2 = 0.872$.

k_d = apparent disappearance constant (h^{-1}), see table II.1.

Q = calculated enzyme release in plasma, see table II.1.

k = clearance rate of GPI as obtained from the fit (equation (2)).

res = quality of fit expressed as the mean residual deviation per data point expressed as a percentage of measured activity.

dk , $d\rho$, dk_d = 95% confidence limits, expressed as a percentage of the parameter value.

rec_1 = recovery, *i.e.* calculated amount of enzyme, as a percentage of the amount of enzyme infused, computed with individual values of PV and k .

rec_2 = recovery, computed with the mean PV value, and individual values of the clearance constants.

rec_3 = recovery, *i.e.* calculated amount of enzyme, as a percentage of the true amount of enzyme infused, computed with a fixed k for GPI: $k_{AST} = 0.38 h^{-1}$ and mean PV value.

Complete data see appendix I; data of chapter VI, table I.

4.7%, $95 \pm 13.2\%$ and $98 \pm 20.6\%$ (mean $\pm$ SD, $n = 12$) (*cf.* rec_1 and rec_4 columns tables II.1. and II.2.). These results were not significantly different from the results obtained with a mean plasma volume value (*cf.* rec_3 ALT and rec_2 AST in table II.1. and rec_2 GPI, table II.2.). The use of fixed mean values for the clearance constants *i.e.* $k_{ALT} =$

0.0342 h^{-1} , $k_{\text{AST}} = 0.19 \text{ h}^{-1}$ and $k_{\text{GPI}} = 0.38 \text{ h}^{-1}$ resulted in variations of 17% and 24% for the mean recoveries of respectively AST and GPI activities in plasma (*cf.* rec_3 columns in tables II.1. and II.2.). Comparison of these values with variations of respectively 12% and 17% obtained with individual estimates of the clearance constants of AST and GPI (*cf.* rec_2 columns in Tables II.1. and II.2.) and variations of respectively 11% and 9% due to errors in the fitting procedure (*cf.* computer simulations, table III) we conclude that for AST, as well as GPI the use of individual estimates of clearance constants results in reduced variation in the calculated recovery.

Table III Computer simulations.

	ρ (GPI/AST)	k_{GPI} (h^{-1})	res (%)	ρ (AST/ALT)	k_{AST} (h^{-1})	res (%)
Parameter values of the simulated curves	0.80	0.39	-	1.70	0.21	-
Mean parameter values after 25 simulations	0.78	0.40	4.8	1.65	0.22	4.8
CV (%)	6.9	8.8	20.3	5.2	11.4	17.0

For AST the remaining errors can satisfactorily be explained by errors from the fitting procedure. Although the improvement in the calculated infused quantities from 17% (rec_3 AST) to 12% (rec_2 AST) is not statistically significant, improvement of the procedure by increase of the number of samples seems promising.

For GPI however additional variation in plasma levels is present. The random error as used in the computer simulations forms only part of the variation found in the actual data.

Fig. III shows plasma enzyme pattern and computed enzyme release

Fig. III
Plasma enzyme pattern and computed enzyme release function (Q) after intramuscular injection.

function (Q) after intramuscular injection of AST and ALT activity in the dog. Table IV shows the recoveries of AST and ALT activities, after intramuscular injection of the enzyme activity deep into the gluteus muscle. The recovery in plasma is expressed as a percentage of the true amount of enzyme injected into the gluteus muscle.

Table IV Intramuscular injections

dog number	prep. number	One-compartment model		Two-compartment model	
		rec AST(%)	rec ALT(%)	rec ALT(%)	rec AST(%)
D3	P1	99	68	86	98
D3	P2	72	67	72	74
D10	P2	94	64	69	94
D11	P2	80	70	77	82
Mean		86.3	66.8	75.5	87
CV(%)		14.4	17	10.5	12.7

rec = amount of enzyme released into plasma after intramuscular injection, expressed as a percentage of the activity administered into the gluteus muscle of the dog.

Complete data, see appendix I; data of chapter VI, table III.

Discussion

In the literature both good and poor correlations have been observed between total enzyme release in plasma and infarct size. A number of factors may interfere in the final relationship between cell death and enzyme release in plasma thus accounting for the occasionally observed poor correlations. In most experimental studies EIS has been related to HIS. The latter method may however have a large inherent error of itself and errors in HIS may wrongly suggest bad results for EIS.

In some other studies the validity of the results on EIS have been checked by estimating infarct size for at least two different enzymes (Witteveen 1972, Norris *et al.*, 1975 and Willems *et al.*, 1979a). Comparable results for different enzymes have been interpreted as an indication for a reliable estimation of infarct size, although it must be remembered that systematic errors interfering in the same extent in estimations of infarct size as calculated with different enzymes will not disturb the observed correlations.

In our study plasma enzyme patterns comparable to those after myocardial infarction, although stretched over a considerably shorter period, were obtained by varying the rate of the infusion pump. The total amount of enzyme infused was estimated from plasma enzyme levels according to the method of Willems *et al.* (1979b). This method has already been applied to plasma enzyme levels in patients after AMI. In this study HBD was used as a reference enzyme in order to estimate individual clearance rates of CK, AST and GPI. Obtained values for the clearance rates were used to calculate EIS. Direct validation of this method in the dog, using the same enzymes as mentioned above was not possible, because in the dog HBD is cleared at a rate comparable to the clearance rates of CK, AST and GPI. One of the requirements of the method is that the reference enzyme is cleared at a rate considerably slower as compared to the clearance rate of the other enzymes. For enzymes in dog heart no such large differences in clearance rates were measured and therefore enzyme preparations obtained from liver tissue have been used in our study.

ALT from dog liver is cleared at a rate about 10-15 times slower than AST and GPI and was therefore used as a reference enzyme to allow estimation of individual clearance rates of AST and GPI.

As has been demonstrated in chapter V, behaviour of enzymes cleared as fast as AST and GPI can be approximated by a one-compartment model. For these enzymes the clearance rate from the circulation is very much larger than the rate of diffusion to the extravascular compartment (fig. I.). From bolus injections of ALT it has been estimated that the half-life for equilibration of ALT between different compartments is about 20 hours (TER = 3.4%). Comparable results have been obtained from a study by Wakim *et al.* (1963).

As the blood-lymph barrier does not discriminate in the passage of molecules with molecular weights exceeding 40,000 Daltons (Grotte, 1956; Pappenheimer, 1953 and Rutili, 1977) comparable transcapillary escape rates are to be expected for AST and GPI. These enzymes are however cleared from the circulation before equilibration between different compartments could have occurred (fig. I.2.). Therefore clearance of these enzymes from the circulation is best described by a mono-exponential function; *i.e.* a one-compartment model. During infusion experiments, enzyme preparations were administered for about 3-6 hours. Within this period equilibration between different compartments could not have occurred for either of the enzymes and the

reference k of ALT was therefore best approximated by the apparent clearance constant k_d of ALT, consisting of both clearance and diffusion to V_e . It was demonstrated that using ALT as a reference enzyme, AST infusions can be quantitated within 12% accurately, whereas for GPI much larger variations were observed. From computer simulations it can be concluded that variations found for AST can be fully explained by experimental error, whereas for GPI much unexplained variance is left.

It was also shown that for AST and GPI quantitation of the infusions with individual clearance constants as obtained from the fit did result in a reduction of the total variation in the recovery of these enzymes, although it was not significant. The best results were however obtained for ALT, using the mean value of the apparent clearance rate, as obtained from separate bolus injections. The advantage of a slowly metabolized enzyme for quantitation purposes, becomes apparent when it is considered that the contribution of the clearance term will be much smaller for such an enzyme and errors in the estimated clearance rate will therefore influence the final quantitation much less than in case of a rapidly cleared enzyme.

In order to obtain an independent check on the estimated individual clearance rates of AST and GPI, apparent clearance rates were estimated from enzyme activities in samples taken during 1-2 hours after the infusion pump was set off. Values for the apparent clearance constants (k_d) correspond to values as obtained from the fit (k). From 5-7 sample points only a rough indication of the apparent clearance constant is obtained, as becomes apparent from the 95% confidence limites.

We conclude that the method of Willems *et al.* can indeed be used to obtain individual values for AST and GPI clearance rates using ALT as a reference enzyme, even when there is still release of enzyme into the circulation.

Estimation of individual clearance rates gave an improvement of the results for both AST and GPI.

In 1975 Roberts *et al.* demonstrated that 63% of the original CK activity present in dog heart was not recovered in plasma after myocardial infarction. For the quantitation of tissue damage from plasma enzyme levels, it is of great importance to know the total amount of enzyme that will be released in plasma and to know the inter-individ-

ual variations in the recovery in plasma between individuals. In the present study intramuscular injections were given in order to estimate the amount of enzyme recovered in plasma. In 1963 Wakim *et al.* observed that after intramuscular injection of AST in the dog the maximal activity in plasma corresponded to one fifth of the activity of this dose injected, as is in agreement with our results. These authors did not calculate the total integrated amount of enzyme recovered in plasma. In the present study the total integrated recoveries of the amount of AST and ALT in plasma, were respectively 95% and 75%, using the mean values for the clearance constants as obtained from bolus injections.

These results are in striking contrast with the low recovery observed for CK in plasma after acute myocardial infarction (Roberts *et al.*, 1975), which is probably introduced because CK shows accelerated denaturation in dog lymph (Robison, 1975).

We conclude that in order to minimize errors in calculations of tissue damage one should select enzymes that are slowly metabolized and show a complete washout from the injured tissue, *i.e.* that do not show accelerated denaturation in lymph.

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CHAPTER VII

In vitro thermal inactivation of different enzymes. Relation with in vivo plasma clearance rates

Summary

In vitro thermal inactivation of CK, AST, GPI, ALT and LD was studied in enzyme preparations obtained from dog heart or dog liver. For CK, GPI and ALT monophasic inactivation curves were obtained, while AST in some cases and LD consistently showed biphasic denaturation, indicating fractions of different thermal stability. For LD these fractions probably correspond to the quantities of isoenzymes LD-1 and LD-2, but for AST no relation could be demonstrated with relative amounts of cytoplasmic and mitochondrial isoenzymes, cAST and mAST.

Inactivation rates of enzyme preparations obtained from different animals were compared. For CK (49 °C and 53 °C), GPI (53 °C) and ALT (62 °C) variation coefficients of thermal inactivation rates of different preparations were respectively 4%, 4% and 19% against 3%, 2.5% and 3.3% for mean variation coefficients within one and the same batch. Corresponding values for the slow phase of LD inactivation (62 °C) and mono-exponential approximation of AST inactivation (62 °C) were respectively 9.9% and 18.7% against 4.0% and 3.2% within batches.

For CK, GPI and ALT thermodynamic activation parameters, *i.e.* the free energy $\Delta G^\ddagger$, enthalpy $\Delta H^\ddagger$ and entropy $\Delta S^\ddagger$ of activation, were determined from inactivation rates measured at different temperatures.

Finally it was investigated whether variations in *in vivo* clearance rates of enzymes can be explained by differences in thermal stability of the enzyme preparations.

With the same CK and AST preparations as used for *in vitro* studies, *in vivo* clearance rates in dogs were measured. For different AST preparations a relation between *in vitro* thermal inactivation rate and *in vivo* clearance rate was observed. Variation in *in vivo* clearance rates of AST was drastically reduced by using preparations which are homogeneous and of equal stability with respect to thermal inactivation.

For CK the variation in *in vivo* clearance rates is much smaller and cannot be explained by differences in the preparations, because all CK preparations were homogeneous and of equal thermal stability. The remaining variation in CK clearance rates seems to be animal dependent.

Introduction

In chapter V we mentioned that besides the inter-individual variation in enzyme clearance rates, there is also a considerable variation in the biological half-life of an enzyme injected several times in the same dog (intra-individual variation). An explanation for this phenomenon could not be given, because the mechanisms responsible for protein removal from the circulation are still poorly understood. It appears that subtle changes in molecular properties may introduce instabilities and inhomogeneities with respect to *in vivo* clearance from the circulation.

Albumin preparations were found to have increased clearance rates after isolation and labeling (Freeman, 1958 and 1959; Yalow *et al.*, 1956 and Bloom *et al.*, 1958).

Berson *et al.* (1953) and Rossing *et al.* (1967) demonstrated the relative insensitivity of electrophoretic, chromatographic and ultracentrifugal methods in the detection of alterations in the albumin molecules. Only heavily labeled albumin gave some evidence on heterogeneity on electrophoretic or ultracentrifugal analysis (Berson *et al.*, 1953 and Fahey *et al.*, 1958).

One well documented molecular change, which leads to increased clearance rates from the circulation is the removal of sialic acid residues from glycoproteins (Morell *et al.*, 1971; Rijk *et al.*, 1976 and Sodetz *et al.*, 1977).

Grütter *et al.* (1979) demonstrated that differences in thermal stability may be due to changes in hydrophobic interactions or hydrogen bonds and do not necessitate large changes in the tertiary structure. He showed that the tertiary structure of lysosyme from bacteriophage T4, as determined by X-Ray diffraction was virtually identical to the tertiary structure of a temperature sensitive mutant of T4 phage lysosyme. It was shown that except for the replacement of a partially exposed arginine to a histidine the amino acid sequence of the temperature sensitive lysosyme is identical to the amino acid sequence

of the wild type lysosyme. This amino acid replacement resulted, however in a completely different thermal stability of the molecule.

Failure of detecting molecular changes by existing physical techniques has resulted into "biological screening", *i.e.* intravenous injection and successive harvesting of the injected substance from plasma (McFarlane *et al.*, 1963).

In the present study thermal inactivation of enzymes is investigated as a possible means of detecting inhomogeneities in enzyme preparations used for *in vivo* clearance studies. If the variation in *in vivo* clearance rate could be partially predicted by *in vitro* characterizations, this technique could find an application in quantitation of organ damage by means of plasma enzyme levels. These quantitations are depending on values of clearance constants which show large inter-individual variations and can only be estimated by analyzing clearance curves for several days. If a reasonable estimate of the clearance constant could be made from *in vitro* thermal inactivation of a plasma sample the procedure could be drastically simplified.

Materials and methods

Mongrel dogs of both sexes and 15-35 kg of body weight were used for all *in vivo* experiments.

Enzyme preparations used for intravenous bolus injections and thermal inactivations were obtained from dog heart or dog liver as described in chapter IV. Collection of blood samples and administration of enzyme preparations was also performed as described in chapter IV. Bolus injections of enzyme in the dog were sampled during 2.0-2.5 hours, taking samples every 10 minutes. During this period enzyme clearance curves were best described by a mono-exponential function (see chapter V).

Enzyme activities were determined as described in chapter III. CK determinations were performed, using the autoanalyzer continuous flow system (37 °C). For the other enzymes the Eppendorf spectrophotometer was used (25 °C).

For the separation of AST isoenzymes, *i.e.* cytoplasmic AST (cAST) and mitochondrial AST (mAST), a column chromatographic method was used (Sampson *et al.*, 1978). The analytical recovery of AST activity from the column was $97.4 \pm 2.4\%$ (mean $\pm$ SD, $n = 33$). For 5 separations of the same preparation (P6) the results are $19 \pm 2.3\%$ mAST. The analyt-

ical recovery of AST activity from the column was $94 \pm 6.9\%$ (mean $\pm$ SD, $n = 5$). All further separations were carried out at least in duplicate.

Isoenzyme separation of CK and LD was performed according to the method of Mercer (1975). For CK $98 \pm 0.4\%$ (mean $\pm$ SD, $n = 8$) of total activity in heart preparations was of muscle type CK-MM. The analytical recovery of total CK activity from the column was $99.3 \pm 3.0\%$ (mean $\pm$ SD, $n = 8$). For LD $5.8 \pm 0.4\%$ (mean $\pm$ SD, $n = 8$) of total activity consisted of the heat labile isoenzymes LD-3,4,5. The analytical recovery of LD from the column was $98 \pm 2.1\%$ (mean $\pm$ SD, $n = 8$). The isoenzymes LD-1 and LD-2 could not be separated with this method.

For the *in vitro* thermal inactivations a closed circulating waterbath with thermostat was used (Lauda Electronic). Before thermal inactivations were started the enzyme preparations were diluted in a pasteurized plasma protein solution (see chapter III). Thermal inactivations of different enzyme preparations were performed simultaneous, in order to prevent day to day calibration errors of the waterbath. Each preparation was divided over a number of closed tubes and for all preparations 8-16 tubes were placed simultaneous into the waterbath at zero time. At different times a sample was taken from all preparations and immediately frozen at -20°C . At zero time 4-5 samples were frozen from each preparation, for an accurate determination of enzyme activity before thermal inactivation was started (C_0). In order to obtain variations in thermal inactivation rates within one and the same batch the above described procedure was performed in duplicate or triplicate.

For thermal inactivations at different temperatures 4 tubes of each enzyme preparation were placed into the waterbath at zero time and were inactivated during approximately 25 minutes. Hereafter the tubes were immediately frozen at -20°C . Activity at zero time was determined as described above. At each temperature the same procedure was followed and thermal inactivation rate was estimated at 10 different temperatures.

The apparent k_{+1} (sec^{-1}) was computed from the following formula:

$$k_{+1} = (\ln C/C_0)/t$$

C : activity after approximately 25 minutes of thermal inactivation (U/l).

C_0 : activity before thermal inactivation was started (U/l).

t : number of seconds inactivated (sec).

k_{+1} : apparent inactivation rate (sec^{-1}).

From the apparent k_{+1} 's at different temperatures thermodynamic activation parameters can be computed as described in appendix III.

$$\Delta G^\ddagger = RT \ln \frac{kT}{k_{+1} h}$$

$$\Delta H^\ddagger = -R \frac{d \ln k_{+1}}{d 1/T} - RT$$

$$\Delta S^\ddagger = -\frac{R}{T} \frac{d \ln k_{+1}}{d 1/T} - R - R \ln \frac{k_B T}{k_{+1} h}$$

where, R is the gas constant (8.3143 J/deg.mol)

h is Planck's constant ($6.6256 \cdot 10^{-34}$ J . sec) and

k_B is Boltzman's constant ($1.381 \cdot 10^{-23}$ J/deg.)

Mono-exponential fits in thermal inactivation curves were obtained as described in chapter V. Double-exponential fits were also performed as described in chapter V. The significance of improvements of fits was tested by a Fisher test for the ratio of residues (Beck *et al.*, 1977). For all parameter values 95% confidence limits have been computed.

The coefficient of variation is defined as: $CV = 100 \times (SD/\text{mean})$ (%).

Results

Fig. I shows the thermal inactivation curves for CK at 49°C and 53°C, for GPI at 53°C and for ALT at 62°C. Thermal inactivation curves of these enzymes correspond very closely to a mono-exponential function.

Table I.1 and I.2 present results of mono-exponential fits on thermal inactivation curves of 8 CK batches obtained from different dog hearts at respectively 49°C and 53°C. At both temperatures convergence to a double-exponential function was not obtained or resulted in insignificant improvement of the fit. The coefficient of variation for thermal inactivation rates of different CK batches was 3-4% and the mean variation within one and the same batch was 3%.

Fig. 1

In vitro thermal inactivation of:

CK at 49°C : ●

CK at 53°C : ○

GPI at 53°C : ■

ALT at 62°C : ★

Clearance curves are best described by a mono-exponential function, as indicated in the figure (—).

Table I.1 Mono-exponential fits after *in vitro* thermal inactivation of different CK preparations at 49°C.

Enzyme preparation	C_0 (U/l)	SD (%)	C_{0fit} (U/l)	dC_0 (%)	k_d (h ⁻¹)	dk_d (%)	Res (%)
P1	2567	3.6	2420	3.9	0.25	4.8	5.9
P2	2958	2.2	2873	2.1	0.25	2.5	3.3
P3	3148	1.7	2933	3.3	0.24	4.3	5.0
P4	3494	2.1	3264	4.0	0.24	5.0	6.2
P5	8477	3.2	8368	2.3	0.25	2.7	3.6
P6	3665	1.0	3562	3.0	0.24	3.7	3.7
P7	3143	3.6	3134	5.0	0.27	5.7	6.4
P8	8181	1.0	8129	3.0	0.25	3.5	3.8
Mean	4434	2.3	3983	3.3	0.25	4.0	4.7
CV (%)	54	46	71	29	4.0	28	27

C_0 : Enzyme concentration at zero time estimated from 5 samples taken before thermal inactivation was started (U/l).

C_{0fit} : Obtained best-fit value for concentration at zero time.

k_d : Apparent disappearance constant (h⁻¹).

dk_d dC_0 : 95% confidence limits expressed as a percentage of k_d , C_0 .

Res : Residue: quality of fit expressed as mean residual deviation per data point, expressed as a percentage of measured activity (%).

CV : Standard deviation as percentage of mean value (coefficient of variation).

For complete data see appendix III; data of chapter VII, tables I and II.

Table I.2 Mono-exponential fits after *in vitro* thermal inactivation of 8 CK preparations at 53°C.

Preparation number	C _O (U/l)	SD (%)	C _O fit (U/l)	dC _O (%)	k _d (h ⁻¹)	dk _d (%)	res (%)
P1	9693	1.5	9808	3.9	1.90	3.3	5.1
P2	11754	1.0	11824	3.5	1.86	3.1	4.6
P3	10832	1.3	10779	4.1	1.74	3.8	5.3
P4	12390	1.8	12492	3.5	1.92	2.9	4.5
P5	1351	0.9	1313	7.0	1.86	6.2	8.8
P6	11967	2.0	12078	5.5	1.85	4.8	7.1
P7	10514	2.8	10637	5.0	1.81	4.4	6.4
P8	11962	3.4	12157	2.1	1.89	2.6	2.3
Mean	10058	1.8	10136	4.3	1.85	3.9	5.5
CV (%)	36	47	36	34	3.1	31	35

C_O, C_Ofit, k_d, dk_d and res, see Table I.1.

Table II Mono-exponential fits after *in vitro* thermal inactivation of different GPI preparations, obtained from heart or liver tissue at 53°C.

Enzyme preparation	C _O (U/l)	SD (%)	C _O fit (U/l)	dC _O (%)	k _d (h ⁻¹)	dk _d (%)	res (%)
P1	286	1.6	288	2.3	0.51	2.2	2.4
P2	442	1.0	447	1.8	0.50	1.7	1.9
P3	432	1.1	438	1.7	0.50	1.6	1.8
P4	434	0.7	436	1.8	0.50	1.7	1.9
P5	399	0.4	405	1.9	0.47	2.0	2.0
P6	498	0.5	504	1.7	0.46	1.8	1.7
P7	405	0.5	409	1.8	0.46	1.9	1.9
P8	455	1.1	459	1.5	0.46	1.6	1.6
Mean	419	0.9	423	1.8	0.48	1.8	1.9
CV (%)	15	47	15	23	4.5	12	13
P1L	360	1.1	359	1.9	0.41	2.3	2.0
P2L	354	0.3	354	1.0	0.38	1.3	1.0
P3L	341	0.9	340	1.3	0.40	1.6	1.4
Mean	352	0.8	351	1.4	0.40	1.7	1.5
CV (%)	2.8	54	2.8	33	3.9	30	34

C_O, C_Ofit, k_d, dk_d, dC_O, res and CV, see Table I.

For the complete data see appendix III; data of chapter VII, table III.

Table II presents the results of mono-exponential fits of GPI thermal inactivation curves, obtained after thermal inactivation at 53°C of the same enzyme batches as used for CK thermal inactivations and some preparations obtained from different livers. Convergence to a double-exponential function was never obtained and the coefficient of variation within one and the same batch was only 2.5%. GPI preparations obtained from liver tissue have a significantly different thermal inactivation rate, as compared to GPI preparations obtained from heart tissue.

Table III Mono-exponential fits after in vitro thermal inactivation of different ALT preparations at 62°C.

Enzyme preparation	PC_{O_0} (U/l)	SD (%)	C_{O_0} (U/l)	dC_{O_0} (%)	k_d (h ⁻¹)	dk_d (%)	res (%)
PL1	324	1.5	312	4.9	0.28	6.6	5.3
PL2	219	2.6	215	2.6	0.20	4.2	2.9
PL3	485	2.8	481	5.2	0.19	8.4	5.7
PL4	270	1.6	261	3.6	0.30	3.2	5.0
PL5	554	2.3	554	1.6	0.23	2.5	2.0
PL6	422	2.3	421	1.9	0.21	2.9	2.0
Mean	379	2.2	374	3.3	0.24	4.5	3.8
CV (%)	34	24	35	46	19	22	45

C_{O_0} , C_{O_0} fit, dC_{O_0} , k_d , dk_d , res and CV, see Table I.

For complete data see appendix III; data of chapter VII, table VI.

Table III presents the results of mono-exponential fits on ALT thermal inactivation curves after thermal inactivation at 62°C of 6 different ALT batches, obtained from different dog livers. ALT thermal inactivation curves conformed very closely to a mono-exponential function. Convergence to a double-exponential was never obtained. The coefficient of variation for the thermal inactivation rate of different ALT batches was however 19%, whereas variations within one and the same batch were only 3.3%.

Fig. II shows the thermal inactivation curves of AST and LD from P1 at 62°C. These curves were best described by a double-exponential function.

Table IV.1 presents results of mono-exponential fits on AST thermal inactivation curves of 12 different AST batches after thermal inactivation at 62°C. For the thermal inactivation rate of these prepara-

Fig. II

In vitro thermal inactivation of:

LD at 62°C : □

AST at 62°C : ▲

Curves are best described by a double-exponential function, as indicated in figure (-).

Table IV.1 Mono-exponential fits after *in vitro* thermal inactivation at 62°C of different AST preparations from heart or liver tissue.

Enzyme preparation	C ₀ (U/l)	SD (%)	C ₀ (U/l)	dC ₀ (%)	k _d (h ⁻¹)	dk _d (%)	res (%)	mAST (%)
P1	257	0.5	237	8.6	0.34	9.1	9.1	10
P2	231	1.1	222	3.4	0.21	5.7	3.7	12
P3	194	2.0	184	4.1	0.24	6.0	4.4	7
P4	211	1.4	197	6.4	0.29	7.8	6.8	9
P5	194	2.3	186	3.6	0.20	6.1	4.0	19
P6	145	1.2	130	10.8	0.33	11.7	11.3	19
P7	216	2.0	193	8.8	0.26	12.2	9.4	27
P8	218	0.3	208	3.9	0.22	6.2	4.3	16
P1L	181	1.5	169	6.1	0.29	7.5	6.8	21
P2L	275	1.5	263	3.8	0.24	5.4	4.2	12
Mean	212	1.4	199	6.0	0.26	7.7	6.4	15
CV (%)	17.5	46	18.5	45	18.7	31	43	41

C₀, SD, C₀, dC₀, k_d, dk_d and res, see Table I.

mAST : Percentage of mitochondrial AST as determined from column chromatographic method.

For complete data see appendix III; data of chapter VII, table V.

tions a coefficient of variation of 18.7% was observed. Part of these thermal inactivation curves showed improved double-exponential fits. The results of these fits are presented in table IV.2. A fraction of

different thermal stability is present in these preparations. The inhomogeneity in the AST preparations as observed from the thermal inactivation curves is not related to the relative amounts of cytoplasmic and mitochondrial isoenzymes cAST and mAST. For the thermal inactivation rate of the heat stable fraction in each preparation a coefficient of variation of only 7% was observed, whereas for the heat labile fraction completely different clearance rates were observed (CV = 54%).

Table IV.2 Double-exponential fits after *in vitro* thermal inactivation at 62°C of different AST preparations.

Enzyme preparation	C ₁ (U/l)	dC ₁ (%)	C ₂ (U/l)	dC ₂ (%)	b (h ⁻¹)	db (%)	a (h ⁻¹)	da (%)	Res (%)	C ₁ /C ₁ +C ₂ × 100	mAST (%)
P1	124	22	132	2.2	0.92	24	0.22	16	1.1	48.4	10
P3	24	28	169	3.4	4.88	89	0.22	4.0	1.3	12.4	7
P4	50	39	160	12	1.73	72	0.24	11	2.3	23.8	9
P6	64	27	81	5.0	1.23	41	0.22	19	2.5	44.1	19
P7	52	20	164	1.5	7.27	80	0.22	6.1	2.1	24.1	27
P8	24	0	194	8.2	5.31	45	0.20	0.0	0.8	11.0	16
P1L	41	32	139	9.0	1.98	63	0.25	8.0	2.0	22.8	21
Mean	54	24	148	49	3.33	59	0.22	9.2	1.7	26.7	15
CV (%)	63	51	24	254	74	39	7.2	73	38	54.0	47

C₁, C₂, a, b, dC₁, dC₂, db, da, and Res, see Table IV.

For LD the inhomogeneity of the enzyme batches as observed from the thermal inactivation curves can probably be clarified by the presence of the isoenzymes LD1 and LD2. The results of the double-exponential fits are presented in table V. Variations in the clearance rates of the slow and fast cleared fractions were respectively 9.9% and 14%.

Fig. III.1, 2 and 3 show the thermal inactivation rates of respectively GPI, ALT and LD as measured at different temperatures versus the reciprocal absolute temperature (1/T). For GPI and ALT $\ln k_{+1}$ versus 1/T fits very closely to a mono-exponential function, 95% confidence limits were respectively $\pm 6\%$ and $\pm 4\%$. For CK the 95% confidence limit was somewhat larger $\pm 13\%$, but no systematic deviation from a mono-exponential function was observed. The computed thermodynamic activation parameters are represented in table VI. For AST and LD the thermodynamic activation parameters $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were not

Table V Double-exponential fits after *in vitro* thermal inactivation of different LD preparations at 62°C.

Enzyme prep.	C ₀ (U/l)	SD (%)	C ₁ (U/l)	dC ₁ (%)	C ₂ (U/l)	dC ₂ (%)	b (h ⁻¹)	db (%)	a (h ⁻¹)	da (%)	res (%)	C ₂ /C ₁ +C ₂ × 100
P1	932	2.4	415	9.9	516	6.0	4.19	30	0.077	19	2.1	36
P2	805	1.8	327	9.8	478	5.2	4.34	30	0.087	15	1.9	37
P3	444	0.3	255	9.0	188	8.2	4.11	30	0.066	26	2.8	30
P4	801	1.7	386	8.8	415	5.8	4.76	28	0.084	17	2.2	34
P5	715	3.0	316	10.1	398	5.9	4.51	31	0.088	17	2.2	36
P6	656	2.9	310	12.2	345	7.5	5.96	41	0.095	20	3.0	34
P7	756	1.4	302	9.8	433	5.2	3.85	30	0.093	14	1.8	38
P8	771	1.9	340	8.0	430	4.5	5.03	25	0.087	13	1.8	36
Mean	735	1.9	331	9.7	403	6.0	4.59	31	0.084	18	2.2	35
CV (%)	19	45	15	12.7	25	20	14.4	15	9.9	23	20	7.1

C₁, C₂, a, b: obtained best-fit values for the double-exponential fit (see chapter V).

dC₁, dC₂, da, db: 95% confidence limits expressed as a percentage of obtained parameter values.

For complete data see appendix III; data of chapter VII, table IV.

Fig. III.1

Thermal inactivation rate of GPI at different temperatures.

Fig. III.2

Thermal inactivation rate of ALT at different temperatures.

Fig. III.3

Thermal inactivation rate of CK at different temperatures.

Table VI Thermodynamic activation parameters, as obtained from thermal inactivation rates at different temperatures.

Enzyme	Preparation	$\frac{d \ln k_{+1}}{d 1/T}$	95% conf. limit	$\Delta H^\ddagger$ kJ/mol	$\Delta G^\ddagger$ kJ/mol	$\Delta S^\ddagger$ J/mol.degr.
ALT	P1L	-47355	2.4	391	104	861
ALT	P2L	-51779	6.5	428	107	963
GPI	P1L	-59361	9.5	496	104	1205
GPI	P2L	-59558	6.1	498	105	1205
GPI	P1	-67358	5.4	560	104	1402
GPI	P3	-64093	5.8	536	105	1322
CK	P1	-38944	12.8	326	100	699
CK	P3	-48058	14.2	402	100	933

k_{+1} : thermal inactivation rate (sec^{-1})

T : absolute temperature (K)

$\Delta G^\ddagger$: activation free energy

$\Delta H^\ddagger$: activation enthalpy

$\Delta S^\ddagger$: activation free entropy

For complete data see appendix III; data of chapter VII, table VII up to XI.

determined because these preparations contain fractions of different thermal stability.

Table VII shows that there seems to be no relation between *in vivo* half-life of CK, LD, AST, ALT and GPI in the circulation and molecular weight, thermal inactivation rate or thermodynamic activation parameters.

Table VIII shows the mean *in vivo* clearance rates of CK and AST as obtained after repeated bolus injections of different enzyme preparations in four different dogs (Fig. IV).

Table VII Relation between *in vivo* half-life of different enzymes and physical parameters like, molecular weight, thermal inactivation rate or thermodynamic activation parameters.

Enzyme	Source	$T_{1/2}$ <i>in vivo</i> (h)	Temp. °C	$T_{1/2}$ <i>in vitro</i> (h)	MW .10 ⁻³	subunit MW .10 ⁻³	$\Delta G^\ddagger$ kJ/mol	$\Delta H^\ddagger$ kJ/mol	$\Delta S^\ddagger$ J/mol.degr.
ALT	liver	31.	62	2.89	180	90	106	410	912
CAST	heart	3.47	62	2.70	90	45	-	-	-
CAST	liver	3.54	62	2.55	90	45	-	-	-
CK-MM	heart	1.87	53	0.38	81	40.5	100	364	816
LD-1,2	heart	1.58	62	8.3	140	18	-	-	-
GPI	liver	1.58	53	1.73	134	67	105	497	1205
GPI	heart	1.45	53	1.44	48	67	105	548	1362

$T_{1/2}$: half-life (hours)

Temp. : temperatures of thermal inactivation *in vitro* (°C)

MW : molecular weight

$\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ see table VI.

In vivo half-lives of ALT, GPI and AST see chapter VI. For *in vivo* half-lives of AST and CK see chapter V.

Table VIII *In vivo* clearance of four different CK and AST preparations in four different dogs.

Preparation dependent variation					Dog dependent variation				
Prep. number	k_d CK (h ⁻¹)	CV (%)	k_d AST (h ⁻¹)	CV (%)	Dog number	k_d CK (h ⁻¹)	CV (%)	k_d AST (h ⁻¹)	CV (%)
P1	0.31	14.6	0.22	10.1	D1	0.26	5.9	0.22	34
P2	0.31	19.6	0.18	14.1	D2	0.37	8.5	0.22	17
P3	0.35	18.7	0.19	14.3	D3	0.34	10.4	0.22	19
P4	0.32	11.1	0.27	13.9	D4	0.32	10.7	0.19	21
Mean	0.32	16.0	0.22	13.2		0.32	8.8	0.21	23
CV (%)	5.9		18.8			14.4		7.1	

k_d and CV see table I.

For complete data, 95% confidence limits of the parameters and residues see appendix III; complete data chapter VII, table XIII.

The AST preparations, which are least sensitive to thermal inactivation *in vitro* (P2 and P3), also appeared to be more stable upon injection *in vivo*. The *in vitro* more unstable AST preparations P1 and P4 (cf. table IV.1) were also more rapidly cleared upon injection *in*

Fig. IV

Clearance rates of CK and AST, measured simultaneous after intravenous bolus injections of enzyme preparations obtained from heart tissue.

vivo (cf. table VIII). By contrast to AST, variation in simultaneous measured CK clearance rates was largely animal dependent. *In vitro* no differences in thermal stability could be detected for these different CK preparations (cf. table I). The intra-individual variation in CK clearance rates was only 5-9%, whereas the inter-individual variation was 16%.

Apart from the preparation dependent variation in AST clearance rates, inter-individual variation in AST clearance rates was only 7% (table VIII).

Discussion

In vivo clearance from the circulation of partially denatured proteins appears to be completely different from *in vivo* clearance of native proteins. Therefore it is necessary to ascertain that the properties of protein preparations used for turnover studies are identical to those of the native proteins. Fahey *et al.* (1958) demonstrated that the chromatographic elution profile of radioiodinated human serum albumin, as obtained from commercially available preparations was significantly different from the elution profile of native human serum albumin. However these authors did not relate these inhomogeneities to *in vivo* differences in clearance rates from the circulation as mentioned in the introduction. It has been demonstrated that alterations in the *in vivo* clearance rates were not always accompanied by molecular changes detectable by physical or chemical methods. Yalow *et al.* (1935) already emphasized the relative insensi-

tivity of electrophoretic analysis in the detection of apparently subtle alterations in the albumin molecules, which have a higher clearance rate in the circulation. Rossing *et al.* (1967) compared two different albumin preparations, one prepared by alcohol fractionation (SS-albumin) and the other prepared by salt fractionation (BW-albumin). The preparations were analyzed both by *in vivo* and *in vitro* studies. Paper-, immuno-, moving boundary electrophoresis, gel filtration and ultracentrifugation revealed that both preparations contained at least 90% monomeric albumin. Simultaneous injections of SS-¹²⁵I-albumin and BW-¹³¹I-albumin and BW-¹²⁵I-albumin resulted however in a significantly higher metabolic clearance rate and distribution volume for the SS-albumin ($p < .0001$). The results appeared to be independent of the label used.

Posen *et al.* (1965) advocated the determination of thermal inactivation at 56°C to differentiate between alkaline phosphatase isoenzymes from liver, bone and bile. This method could even be used to determine the relative amounts of the isoenzymes present in serum of patients. Differences in thermal stability of isoenzymes are known for several enzymes. Thermally labile isoenzymes of CK (Nealon *et al.*, 1976) appear to be more labile upon injection *in vivo* (Rapaport, 1975), as compared to the thermally more stable CK isoenzymes. *In vivo* clearance of LD-3,4,5 *i.e.* the heat labile isoenzymes, is also fast as compared to the *in vivo* clearance rate of heat stable LD1 and LD2 (Boyd *et al.*, 1969).

We used thermal inactivation curves to analyze the homogeneity and stability of different enzyme batches. We considered the possibility that variations in the molecular conformation, leading to a higher clearance rate *in vivo*, may be reflected in a difference in thermal stability of the molecules.

For CK and GPI enzyme batches appeared to be homogeneous and no significant differences were observed between thermal inactivation rates of 8 different batches obtained from heart tissue. GPI preparations obtained from liver tissue were also homogeneous, but the thermal inactivation rate was significantly different as compared to GPI from heart tissue. It is tempting to speculate that liver GPI and heart GPI are different isoenzymes in analogy with alkaline phosphatase, CK and LD. However caution is needed as will be discussed below.

Biphasic thermal inactivation as observed for AST, seems not to be related to the relative amount of mitochondrial AST. Other mechanisms

leading to relatively stable or labile fractions are possible; for instance binding of enzyme to other constituents in plasma, dimerisation of the protein, exposure to lysosomal enzyme activity depending on the preparation procedures or multistep denaturation.

ALT preparations were also homogeneous, with respect to thermal inactivation, but significant differences between thermal inactivation rates of different enzyme batches were observed (CV 18.7%).

For LD two fractions of completely different thermal stability were present in our preparations, probably corresponding to the amount of LD1 and LD2 in these preparations. The relative amounts of these fractions are practically the same in all preparations (CV = 7%), as is in contrast to fractions present in only part of the AST preparations, where extremely large variations have been observed (CV = 54%).

Values of $\Delta G^\ddagger$ indicate that the change in free energy associated with the formation of the activated complex is of the same order of magnitude for all enzymes studied here. Values of $\Delta G^\ddagger$ also correspond very closely to values as obtained from the literature for trypsin, trypsin kinase, thyroglobulin, lobster haemoglobin and enteropeptidase (Joly *et al.*, 1965).

The large value of $\Delta S^\ddagger$ for GPI indicates that the transition from native state to the activated complex is accompanied by a large increase in degrees of freedom, unfolding, compared to this transition for ALT and CK. It should be noted however that thermal inactivation need not be a physiological process and values of for instance $\Delta G^\ddagger$ for biological denaturation can be largely influenced by the presence of biological activators such as enzymes.

McLendon *et al.* (1978) demonstrated a relation between the free energy of unfolding ΔG and the intracellular turnover rates of different enzymes. From results we obtained until now it seems that there is no relation between thermodynamic activation parameters $\Delta G^\ddagger$, $\Delta H^\ddagger$, $\Delta S^\ddagger$ and *in vivo* clearance rates. It should be noted however that in order to establish such a relationship above mentioned parameters should be determined for considerably more enzymes, but even the trend did not become apparent from our data.

In 1970 Dehlinger *et al.* found that the intracellular turnover rate of proteins is related to the natural logarithm of their subunit molecular weight. A higher intracellular turnover rate was found for the larger proteins. This observation led these workers to propose that a relation between the size of a protein and its rate of degra-

dation is based on the overall chance of a protein to be hit by a protease, producing an initial rate limiting peptide bond cleavage. From our results it seems that in dogs CK, AST, ALT, LD and GPI clearance rates from the circulation are not related to the natural logarithms of their respective subunit molecular weights. Moreover for isoenzymes of the same molecular weight completely different clearance rates have been observed (Rapaport, 1975 and Boyd *et al.*, 1967). From this point of view a relation between molecular weight and *in vivo* clearance rate is not to be expected at all.

For different enzymes we observed that there is no relation between the thermal stability and the *in vivo* clearance rate from the circulation. However for any given enzyme the thermal stability of different enzyme preparations (AST) or of different isoenzymes (CK, LD and alkaline phosphatase) can be related to the *in vivo* stability. In these cases differences in molecular conformation of the enzyme are probably reflected in a different thermal stability.

It would be interesting to see whether variations in enzyme clearance rates as obtained after organ damage are introduced by variations as observed in our case for CK, *i.e.* animal dependent variation or by variations as observed for AST, *i.e.* preparation dependent variation, caused for instance by molecular differences in AST molecules. The latter variation could be easily eliminated by analysis of plasma samples *in vitro* and would allow *in vitro* estimation of AST clearance rates.

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CHAPTER VIII

Conclusions and summary

Normally only very small amounts of tissue enzymes are present in the circulation, but after tissue injury the levels of tissue enzymes in plasma increase, due to leakage of enzymes from damaged cells. In this thesis the behaviour of tissue enzymes in the circulation is studied in order to validate and improve methods presently used for the quantitation of tissue damage on the basis of enzyme levels in plasma.

A considerable part of the results presented in this thesis is obtained from intravenous and intramuscular injections and intravenous infusions of enzymes in the dog. These animals are commonly used in experimental models to study enzymatic infarct quantitation. Enzyme preparations as used for the experiments were obtained from dog heart or liver. Pieces of heart or liver tissue were incubated in dog plasma under hypoxic conditions. Under these conditions enzymes leak from the tissue into the plasma, which was hereafter centrifuged and used for the experiments.

In most studies enzymatic infarct size is expressed in grams of necrotic tissue. In order to obtain the weight of necrotic tissue in grams the calculated total activity of enzyme released into the circulation has to be divided by the mean enzyme activity per gram of normal tissue. Selection of enzymes with small intra- and inter-individual variations in tissue enzyme content will minimize errors in this procedure.

We observed that for (1) CK, LD, GPI and (2) ALT enzyme activity is rather homogeneously distributed throughout respectively heart and liver tissue of the dog. Variations in enzyme activity per gram of normal tissue, in samples taken from various sites of the same organ are less than 5-7%. Inter-individual variations in enzyme activity per gram of normal tissue were 5-10%. The use of a mean value for the enzyme content of normal tissue will therefore introduce only a small error in the quantitation of acute heart or liver tissue damage.

Since 1971 creatine kinase (CK) is commonly used in diagnosis and quantitation of acute myocardial infarction. A correct estimation of CK clearance rate from the circulation is essential for a reliable

estimation of enzymatic infarct size. When the work for this thesis was started there existed a controversy in the literature on the model to describe the clearance and distribution of CK activity after intravenous injection in the dog. With the experiments described in chapter V we were able to solve this controversy. Infusion of enzymes at a constant rate allowed us to a model independent estimation of enzyme clearance constants.

We conclude that for rapidly cleared enzyme, *i.e.* half-lives of less than 3-5 hours, with molecular weights larger than 40,000 - 60,000 Daltons, diffusion to the extravascular space can be neglected, as compared to the clearance rate from the circulation and a one-compartment model is sufficient to describe the clearance and distribution of these enzymes. For slowly metabolized enzymes like ALT diffusion to the extravascular space cannot be neglected and clearance and distribution of such enzymes is best described by a two-compartment model (*cf.* chapter V).

From the literature (Tables I and II, chapter II) and from the results presented in chapters V and VI we conclude that enzymes with molecular weights exceeding 40,000 - 60,000 Daltons extravasate with a rate of 1-4% per hour. Extremely fast extravasation of enzymes with high molecular weights, *i.e.* transcapillary escape rates of 20-50% per hour as have been reported even in the recent literature (Table II, chapter II), are attributed to the presence of quickly metabolized, partly denatured fractions of enzyme activity in the preparations and not as has been suggested to a distribution of enzyme activity over different compartments.

Part of the contradictions in earlier work on the clearance of enzyme activity from the circulation were caused by use of different and/or inferior enzyme preparations. In chapter VII we describe a method to investigate the stability and homogeneity of enzyme preparations used for *in vivo* studies. *In vitro* thermal inactivation of enzymes at higher temperatures was used to analyze the stability and homogeneity of the enzyme preparations. It was assumed that differences in molecular conformation of an enzyme would be reflected in a different thermal stability of the enzyme. If this assumption is correct thermal inactivation curves at higher temperatures could be used to detect inhomogeneities and instabilities in the enzyme preparations. We demonstrated a preparation-dependent variation in the *in vivo* clearance constant of AST. Using enzyme preparations of the same

thermal stability the inter-individual variation in AST clearance rate could be reduced to 7-10%. Variation in CK clearance rates appeared to be mainly animal dependent and not preparation dependent. All CK preparations used were of equal thermal stability. From the results presented in chapter VII we conclude that there is no relation between physical parameters like molecular weight, thermal stability or thermodynamic activation parameters and the *in vivo* clearance rate of enzymes from the circulation. It should be noted however that in order to establish such a relationship above mentioned parameters should be determined for considerably more enzymes, but even the trend did not become apparent from our data.

In most experimental studies enzymatic infarct size has been related to histologically assessed infarct size. The latter method is based upon specific staining techniques, marking either the damaged or the undamaged tissue. After excision and weighing of the damaged tissue the amount of tissue injury can be expressed in terms of grams of injured tissue. Histological assessment of infarct size has however a considerable experimental error, which may cause a poor correlation with enzymatic infarct quantitation.

Therefore in our study plasma enzyme patterns comparable to those obtained after myocardial infarction were simulated by varying the rate of intravenous infusion of the enzyme preparation. The amount of enzyme infused was quantitated from the enzyme levels appearing in plasma and compared to the true amount of enzyme administered. This experimental set-up allows a very reliable check on methods presently used to estimate tissue damage from plasma enzyme levels.

To quantitate the total activity infused a method was used, which allows estimation of individual clearance constants of rapidly cleared enzymes (AST or GPI), using a simultaneously measured slowly cleared enzyme (ALT) as a reference.

The use of fixed mean values of the clearance constants of these enzymes, *i.e.* $k_{ALT} = 0.034 \text{ h}^{-1}$, $k_{AST} = 0.19 \text{ h}^{-1}$ and $k_{GPI} = 0.38 \text{ h}^{-1}$ resulted in errors of respectively 7.7%, 17 % and 24% for the mean recoveries of ALT, AST and GPI activities in plasma.

Comparison of these values with variations of respectively 12% and 17% obtained with individual estimates of AST and GPI clearance rates and variations of respectively 11% and 9% due to errors in the fitting procedure, as judged from computer simulations, we conclude that for AST as well as GPI the use of individual estimates of clearance rates

results in a reduced error in the calculated recovery. The best results were however obtained with the slowly metabolized enzyme ALT, using the mean value of the clearance constant.

Some additional factors, which may affect enzymatic quantitation of tissue damage are: (1) incomplete release of enzyme activity from necrotic cells, (2) local inactivation of enzyme activity at the place of tissue injury and (3) extravascular inactivation of enzyme activity.

In the present study intramuscular injections of AST and ALT activity in the gluteus muscle of the dog were used to estimate the amount of enzyme activity eventually recovered in plasma.

We demonstrated that after intramuscular injections at least 75% of the ALT activity administered was recovered in plasma, whereas the recovery for AST was 86%.

These results are in striking contrast to the low recovery of CK activity in plasma after acute myocardial infarction, as has been reported in the literature. This low recovery is probably introduced because CK shows accelerated denaturation in dog lymph. After intramuscular injection ALT and AST release from muscle continued up to 40-50 hours after injection. These results indicate that especially for AST, an enzyme with a half-life of 3.5 hours, as estimated from plasma activity curves after intravenous injection of the activity, there is very few local inactivation at the site of injection or during transport through the interstitial fluid. The high recoveries in plasma indicate that catabolism of these enzymes occurs in the vascular pool or in immediate contact with this pool and not in the extravascular space.

Conclusies en samenvatting

Dit proefschrift handelt over het gedrag van weefselenzymen in de circulatie. Weefselenzymen catalyseren specifieke biologische reacties in de cel. Onder normale, niet-pathologische omstandigheden komen deze enzymen slechts in zeer geringe concentraties voor in de circulatie, de zogenaamde normaalwaarde.

Storingen in de celfunctie, veelal veroorzaakt door een tekort aan voldoende cellulaire energie om de normale physiologische processen in de cel te laten verlopen, leiden tot een verhoogde permeabiliteit van de celwand. Dit geeft aanleiding tot het vrijkomen van intracellulaire enzymen en andere stoffen in de interstitiële ruimte en in de circulatie. Het feit dat elk enzym meestal slechts één biologische reactie catalyseert maakt het mogelijk om met een grote gevoeligheid en specificiteit de concentratie van een enzym, in bijvoorbeeld een plasmamonster te bepalen.

Elk celtype heeft een karakteristiek enzympatroon. Levercellen bijvoorbeeld, vervullen een belangrijke rol in de afbraak en synthese van eiwitten en bevatten dan ook een relatief hoge concentratie transaminases, die nodig zijn bij de omzetting van aminozuren. Spiercellen daarentegen bevatten relatief weinig transaminases, maar wel een hoge concentratie creatine kinase, een enzym dat verantwoordelijk is voor de opslag van energie in de vorm van creatine fosfaat. Beschadiging van een bepaald celtype, geeft aanleiding tot een karakteristiek enzympatroon in het plasma, dat kan worden gebruikt om de plaats van de beschadiging vast te stellen. Weefsel specifieke enzymen, isoenzymen en karakteristieke enzymquotienten in het plasma zijn een belangrijk hulpmiddel geworden bij de diagnose van een groot aantal ziekten.

Het uiteindelijke niveau van intracellulaire enzymen in het plasma is afhankelijk van het aantal beschadigde cellen, maar ook van de afbraaksnelheid van het enzym in de circulatie en de duur van de uitstorting uit het weefsel.

In een aantal studies is een significante, hoewel niet indrukwekkende relatie gevonden tussen de maximale enzymactiviteit in het plasma en de histologisch bepaalde infarctgrootte. De histologische infarctgrootte wordt bepaald met behulp van specifieke kleuringstechnieken, die ofwel het necrotische dan wel het niet-necrotische (vitale) weefsel kleuren. Na uitsnijden en wegen wordt de totale weefselschade

uitgedrukt in het aantal grammen necrotisch weefsel. Het feit dat de correlaties tussen de maximale enzymactiviteit in het plasma en de histologische infarctgrootte onvoldoende zijn voor een nauwkeurige schatting van de infarctgrootte uit plasmamonsters in individuele gevallen, is het gevolg van factoren als het nemen van onvoldoende plasmamonsters, individuele verschillen in de verdwijningsconstanten, maar ook van experimentele fouten bij het bepalen van de histologische infarctgrootte.

In modellen die de verdwijning van eiwitten uit de circulatie beschrijven moet dan ook rekening worden gehouden met verschijnselen als: het diffunderen van eiwit naar de extravasculaire ruimte, terugkeer van eiwit vanuit de extravasculaire ruimte en afbraak in de circulatie. Enzymen verdwijnen uit de circulatie met snelheden die karakteristiek zijn voor zowel het enzym als voor de species. Daarnaast bestaat binnen één species ook nog een belangrijke interindividuele variatie in de verdwijningssnelheid van een enzym.

Sinds 1971 wordt gebruik gemaakt van verschillende modellen om acute weefselschade te quantiteren aan de hand van enzymniveaus in het plasma. Tot op heden zijn zowel redelijk goede als slechte resultaten verkregen.

Het doel van dit onderzoek is de bestaande methodes om acute weefselschade te quantiteren te valideren en verbeteren. Het merendeel van de resultaten werd verkregen uit intraveneuze en intramusculaire injecties en intraveneuze infusen in de hond. De hond is het standaard proefdier in experimentele modellen om enzymatische infarctgrootte te bestuderen. De enzympreparaten werden verkregen door stukjes hart of leverweefsel onder hypoxische omstandigheden te incuberen in hondenplasma, waarbij de enzymen uit het weefsel lekken en in het plasma komen. Het plasma werd vervolgens afgedraaid en gebruikt voor de experimenten.

De enzymatische infarctgrootte wordt meestal uitgedrukt in het aantal grammen necrotisch weefsel. Dit is uitsluitend gerechtvaardigd voor die enzymen waarvoor zowel de intra- als de interindividuele variaties in de enzyminhoud per gram weefsel klein zijn. Wij vonden dat voor: (1) CK, LD, GPI en voor (2) ALT de enzymactiviteit redelijk homogeen verdeeld is over respectievelijk hart- en leverweefsel. De variaties in de enzymactiviteit per gram weefsel, in monsters genomen op verschillende plaatsen in hetzelfde orgaan liggen rond de 5-7%. Interindividuele variaties in de enzymactiviteit per gram weefsel

liggen rond de 5-10%.

Het gebruik van de gemiddelde waarde voor de enzymactiviteit per gram normaal weefsel, in plaats van individuele waarden zal derhalve voor de bovengenoemde enzymen slechts een zeer geringe fout introduceren bij de quantitering van acute hartspier of leverschade.

Bij de berekening van de enzymatische infarctgrootte en meer algemeen bij de studie van het eiwitmetabolisme speelt de afbraaksnelheid van een enzym, meestal uitgedrukt in de eerste-orde verdwijningsconstante k , een belangrijke rol.

Bij de diagnose en quantitering van acute hartspierschade wordt veelal gebruik gemaakt van het enzym creatine kinase. Toen met het werk voor dit proefschrift werd aangevangen bestond er in de literatuur een belangrijke controverse over het model om de verdwijning van CK uit de circulatie zo exact mogelijk te beschrijven. De experimenten beschreven in hoofdstuk V brachten de bestaande controverse tot een oplossing. Een methode waarbij enzymen met een constante snelheid werden geïnfundeerd stelde ons in staat tot een model onafhankelijke schatting van de verdwijningsconstante van een enzym.

Wij concluderen dat voor enzymen, die relatief snel worden afgebroken, een halfwaardetijd kleiner dan 3-5 uur, en een molecuulgewicht groter dan 40.000-60.000 Daltons hebben, de diffusie naar de extravasculaire ruimte verwaarloosbaar is t.o.v. de afbraaksnelheid van deze enzymen in de circulatie en een één-compartiment model is voldoende om de verdwijning van deze enzymen uit de circulatie te beschrijven. Voor langzaam afgebroken enzymen, zoals ALT is de diffusie naar de extravasculaire ruimte niet meer te verwaarlozen en is een twee-compartimenten model noodzakelijk.

Uit de resultaten beschreven in hoofdstuk VI is geconcludeerd dat eiwitten met een molecuulgewicht groter dan 40.000-60.000 Daltons met een snelheid van 1-4% per uur naar de extravasculaire ruimte diffunderen. Dit resultaat is in overeenstemming met studies van radioactief gelabelde plasma-eiwitten, maar zelfs in zeer recente literatuur worden nog transcapillaire verdwijningssnelheden van 20-50% per uur gevonden voor enzymen. Deze resultaten zijn waarschijnlijk toe te schrijven aan de aanwezigheid van snel gemetaboliseerde fracties in de enzympreparaten en niet aan de gesuggereerde verdeling van de enzymen over meerdere compartimenten.

Het reticuloendotheliale systeem blijkt een belangrijke rol te spelen bij de verdwijning van niet-natieve eiwitmoleculen uit de

circulatie. De snelle verwijdering van beschadigde eiwitmoleculen uit de circulatie kan worden gezien als een bescherming van het organisme tegen beschadigde of anderszins veranderde moleculen.

In hoofdstuk VII is derhalve gezocht naar een methode om de samenstelling van de gebruikte enzympreparaten te analyseren. *In vitro* thermische inactivering van de enzymen bij hoge temperatuur is gebruikt om de homogeniteit en de stabiliteit van de enzympreparaten te bepalen. Verschillen in moleculaire conformatie van een enzym zouden immers mogelijk tot uiting kunnen komen in een veranderde thermische stabiliteit. Wanneer deze veronderstelling correct is zouden thermische inactiverings curves kunnen worden gebruikt om de homogeniteit en stabiliteit van de enzympreparaten te bepalen.

Voor AST werd een preparaat afhankelijke variatie in de *in vivo* verdwijningssnelheid gevonden. Indien gebruik gemaakt wordt van preparaten van identieke thermische stabiliteit kan de interindividuele variatie in de AST verdwijningsconstante worden beperkt tot 7-10%. Er was geen verschil in de *in vitro* thermische stabiliteit van de CK preparaten. De variatie in de CK verdwijningsconstante blijkt eerder hond- dan preparaat-afhankelijk.

Uit de resultaten vermeld in hoofdstuk VII hebben wij geconcludeerd dat er voor de bestudeerde enzymen geen relatie bestaat tussen fysiologische parameters als molecuulgewicht, thermische stabiliteit of thermodynamische activeringsparameters en de *in vivo* verdwijningssnelheid uit de circulatie.

Zoals vermeld kunnen fouten in de bepaling van de histologische infarctgrootte slechte correlaties met de enzymatische methode veroorzaken. Daarom werden in onze studie enzympatronen in plasma, vergelijkbaar met die na een hartinfarct, gesimuleerd door de snelheid waarmee de preparaten werden geïnfundeerd te variëren. De hoeveelheid geïnfundeerde enzymactiviteit werd vervolgens geschat op basis van de enzymniveaus in het plasma en direct vergeleken met de in werkelijkheid toegediende hoeveelheid enzymactiviteit. De totale hoeveelheid geïnfundeerde enzymactiviteit werd bepaald aan de hand van de enzymniveaus in het plasma, met behulp van een methode die ons in staat stelt de individuele verdwijningsconstanten van snel afgebroken enzymen (AST en GPI) te bepalen, gebruik makend van een gelijktijdig gemeten referentie enzym (ALT).

Het gebruik van vaste gemiddelde waarden voor de verdwijningsconstanten van deze enzymen, *i.e.* $k_{ALT} = 0.034 \text{ h}^{-1}$, $k_{AST} = 0.19 \text{ h}^{-1}$ en GPI =

0.38 h^{-1} resulteerde in fouten van respectievelijk 7.7%, 17% en 24% voor de geïnfundeerde hoeveelheid ALT, AST en GPI. Vergelijken we deze waarden met variaties van 12% en 17% verkregen met individuele schattingen van de AST en GPI verdwijningssnelheden en variaties van respectievelijk 11% en 9% geïntroduceerd door fouten in de procedure (geschat m.b.v. computer simulaties) dan kunnen we concluderen dat zowel voor AST als voor GPI het gebruik van individuele schattingen van de verdwijningsconstanten resulteert in een geringere fout. De beste resultaten werden echter verkregen met het langzaam afgebroken enzym ALT, zelfs indien gebruik werd gemaakt van de gemiddelde waarde voor de ALT verdwijningsconstante, d.w.z. de interindividuele variaties in deze grootte worden verwaarloosd.

Andere factoren die een invloed kunnen hebben op de enzymatische quantitering van weefselschade zijn: (1) onvolledige uitstorting van enzymactiviteit uit necrotische cellen, (2) locale inactivering van enzymactiviteit op de plaats van de weefselbeschadiging en (3) extra-vasculaire afbraak van enzymactiviteit.

Intramusculaire injecties, diep in de gluteus spier van de hond, werden gegeven om de totale hoeveelheid enzymactiviteit die uiteindelijk vanuit de spier in de circulatie terechtkomt te schatten. Na een intramusculaire injectie wordt gedurende 40-50 uur enzymactiviteit in het plasma uitgestort. Het bleek dat na intramusculaire injectie ten minste 75% van de totaal toegediende hoeveelheid ALT uiteindelijk in het plasma terechtkomt, terwijl voor AST tenminste 86% van de geïnjecteerde activiteit in het plasma verschijnt. Deze resultaten staan in sterke tegenstelling tot de lage opbrengst zoals in de literatuur beschreven voor CK, na een acuut myocardinfarct. Deze lage opbrengst is vermoedelijk te verklaren door een snelle inactivering van CK activiteit in de lymfe. Deze resultaten bevestigen dat speciaal voor AST er zeer weinig inactivering is op de plaats van injectie, maar ook tijdens het transport van de spier via de lymfe en/of de interstitiële ruimte naar het plasma. De hoge opbrengsten in plasma voor AST en ALT duiden aan dat de afbraak van deze enzymen in het plasma compartiment of in de onmiddellijke nabijheid van dit compartiment plaatsvindt.

It is a capital mistake to theorise before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts.

By Sir Arthur Conan Doyle, 1928
SHERLOCK HOLMES, Short Stories,
A Scandal in Bohemia,
twenty-first impression, London,
John Murray, 1976, page 7.

APPENDIX I

Presentation
of
complete data

Table I.1, I.2 AST, ALT and GPI contents of different dog livers, expressed in units per gram wet weight (U/gr) (complete data of chapter III, table I).
CV = coefficient of variation i.e. standard deviation expressed as a percentage of the mean. Samples are taken from:
- Right lobe (RL)
- Left lobe (LL)
- Quadrate lobe (QL)
- Caudate lobe (CL)

Table I.1

sample site	liver 1				liver 2				liver 3				liver 4				liver 5			
	AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI	
RL 1	119.5	79.8	85.0		123.0	86.1	101.1		107.5	80.2	166.0		113.4	83.6	190.5		124.2	87.0	108.0	
RL 2	129.0	87.6	96.2		124.1	88.2	98.5		101.0	75.3	159.8		112.6	87.9	124.2		118.5	82.3	105.1	
RL 3	129.9	87.9	93.5		119.6	83.1	106.0		99.2	71.9	153.1		102.7	82.3	128.0		115.6	80.7	101.4	
RL 4	119.5	80.5	94.4		122.5	80.4	102.7		104.5	73.1	166.6		108.1	83.8	119.4		120.8	86.5	109.8	
LL 1	131.2	81.7	82.1		127.2	79.5	109.3		103.7	78.5	164.6		125.1	91.9	127.3		123.2	84.4	106.2	
LL 2	121.6	83.7	92.6		135.8	85.6	112.1		105.4	81.7	174.2		127.2	87.6	136.9		119.0	80.1	99.2	
QL 1	135.6	79.3	92.1		130.3	89.8	112.8		103.2	76.2	155.2		106.2	79.1	125.9		125.4	88.2	106.3	
QL 2	131.0	85.0	96.5		125.7	83.2	107.5		99.7	73.5	153.5		111.0	86.0	125.9		117.0	82.8	101.7	
CL 1	124.3	81.9	91.4		126.2	87.1	103.2		96.3	69.3	145.3		110.7	86.5	122.1		116.8	85.0	102.5	
CL 2	123.3	78.9	86.8		119.4	79.0	106.0		102.5	74.6	161.2		119.2	90.9	119.3		123.1	89.0	106.1	
mean	126.7	82.6	91.4		125.4	84.2	106.1		102.3	75.4	160.0		113.7	86.0	126.0		120.4	84.6	104.6	
CV	4.1	4.1	5.1		4.1	4.4	4.4		3.2	5.1	5.4		7.0	4.4	4.1		2.8	3.8	3.2	

Table I.2

sample site	liver 6				liver 7				liver 8				liver 9				liver 10			
	AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI		AST	ALT	GPI	
RL 1	96.6	96.9	98.0		97.4	82.4	123.4		120.8	89.6	101.1		86.3	102.2	123.2		115.7	93.5	95.4	
RL 2	96.2	95.6	98.5		102.5	81.9	115.6		115.7	89.6	104.3		78.5	86.8	109.5		110.3	95.6	109.6	
RL 3	116.7	109.7	94.2		101.5	78.5	114.0		123.1	101.0	128.4		87.3	105.4	117.3		112.2	98.9	96.0	
RL 4	97.6	109.4	108.7		94.2	73.2	108.0		121.0	89.8	109.2		88.5	110.0	123.0		119.4	90.0	94.5	
LL 1	99.0	96.8	91.6		95.8	85.7	111.5		129.2	104.5	115.0		90.1	95.3	129.1		121.5	111.5	102.7	
LL 2	100.2	107.6	106.7		96.0	76.7	103.5		132.0	100.5	116.4		86.1	99.5	113.2		121.9	107.3	101.8	
QL 1	93.1	93.2	92.1		93.1	73.1	105.7		111.0	92.6	104.5		83.1	91.3	117.5		115.8	91.2	94.9	
QL 2	109.0	110.1	98.0		91.5	74.2	114.3		119.3	96.8	105.1		89.2	100.2	120.2		118.2	97.0	96.2	
CL 1	113.9	92.6	112.8		97.6	75.7	108.0		120.9	91.5	103.0		90.4	104.3	128.0		121.0	102.6	109.9	
CL 2	108.1	94.3	103.1		86.2	66.1	97.4		116.0	86.5	110.1		91.7	103.2	130.2		109.3	93.4	101.0	
mean	103.2	100.6	100.4		95.6	77.0	110.5		121.1	94.2	109.7		87.1	99.8	121.1		117.3	98.1	100.8	
CV	7.9	7.6	7.3		6.0	7.0	6.6		5.1	6.3	7.6		4.4	7.0	5.7		4.1	7.3	6.3	

Table II.1, II.2

CK content of 10 different dog hearts, expressed in units per gram wet weight (U/gr) (complete data of chapter III, Table III). Samples taken from:

- Left ventricle (LV)
- Septum (SE)

Table II.1

CK sample site	heart 1			heart 2			heart 3			heart 4			heart 5		
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3
LV epi	3526	3450	3553	3510	4038	3561	3334	3644	3776	3508	3934	3739	3641	3894	3752
LV middle	2930	3122	3186	3079	3526	3279	3083	3296	3315	3278	3479	3357	3671	3648	3625
LV endo	2800	3016	3065	2960	3505	3083	3092	3227	3434	3596	3581	3537	3365	3287	3513
SE left	3288	3246	3321	3285	3498	3016	2982	3165	3694	3648	3767	3703	3848	3677	3507
SE middle	3092	3055	2819	2989	3394	3279	3243	3305	3716	3691	3793	3734	4087	3513	3255
SE right	2818	3148	3223	3063	3451	3497	3559	3502	3653	3790	3733	3725	4362	3614	3796
RV 1	3586	3550	3424	3520	4055	4165	3746	3988	3865	3913	3914	3897	3666	3648	3420
RV 2	3513	3629	3644	3595	3875	3755	3491	3707	3800	4062	3423	3742	3686	3553	3391
RV 3	3353	3380	3330	3354	3663	4054	3648	3788	3676	3820	3810	3744	3403	3591	3807

Table II.2

CK sample site	heart 6			heart 7			heart 8			heart 9			heart 10		
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3
LV epi	4150	4059	4211	4140	4074	3960	4021	4018	3851	3339	3690	3627	3422	3285	3787
LV middle	4014	4000	3725	3913	3937	4004	3646	3862	3366	3487	3581	3478	3404	3563	3367
LV endo	4004	3639	3698	3780	4076	3578	3684	3779	3373	3477	3204	3351	3413	3237	3375
SE left	4045	3636	3932	3871	4080	3926	3868	3958	3125	3356	3326	3269	3397	3417	3501
SE middle	4097	4042	3825	3988	3441	3625	3987	3684	2864	3395	3243	3167	3519	3272	3533
SE right	4100	4125	4042	4089	3421	3564	3653	3546	3639	3347	3660	3549	3431	3299	3550
RV 1	4554	5071	4475	4700	3724	3837	3285	3615	3250	3426	3320	3325	3451	3489	3474
RV 2	4568	4448	4193	4410	3564	3820	3459	3614	3078	3489	3660	3342	3547	3317	3357
RV 3	4376	4806	4383	4521	3562	3858	3407	3678	3620	3240	3125	3328	3267	3513	3209

Table III.1, III.2

LD content of 10 different dog hearts, expressed in units per gram wet weight (U/gr) (complete data of chapter III, table III). Samples taken from:

- Left ventricle (LV)

- Right ventricle (RV)

- Septum (SE)

Table III.1

LD	heart 1				heart 2				heart 3				heart 4				heart 5			
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
LV epi	298	264	303	295	313	277	271	287	358	356	363	359	328	320	333	327	305	307	317	310
LV middle	287	273	283	283	306	317	299	307	353	353	344	350	320	313	324	319	313	289	287	296
LV endo	285	276	263	275	301	311	278	296	335	366	355	352	308	321	313	314	319	322	318	320
SE left	305	312	327	314	319	309	285	304	373	396	375	381	339	354	351	348	297	327	294	306
SE middle	302	293	278	291	300	282	294	292	381	410	391	394	341	352	334	342	327	296	299	307
SE right	287	309	301	299	296	286	314	299	414	377	397	396	350	343	349	347	281	303	321	302
RV 1	268	294	293	285	267	257	266	264	326	349	343	339	297	320	318	312	316	300	302	306
RV 2	287	294	314	302	281	264	264	268	350	366	375	363	324	330	343	332	287	297	267	284
RV 3	303	330	303	312	264	298	267	276	384	370	360	371	343	348	331	341	295	312	280	296

Table III.2

LD	heart 6				heart 7				heart 8				heart 9				heart 10			
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
LV epi	329	325	319	324	286	272	260	273	340	316	323	326	337	323	309	323	290	277	280	282
LV middle	315	330	307	317	328	310	303	313	341	338	346	342	327	328	317	324	303	295	290	296
LV endo	325	311	326	321	314	294	308	305	330	350	323	334	308	325	311	315	302	286	286	291
SE left	320	325	335	327	308	300	324	314	360	378	339	359	329	312	292	311	302	365	331	313
SE middle	325	330	310	322	306	308	302	305	354	373	357	361	298	299	296	298	310	288	295	301
SE right	325	315	315	318	294	328	275	299	323	305	336	321	303	313	274	297	292	309	289	297
RV 1	317	323	332	324	298	305	280	294	309	279	301	296	293	299	304	299	280	300	280	287
RV 2	319	328	319	322	310	272	293	292	321	281	269	290	289	301	306	299	305	283	307	298
RV 3	314	318	322	318	295	309	263	289	308	315	294	306	311	297	288	299	299	325	299	303

Table IV.1, IV.2 AST content of 10 different dog hearts, expressed in units per gram wet weight (U/gr) (complete data chapter III, table III). Samples taken from:
 - Left ventricle (LV)
 - Septum (SE)
 - Right ventricle (RV)

Table IV.1

AST sample site	heart 1			heart 2			heart 3			heart 4			heart 5		
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3
LV epi	164	178	164	169	140	114	145	134	143	128	151	141	154	153	160
LV middle	163	174	157	165	148	138	133	140	146	132	137	138	150	150	147
LV endo	164	157	173	165	148	135	140	141	142	153	154	150	156	156	163
SE left	173	162	186	174	153	137	140	143	150	136	162	149	162	149	174
SE middle	157	176	187	173	134	147	173	137	148	144	151	147	152	161	166
SE right	163	178	186	176	150	145	132	143	151	169	147	155	157	170	162
RV 1	180	182	189	184	160	179	152	164	141	147	171	153	161	165	180
RV 2	191	185	184	187	177	148	167	164	155	162	148	155	155	174	167
RV 3	190	183	189	187	147	200	166	171	153	177	167	166	172	181	178

Table IV.2

AST sample site	heart 6			heart 7			heart 8			heart 9			heart 10		
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3
LV epi	215	213	221	216	190	180	196	189	192	185	214	197	196	192	201
LV middle	211	218	209	213	183	230	172	195	175	185	182	181	208	199	197
LV endo	217	209	219	215	210	188	179	192	179	204	190	191	199	208	197
SE left	210	223	227	220	214	166	178	186	184	195	179	186	194	196	204
SE middle	218	219	212	216	170	194	186	193	159	171	165	165	199	205	205
SE right	221	211	219	217	176	184	185	182	196	186	165	182	211	191	195
RV 1	230	227	229	229	191	203	182	192	183	216	202	200	188	188	191
RV 2	225	231	227	228	194	201	213	203	171	188	180	179	205	191	185
RV 3	221	222	222	222	182	182	192	185	185	171	166	174	204	195	197

Table V.1, V.2 GPI content of 10 different dog hearts, expressed in units per gram wet weight (U/gr) (complete data chapter III, table III). Samples taken from:
- Left ventricle (LV)
- Septum (SE)
- Right ventricle (RV)

Table V.1

GPI	heart 1				heart 2				heart 3				heart 4				heart 5			
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
LV epi	165	169	168	167	162	155	154	157	146	148	153	149	156	157	161	158	165	162	165	164
LV middle	154	156	161	157	145	152	144	147	156	149	127	144	155	152	147	152	165	159	165	163
LV endo	158	155	161	158	155	150	150	152	161	145	150	152	160	148	156	155	153	147	151	150
SE left	182	182	183	183	160	153	147	153	158	140	173	157	170	161	165	165	162	175	159	162
SE middle	185	172	158	172	159	154	136	150	169	177	174	161	165	177	148	163	171	171	156	166
SE right	173	174	168	172	161	147	156	155	150	155	158	154	161	165	162	163	162	156	156	158
RV 1	173	191	187	184	162	158	151	157	157	160	168	162	167	174	169	170	149	146	170	155
RV 2	185	196	189	187	176	161	148	162	165	172	169	169	171	179	169	173	152	146	163	154
RV 3	196	183	185	188	159	179	152	163	167	170	170	169	176	180	168	175	147	169	155	157

Table V.2

GPI	heart 6				heart 7				heart 8				heart 9				heart 10			
	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
LV epi	171	162	168	167	150	154	155	153	147	137	171	152	173	168	169	170	158	164	163	162
LV middle	165	170	162	166	175	192	161	176	150	139	138	142	174	178	171	174	166	174	161	172
LV endo	173	165	172	170	191	171	195	186	140	155	134	143	169	171	174	171	175	163	179	172
SE left	163	169	174	169	192	196	193	194	147	161	142	150	174	180	174	176	190	188	185	188
SE middle	160	172	165	166	168	189	202	186	138	153	144	145	179	172	170	174	175	181	173	176
SE right	165	166	163	165	173	189	182	181	161	149	160	157	172	171	175	173	172	187	172	171
R* 1	174	177	181	177	164	182	155	167	145	143	164	151	166	168	162	165	170	185	173	176
RV 2	179	179	172	177	161	177	173	170	145	154	142	147	165	163	159	162	165	180	179	175
RV 3	173	169	175	172	163	165	144	157	145	152	129	142	160	170	169	166	175	175	169	173

Table I.1, I.2 Complete data constant rate infusions CK and AST, chapter V, table I.

Table I.1

Experiment 1				Experiment 2				Experiment 3				Experiment 4				Experiment 5				Experiment 6			
Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)
0	84	30.3		0	99	30.8	41	0	85	43.5	36	0	38.2	13.8	44	0	33.2	19.2	38	0	80	26.1	25.1
7	2978	316	35	3.5	3758	356		10.5	2268	213	34	10	2295	232	42	5.5	3520	274	36	5	3525	314	
16	2765	312	35	15.5	3450	362	38	20.5	2165	207	34.5	20	2280	225	40	10	3540	280	35	17	3265	290	
29	2475	299	35	30	3370	320	38	30	2135	207	34	30	2123	218	40	21	3350	270	34	30	3043	272	
44	2375	279	35	45	3103	315	38	41	2043	197	33	44	1985	206	40	31	3200	264	34	46	2895	275	
60	2225	266	34	64	3000	284		51	1935	192	33	51	1828	200	41	40	3090	255	34	61	2768	267	
72	1895	245	34	70	2775	275	38	60	1868	193	33	60	1735	197	40	51	3020	252	33	76	2613	255	
79	2017	256	35	77	2675	269	38	70	1698	184	32.5	71	1718	193	40	60	2925	244	31	83	2493	249	
87	1833	247	35	90	2465	265	38	82	1688	-	40	82	1688	-	40	70	2805	237	32	93	2355	237	
207	1518	229	35	160	2170	240		90	1550	180	32	86	1515	186	40	80	2613	233	32	363	2060	263	
278	1533	235	35	170	2185	223	38	120	1550	180	32	90	1495	185	41	90	2558	224	32	370	2018	252	
331	1435	235	35	285	2125	236	38	136	1588	186	32	242	1100	186	41	100	2493	223	30	376	2058	251	
345	1442	245	33	320	2025	223	39	181	1690	198	33	285	990	188	41	120	2368	223	30	381	2103	259	
360	1430	245		330	2135	222	39	212	1690	206	33	303	910	182		132	2390	222	31				
								240	1690	201	32	331	1005	182	40	160	2412	238	31				
								268	1715	208	32	360	973	177	40	190	2460	241	35				
								301	1755	214	31	375	1095	176	40	224	2465	247	36				
								326	1765	220	32	395	1085	180	40	250	2525	255	31				
								356	-	236	31					298	2518	261	31				
																330	2453	262	31				
																360	2445	262	31				
																382	2516	266	31				
																399	2428	265	31				
C _{eq} -C _n	1356	215			1996	193			1700	196			1053	165			2442	246			1963	221	
P	77562	5901			77004	5868			71070	4806			84979	6451			103305	6925			78750	5972	
Q	2943	215			2878	231			2157	162			3799	297			3499	217			3144	244	
C ₀	2952	292			3734	345			2357	173			2439	222			3611	227			3455	281	

- Infusion rate in all experiments : 6.108 ml/h

- C_{eq}, C_n, C₀ see chapter V, analysis of data

- P : concentration of the infused enzyme preparation (U/l).

- Q : total amount of units injected (U).

- Ht: hematocrit value (%).

Table I.2

Experiment 7				Experiment 8			
Time min.	CK (U/l)	AST (U/l)	Ht (%)	Time min.	CK (U/l)	AST (U/l)	Ht (%)
0	61	22	41	0	80	28	39
4	2220	184	39.5	7	1668	175	36.5
7.5	2210	188	39	15	1625	169	38.5
15	2073	172	43	30	1513	163	36
22	1943	168	42	44	1288	160	36
26	1933	166	41	62	1213	153	35
32	1923	166	40	70	1158	149	
42	1563	155		76	1063	149	34.5
52	1445	154	40	82	1035	146	
63	1440	150		90	1035	142	
70	1315	144	39	270	883	149	38
75	1300	145		300	723	152	
81	1168	139		330	795	152	36.2
91	1085	136	38	360	895	152	
122	1055	145		371	895	152	
153	1240	-		382	918	153	35
188	1215	164		398	875	155	
218	1165	164	36	401	935	161	33.5
238	1200	167	35	411	890	152	34
269	1290	179	35.5				
302	1305	183	35				
332	1360	191	38				
364	1415	196	39				
385	1345	200	36				
390	1425	199					
C _{eq} -C ₀				522 125			
P				110056 7696			
Q				4460 293			
C ₀				2311 163			

Table II.1, II.2 Intravenous bolus injections of CK and AST complete data chapter V, table II.

Table II.1

Experiment 1				Experiment 2				Experiment 3				Experiment 4			
Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)
7.5	92.7	98.7		7.0	-	104.3		7	97.2	102.1	35	2.5	100.6	108.7	40
11.0	92.9	98.7		10	98.4	103.4		9	92.8	101.4	34.5	6	92.7	101.8	40
16	90.7	100.4		13	86.8	97.5		13	97.6	94.6	34.5	16	86.4	96.0	40
20.5	86.5	97.0		21	81.9	90.7		20	93.7	91.1	35	20	92.0	95	41
24	83.9	93.8		26	73.9	85.8		35	79.4	83.4	34	28	84.1	91.3	39
29.5	80.4	93.8		33	72.6	85.0		38	74.8	82.3	34	34	83.4	90.8	39
39	74.3	86.2		39	68.0	80.8		50	69.4	83.8	34	43	74.0	83.0	
46	70.6	88.1		47	67.0	77.6		58	70.3	82.8	35	57	66.2	75.6	
50	68.6	89.0		61	58.7	71.2		81	57.9	71.3		63	62.7	72.4	
62	66.3	84.0		90	44.9	63.3		96	51.0	66.4	34	75	50.6	66.6	36
90	55.3	77.2		123	28.9	58.7		125	42.4	56.9		90	43.1	67.3	37
119	43.0	72.3		180	20.1	43.3		177	28.4	46.0		110	34.4	55.0	
178	27.9	60.9		243	15.1	35.6		230	20.9	38.4	35	138	24.4	50.8	
240	20.1	54.2			11.1	29.3		298	12.7	34.8		182	17.1	43.1	37
279	15.9	46.8		301	9.5	27.4		325	9.1	31.3		250	12.1	34.2	
299	14.7	46.6		377	6.7	21.5		337	9.5	28.1		270	10.8	33.9	
357	11.2	43.6		381	6.3	16.7		357	7.5	27.3		277	9.2	29.8	
400	9.4	37.2		401	5.6	16.7		376	7.5	24.0		301	7.2	25.5	
416	8.1	36.0		418	5.3	14.9		393	6.5	18.1		329	6.0	25.8	
450	7.2	32.7		441	5.0	-		414	6.8	17.5		350	6.0	21.7	
461	6.8	31.9		459	4.8	-		428	6.7	17.8		364	5.0	18.4	
479	6.5	30.2		474	4.9	-		444	5.9	15.0		378	3.8	19.6	
492	6.2	30.0						448	4.7			393	-	16.8	36
								468	5.7			405	-	15.0	42
								470	4.7						

- Ht, see table I.1
 - all values are expressed as a percentage of the extrapolated activity at time zero (C₀).

Table II.2 Intravenous bolus injections of CK and AST, complete data chapter V, table II.

Experiment 5				Experiment 6				Experiment 7				Experiment 8				Experiment 9				Experiment 10			
Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)	Time min.	CK (%)	AST (%)	Ht (%)
10	97.0	93.6	42	20	98.1	93.4	37	12	91.3	95.3	38	9	88.3	98.3		12		93.4	43	10	95.1	100.9	
20	82.5	92.9	40	40	87.3	89.3	35	20	85.4	93.8	38	22	85.2	101.4	39	20	91.4	96.7	43	21	85.0	94.3	
37	74.3	86.5		60.5	73.2	82.6	35.5	40	80.2	87.3	38	41	80.5	95.9		42	81.0	92.8	43	41	74.0	91.0	42
44	74.8	83.8	41	81	63.1	76.4	34.5	60	74.0	80.3	37	53	67.4	84.9		60	71.3	87.3	43	62	64.4	85.1	
53	71.6	81.1	41	101	56.7	71.7	33.5	84	67.8	74.8	37	78	63.6	84.3	39	79	67.5	80.7	43	84	53.1	82.6	42
61	64.9	78.4	40	120	48.9	66.4	34	100	56.7	-	35	99	52.4	78.6		99	62.2	77.3	43	103	48.6	78.4	
82	60.1	74.7	40	144	42.9	53.4	34	120	52.4	62.8	36	121	49.2	73.3	38	122	53.3	71.8	43	120	44.4	75.9	
99	51.3	70.2	41	159	39.6	59.9	34	140	47.5	59.3	36	143	40.4	63.7	37	141	46.9	69.1	43	141	38.3	71.8	
121	41.0	62.9	41	181	34.7	57.6	35	160	43.1	55.9	36	171	33.9	56.1		156	42.0	66.8	43	163	33.2	66.4	42
141	36.5	58.3		209	30.4	54.0	33	176	38.4	54.4	36	180	30.9	51.3		181	37.1	63.0	43	181	30.9	65.9	
159	31.9	54.7	41	220	29.7	48.2	35	201	31.6	48.4	35	209	28.4	49.9		202	32.7	60.8	43	199	31.1	62.6	
181	25.8	51.1	41	245	25.9	-	34	220	29.5	45.4	36	240	26.7	49.2		220	31.4	57.5	43	213	24.6	61.8	41
200	23.7	46.5	40	256	23.7	44.6	35	240	26.1	42.9	34	271	24.7	41.7	35	240	28.8	56.9	43	241	22.7	57.6	43
220	20.2	42.4	39.5	280	20.8	41.7	35	264	23.1	40.0	33	304	23.5	38.3		262	26.3	54.7	43	273	16.7	53.4	
240	17.0	39.2	39.5	301	18.9	36.2	35	280	19.2	35.9	33	321	21.6	38.3	36	280	22.3	51.4	44	302	16.4	50.9	42
280	12.9	33.3	39	320	16.5	38.2	33	304	14.9	33.9	33	360	18.9	34.8		301	19.6	48.6	43	317	15.8	47.6	
308	11.3	29.7	39	340	14.9	32.9	33	320	13.9	31.4	32	400	17.2	32.8	35	320	18.4	44.2	43	360	12.0	43.4	41
319	9.9	28.3	40	360	13.7	31.7	34	340	12.9	28.9		423	16.9	32.1		361	14.0	40.9	43	390	9.7	39.2	
335	8.5	27.0	39	384	12.4	31.1		361	10.8	24.9		445	17.2	28.6		398	12.9	35.4	45	420	9.1	37.6	41
360	8.0	25.1	39	404	11.6	30.0	34	382	9.0	25.4	31	454	16.8	29.3		420	12.6	34.3	42	455	8.7	33.4	41
380	6.8	22.0	40	421	10.2	28.2	34	400	9.0	23.9	30	466	15.1	26.6		442	12.8	32.6	43	463	7.8	35.0	
403	5.7	20.6		442	9.2	21.7		421	7.7	21.9	32	480	15.1	27.3		458	12.2	29.8		477	7.6	34.2	
433	4.4	17.9	40	460	8.3	22.9		435	7.6	20.9	32	492	17.6	25.9		476	11.4	28.2		491	7.4	32.5	
445	4.7	17.0		480	7.5	22.9	34	460	7.0	19.0	32	492	14.5	26.6						515	6.7	30.9	
469	3.6	16.1						476	6.4	18.5										518	6.4	30.0	

- Ht, see table I.1

- all values are expressed as a percentage of the extrapolated activity at time zero (C_0).

Table II. Plasma activities of ALT, AST and GPI during and after intravenous infusion of enzyme preparations obtained from dog liver.

Experiment 1					Experiment 2					Experiment 3					Experiment 4				
Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)
0	17.7	9.7	40.2	42	0	17.3	6.7	41.0	38	0	36.5	26.6	118.4	45	0	24.0	8.5	37.7	39
5	23.7	13.6	41.0	40	5	21.6	9.1	44.0	38	5	48.8	36.0	125.8	45	5	34.7	12.5	48.7	38
10	32.1	17.6	53.5	39	9.5	26.5	12.4	50.7	37	10	48.0	36.1	127.2	45	10	44.1	18.6	53.2	39
16	40.6	23.4	63.8	40	15	32.9	16.3	49.8	39	16	59.6	37.4	133.4	45	20	65.3	29.0	67.9	39
21	48.4	26.7	73.2	39	21	42.5	20.6	68.7	38	20	63.5	38.7	135.1	44.5	30	92.2	38.2	85.7	39
31	63.9	36.3	97.3	39	27	48.8	24.9	75.6	38	30	81.7	47.8	165.2	45	42	110.2	49.1	111.8	39
42	84.7	48.0	127.9	39	36	61.3	30.5	91.9	41	41	93.9	53.6	174.4	45	60	153.6	67.3	138.6	38
61	113.7	63.2	162.9	38	46	72.9	37.3	105.3	38	52	110.6	61.9	184.4	44.5	93	206.2	92.8	190.2	38
82	135.9	77.6	191.8	38	59	91.5	49.2	122.5	37	61	123.7	68.3	-	44.5	101	242.9	100.3	199.3	38
90	151.9	83.5	199.9	38	81	58.6	149.2	122.5	37	82	153.4	79.2	-	44.5	121	249.4	119.9	224.0	38
107	181.7	93.3	216.9	38	107	76.5	186.6	186.6	38	100	178.8	87.4	-	44.5	151	343.4	138.0	257.8	38
120	-	106.6	244.2	39	122	85.7	195.2	38	120	200.6	95.0	220.2	45	166	349.6	150.4	278.5	38	
142	219.8	120.5	259.1	39	173	113.0	244.8	37	140	235.1	108.0	236.6	45	181	384.5	156.6	288.8	38	
161	250.6	127.3	278.9	38	200	123.6	263.1	36	143	243.9	109.1	235.6	45	195	445.1	163.8	306.6	38	
180	276.2	139.7	301.2	39	209	135.0	276.4	36	167	268.1	116.7	253.0	45	210	470.9	167.4	313.7	38	
202	315.6	145.9	318.2	39	215	131.6	264.4	36	181	276.7	120.7	258.6	45	220	462.2	165.8	307.9	38	
210	310.8	153.6	315.5	35	227	120.2	260.2	35	195	301.0	127.1	261.1	44.5	230	489.5	160.1	296.5	38	
215	323.0	150.1	323.7	37	247	266	111.9	231.5	35	209	324.9	133.5	268.3	45	242	435.3	159.3	296.5	38
221	308.3	144.9	304.7	37	256	-	227.2	227.2	36	221	312.4	128.5	248.5	45	260	422.6	151.4	255.0	37
226	317.7	-	306.7	38	279	266	105.4	206.7	35	230	315.5	122.7	248.2	44	270	408.4	140.6	253.6	37
241	308.7	130.8	277.4	38	287	263	104.1	176.1	35	240	317.6	116.6	227.9	45	300	415.3	137.6	233.6	37
257	303.2	126.8	263.8	39	300	271	99.9	182.8	39	251	307.2	111.5	220.9	44.5	303	416.1	131.9	215.1	37
269	319.2	123.8	238.7	39	319	272	96.5	168.8	39	271	307.2	106.1	200.9	45	317	410.6	124.4	202.3	37
282	300.7	117.5	226.7	39	320	267	95.9	162.3	39	286	306.5	101.3	190.3	45	330	-	122.6	183.7	37
291	299.2	118.3	207.5	39	327	273	94.0	150.5	39	303	304.1	93.9	170.0	45	336	399.7	-	180.9	37
304	310.1	112.1	198.0	39						316	304.1	90.8	167.1	44.5					
341	315.6	115.4								330	283.4	87.5	160.3	45					
										331	282.3	85.0	157.4	45					
										350	287.4	79.4	151.7	45					
I	352.4	206.4	412		I	430.3	252	503		I	430.6	252.1	503.5		I	434.9	254.7	508.4	
PV	1.140				PV	1.599				PV	1.462				PV	0.960			
BW	23				BW	32				BW	33.5				BW	22			
PV Plasma volume (l) BW Body weight (kg) Ht Hematocrit (%) I number of units infused (U)																			

Table 12. Plasma activities of ALT, AST and GPI during and after intravenous infusion of enzyme preparations obtained from dog liver.

Experiment 5					Experiment 6					Experiment 7					Experiment 8				
Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Ht (%)
0	30.4	16.9	77.3	45	0	13.7	10.1	50.2		0	24.9	7.5	36.2	38	0	30.3	10.5	40.0	40.5
22	66.0	30.1	88.1	44	5	18.1	13.5	52.1	52.1	10	35.9	12.4	44.7	36	10	34.6	15.1	47.6	40.5
31	75.4	38.8	105.3	44	11	26.1	15.4	58.6		16	37.4	13.9		36	20	41.6	18.0	58.0	40.5
44	95.8	47.2	114.7	43.5	16	33.8	19.3	60.4		22	38.4	15.2	44.4	36	30	41.8	22.0	63.4	41
63	122.6	62.4		43.5	21	39.9	23.5	74.3		32	47.8	18.2		35	50	59.3	29.3	71.5	40
81	149.4	80.8	152.3		30	50.8	29.0	89.8		37	47.1	18.3	50.8	35	70	64.8	33.4	72.8	39.5
97	170.0	88.3	164.2	43.5	42	62.1	32.6	95.6		47	51.6	21.1	51.9	35	90	81.4	42.1	88.5	39
102	177.7	88.3	180.7	43.5	60	62.1	32.6	88.7		56	57.2	22.5	53.4	35	110	105.1	57.6	112.0	40
120	203.8	95.3	198.1	43	77	69.0	40.7	97.6		72	65.1	25.9		35	131	138.3	75.2	142.2	38
142	224.9	105.3	220.5	42.5	91	81.3	44.7	111.7		89	73.9	28.8	65.2	35	151	166.9	91.0	162.4	38.5
164	262.4	120.6	234.6	42	110	108.9	58.3	128.2		106	96.4	38.0	75.0	34	170	193.5	99.3	197.0	
182	283.5	125.5	248.3	47	130	125.2	68.5	144.1		121	111.5	45.9	92.1		181	209.9	98.5	182.6	
191	293.9	130.1	250.6		150	160.8	79.3	179.7		140	126.3	56.4	106.3		200	214.5	90.4	179.2	
201	307.9	133.2	258.3	43	171	189.1	94.2	187.7		159	156.1	66.4	124.0	35	220	218.3	111.5	174.7	
209	322.3	136.0	255.9	44.5	185		104.7	212.0		170	180.8	72.3	130.4	35	241	230.0	114.8	175.8	
215	320.8	136.1	252.6	43.5	195	207.6	99.8	207.1		179	190.2	75.3	127.2		262	236.5	119.1	177.0	
222	320.9	130.9	249.2	44	205	205.8	96.1			192	197.9	79.1	135.6		284	241.4	117.0	169.1	
234	322.1	124.8		43.5	216	206.1	93.0	178.3		200	199.0	76.3	128.8	34.5	299	242.9	103.8	170.2	36.5
249	310.7	121.0		43.5	227	203.6	90.1	174.0		205	199.0	76.3	128.8		312	245.9	102.2	157.9	36
263	315.5	114.5	214.3	43	240	196.7	86.5			215	200.3	78.6	125.2	35	325	238.0	100.8	147.8	37
273	306.0	105.8	201.9	43.5	252	196.0	84.0	155.8		235	213.1	81.1	126.9	35	343	252.9	99.3	142.2	
283	311.0	109.0	194.6	43	261	199.2	84.5	148.4		254	217.1	82.5	126.6	34	362	247.2	95.7	138.9	
303	298.4	96.2		43.5	266	199.2	83.5			270	230.2	81.3	122.7	34	371	230.0	94.8	137.8	
318	300.1	94.4	169.6	43	272	198.9	79.8	138		285	229.1	81.0	115.3	33	386	228.7	94.9	133.3	
331	300.9	90.7		43	304	228.0	80.6	108.3		304	228.0	80.6	108.3						
344	289.9	87.5	151.9	44	320	231.3	80	107.7		320	231.3	80	107.7						
358	295.7	86.9	145.5	43	333	235.6	79.7			333	235.6	79.7		33					
					353	237.8	76.1	99.2		353	237.8	76.1	99.2						
					360	240.0	76.2			360	240.0	76.2							
I	402.8	236	471		I	330.3	193.6	386		I	378.3	221.5	442.1		I	271	159	317	
PV	1.395				PV	1.650				PV	1.650				PV	1.190			
BW	32				BW	33				BW	33				BW	24			

PV, BW, Ht, I, see Table 11.

Table 13. Plasma activities of ALT, AST and GPI during and after intravenous infusion of enzyme preparations obtained from dog liver.

Experiment 9				Experiment 10				Experiment 11				Experiment 12				
Time min.	ALT (u/l)	AST (u/l)	GPI (u/l)	Time min.	ALT (u/l)	AST (u/l)	GPI (u/l)	Time min.	ALT (u/l)	AST (u/l)	GPI (u/l)	Ht. (%)	Time min.	ALT (u/l)	AST (u/l)	GPI (u/l)
0	18.3	9.1	39	0	23.7	16.2	118.5	0	32.5	21.9	59.0	51	0	82.3	10.4	138.3
22	36.3	17.6	51.9	28	56.1	29.0	143.4	7	36.0	22.9	61.2	48	24	68.5	12.2	
31	42.1	19.4	61.3	41	64.0	34.4	158.7	18	44.2	25.7		49	40	102.5	18.7	155.9
40	50.4	24.7	67.6	51	74.6	37.9	169.8	22	45.4	27.9	63.7		52	112.1	23.5	170.1
52	64.6	28.7	77.4	61	82.3	42.9	171.1	29	48.6	28.2			61	115.2	25.3	196.3
60	70.3	32.6	83.1	67	94.1	49.5	173.9	44	59.6	29.7	69.1	47	72	136.4	33.4	200.0
71	89.3	43.1	108.9	73	109.5	55.9	199.2	50	62.9	32.0	73.7	47	80	149.5	41.0	207.5
93	145.5	65.3	199.4	90	167.0	74.4	227.7	60	67.5	38.2	80.2	47	90	179.1	53.7	213.5
110	192.4	81.0	211.6	105	199.1	88.5	266.0	80	87.4	45.2			103	197.8	64.8	244.9
131	231.6	114.2		122	250.2	107.5	309.4	96	106.0	56.3	103.7		124	249.8	80.3	279.3
150	268.7	122.7	279.9	143	294.4	119.8	355.3	110	108.7	60.4			135	259.5	85.9	289.7
156	287.5	128.4	290.1	144	297.9	120.7	334.5	136	143.4	72.1	135.2	47	149	288.4	94.5	312.9
175	280.8	130.9	289.9	151	287.6	114.3	325.0	155	168.9	87.0			166	305.6	104.5	306.2
190	285.8	120.3	289.8	163	284.6	107.8	313.9	172	210.9	104.9	198.0		178	311.5	106.2	290.5
202	306.0	125.3	279.7	175	306.5	112.8	331.4	210	139.8	263.8			203	324.9	106.4	263.8
210	307.2	125.9	286.2	191	325.0	118.4	326.4	227	345.6	160.0	282.4		242	332.4	103.5	242.3
220	308.2	126.3	256.1	210	342.6	118.0	334.7	240	367.0	172.5	307.1		253	331.4	101.2	229.9
230	303.1	126.3	278.0	216	336.1	120.0	330.4	255	406.6	195.1	332.3		269	325.8	94.6	214.6
240	308.0	122.8	282.4	225	320.3	113.0	290.8	265	397.0	193.8	320.5		280	319.9	91.1	205.6
252	304.6	114.7	272.3	232	323.8	108.1	280.9	278	401.6	192.5	313.9		300	311.8	85.3	191.8
263	301.9	113.5	213.5	249	322.7	104.4	256.8	284	403.4	182.1	284.4		313	308.1	83.3	185.1
270		196.5		261	323.1	101.1	242.9	292	356.3	174.4	275.2		329	305.3	80.8	175.7
279	289.8	109.1	182.4	270	322.0	96.4	239.1	299	399.0	172.3	265.8		360	302.1	77.1	164.5
286	300.3	102.1	179.9	271	317.0	96.6	224.6	310	392.0	157.3	254.9		390	299.6	73.5	157.4
290	285.5	101.6	173.5	305	314.0	97.3	210.4	325	386.0	152.2	234.3					
				306	310.5	93.9	201.5	339	395.4	150.1	209.1					
								346	382.2	143.0	206.1					
I	436	217	500	I	485	241	556	I	560	328	655		I	365	182.1	420
PV	1.353			PV	1.395			PV	1.352				PV		1.303	
BN	27			BN	32			BN	31				BN	32		

PV, BN, Rt. I, see Table 11.

Table II Intravenous bolus injections of enzyme preparations obtained from liver tissue.

Experiment 1				Experiment 2				Experiment 3				Experiment 4				Experiment 5			
Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)	Time min.	ALT (U/l)	AST (U/l)	GPI (U/l)
0	22.1	11.4	51.0	0	16.3	7.8	14.2	0	7.8	14.2		0	42.2	9.5	36.9	0	11.9	11.2	
10	393.0	218	532	5	509.0	5	406.8	224	5	406.8	224	10	559	121.0	251	4	512	194	
20	381.7	207.8	504	45	509.3	45	402.0	193	45	402.0	193	20	535	115.8	247	30	499	169	
30	374.1	194.2	501	115	472.0	85	404	162	85	404	162	30	548	109.0	244	83	452	165	
40	369.8	182.5	481	177	437.5	125	402	147	125	402	147	40	537	104.9	225	145	436	145	
55	362.2	167.5	458	243	415.5	180	382	125	180	382	125	50	536	102.3	220	212	430	122	
61	357.1	165.5	442	303	395.0	234	338	103	234	338	103	60	536	94.8	212	293	402	99	
87	352.3	156.7	415	362	385.0	300	341	84.5	300	341	84.5	120	510	86.3	166	384	396	87	
120	344.1	128.8	349	420	344.5	364	341	77.6	364	341	77.6	178	503	86.3	166	426	367	76	
138	338.5	122.5	322	510	325.0	426	336	52.6	426	336	52.6	240	477	59.0		493	354	69	
486	249.2	108.8		600	311.0	488	315	47.0	488	315	47.0	275	466			555	346	62	
663	223.2			690	294.7	540	318	43.9	540	318	43.9	300	46			595	350	59	
773	219.2			745	262.0	656	293	34.9	656	293	34.9	315	450			1400	268	18	
1225	169.4			1252	225.5	722	271	25.5	722	271	25.5	360	444			1458	248	17	
1443	160.0			1340	223.5	782	263	26.3	782	263	26.3	390	436			1520	240	16	
1685	149.5			1410	223.0	1382	320		1382	320		421	428			1641	233	12	
1917	135.3			1500	211.0	1497	213		1497	213		1477	269			1697	227	10	
2035	127.5			1610	220.8	1612	211		1612	211		1510	281			1809	219		
2093	129.4			1740	204.4	1742	214		1742	214		1530	282			1891	212		
2665	114.5			1852	198.9	1858	208		1858	208		1559	288			1935	209		
3127	105.3			2025	186.5	1982	198		1982	198		1584	276			2865	169		
3200	106.1			2092	178.9	2086	188		2086	188		1603	276			3000	157		
3503	100.3			2205	175.8	2204	183		2204	183		1684	269			3080	156		
3577	100.1			2740	157.0	2848	162		2848	162		1769	269			3225	152		
4105	89.0			2855	150.5	3066	155		3066	155		1918	262			3366	149		
4318	93.4			3064	150.0	3121	150		3121	150		2900	225			4320	119		
4370	79.3			3120	149.3	3243	144		3243	144		3115	212			4470	113		
4733	78.2			3305	141.0	3359	131		3359	131		3220	211			4630	107		
4836	82.2			3338	133.7	3487	130		3487	130		3500	204			4740	105		
5036	63.2			3560	124.9	3629	135		3629	135		3775	196			5770	90		
5813	63.2			4275	107.7	4342	106		4342	106		3810	202			5983	81		
5760	64.1			4210	103.0	4440	104		4440	104						6150	87		
6005	58.7			4441	105	4441	105		4441	105									
6140	58.9			4970	100.7	5905	83		5905	83									
				5720	86.9	6025	52		6025	52									
				5940	80.7														
Plasma volume (l) 1.080 1.048 1.056				1.048				1.392 1.404				1.005 1.040 1.089				1.396 1.411			
Body weight (kg) 23				23				27				22				24			

COMPLETE DATA CHAPTER VI
Table III. Intramuscular injections of AST and ALT.

Experiment 1				Experiment 2				Experiment 3				Experiment 4				Experiment 5			
Time min.	AST (U/l)	ALT (U/l)	CK (U/l)	Time min.	AST (U/l)	ALT (U/l)	CK (U/l)	Time min.	AST (U/l)	ALT (U/l)	CK (U/l)	Time min.	AST (U/l)	ALT (U/l)	CK (U/l)	Time min.	AST (U/l)	ALT (U/l)	CK (U/l)
0	10.0	17.3	4.9	0	8.2	46.5	13.1	0	9.9	11.8	12.5	0	9.0	9.3	15.7	0	12.7	54.4	15.9
32	10.2	19.1	6.7	53	25.6	85.7	17.1	29	9.9	20.9	10.6	25	10.0	9.2	10.1	50	41.2	127.0	22.3
59	10.2	18.7	7.3	120	32.1	101.1	13.4	60	15.0	31.3	12.4	70	28.2	59.0	14.3	70	45.7	134.3	20.2
88	10.2	19.4	6.0	170	37.2	117.8	17.3	99	22.6	56.3	13.9	117	30.6	67.9	14.6	85	44.3	140.6	17.0
125	10.2	21.9	5.7	212	38.9	121.8	9.0	162	22.1	58.1	13.2	180	33.1	78.8	13.1	121	53.9	158.4	19.1
154	13.7	23.9	5.0	292	37.7	139.1	5.1	221	31.1	91.8	12.4	240	41.3	99.2	12.6	179	68.8	204.1	20.2
201	14.1	30.6	7.6	362	42.2	142.2	4.4	280	31.6	91.7	14.2	299	46.6	113.4	12.0	260	86.8	257.1	20.9
215	14.9	32.4	5.0	407	43.9	145.7	7.7	346	29.7	92.2	9.8	363	50.9	128.3	13.4	301	86.2	282.8	20.7
242	16.4	34.3	4.7	487	42.8	149.4	4.4	405	30.8	100.5	14.4	504	50.1	133.0	18.4	364	78.6	282.2	
270	23.4	46.8	7.2	535	41.4	155.1	10.1	484	33.0	114.6	15.7	601	53.4	150.7		436	76.7	301.0	24.5
308	24.7	52.3	12.5	602	38.2	152.8	10.1	561	38.6	135.0	18.4	668	52.9	185.9	18.4	540	64.6	285.0	18.5
330	27.0	55.4	17.3	660	40.1	160.0	11.1	651	46.9	155.6	19.9	794	56.5	182.2	20.6	607	74.4	316.6	20.8
362	28.6	57.6	22.0	725	42.2	166.6	11.0	777	43.9	167.9	13.1	902	57.7	195.2	22.7	731	61.7	311.2	20.9
380	28.6	60.7	18.6	1235	35.4	183.5	9.7	886	48.8	183.0	10.0	993	58.4	199.2	22.6	850	61.0	332.2	30.6
530	54.9	109.2	23.4	1270	30.1	191.9	8.7	997	50.0	196.4	10.0	1446	51.5	238.6	10.5	851	60.3	325.6	29.9
660	53.6	121.2	28.2	1445	28.6	191.7	12.4	1447	40.3	220.6	11.8	1562	47.2	239.6	17.9	925	56.5	323.0	
740	48.9	126.0	29.2	1565	27.3	185.5	13.4	1527	40.3	224.9		1692	44.9	227.5	27.3	1403	48.7	355.2	32.4
1570	28.9	147.7	20.7	1670	26.0	189.2	12.1	1693	38.1	233.8		1857	38.4	227.5		1496	40.0	339.3	17.4
1690	24.8	148.1	19.3	1810	24.8	178.7	12.7	2218	31.3	230.0		2236	32.6	230.0	15.7	1639	38.5	347.1	24.6
				1962	24.9	162.0	8.7	2870	19.0	213.0		2880	22.6	233.6	13.6	1806	36.6	343.2	29.1
				2075	20.4	178.5		2870	13.0	187.0		3133	18.5	210.9		1857	31.2	344.0	18.2
				2760	17.6	176.0		3127	8.7	181.6		3362	16.3	201.9		1980	34.1	345.0	16.4
				2934	15.2	163.9		3348	6.6	170.1		3653	16.3	190.4	13.8	2170	26.8	339.0	13.9
				3080	12.1	158.1		3532	5.6	157.1		3855	15.1	169.8	14.8	2472	21.9	336.7	
				3223	13.7	169.6		4325	7.3	136.4		4344	10.3	155.9	17.4	2814	14.9	296.4	13.9
				3345	16.1	163.4		4718	6.0	136.4		4734	10.3	165.9	17.4	3063	14.1	290.0	
				4280	14.2	136.4		4970	6.0	136.4		5095	9.5	141.1	17.8	3298	13.9	279.0	20.9
				4470	10.1	125.7		5815	6.0	112.7		5932	9.0	128.1	12.9	3580	11.8	268.5	
				4705	13.4	110.9		6125	7.3	105.3		6076	9.3	120.5		3552	13.8	273.3	16.0
								6225	7.3	98.4		6926	8.8	116.5		4340	11.9	227.0	
								6278	7.1	94.4		7306	10.4	99.3		4675	11.8	207.5	
								7280	6.1	88.5		7470	8.1	94.5		5000	9.9	192.6	15.7
								7420	6.1	88.5		7706	8.1	94.5		5767	9.9	166.4	
								8480	7.4	72.8		8758	9.0	77.7	15.8	6145	8.9	163.4	
								10130	6.5	50.2		10146	9.3	57.7	6.4	7235	9.9	136.4	
																8695	9.9	108.1	
Q	243.4	415.6	54.0	314	536	69		370	857	84		Q	378	875	96	Q	363	841	
PV	1.381			1.37				1.210				PV	1.045			PV	0.987		
BW	27							24				BW	22			BW	23		

Q

number of units injected (U)

PV plasma volume (l)

BW body weight (kg)

Table I *In vitro* thermal inactivation of different CK preparations at 49° C.

Time minutes	P1	P2	P3	P4	P5	P6	P7	P8
0	2510	2976	3085	3508	8354	3640	3159	8178
0	2706	2980	3178	3559	8670	3701	3073	8301
0	2616	3024	3113	3511	8622	3668	3095	8192
0	2526	3003	3223	3523	8773	3683	3333	8168
0	2479	2927	3141	3368	8566	3614	3053	8068
15	2253	2748	2749	3137	8046	3434	2898	7929
23	2162	2642	2499	2666	7668	3199	2835	7078
30	2079	2492	2472	2958	7490	-	-	-
41	1970	2353	2482	2670	7281	2878	2718	6828
53	1829	2258	2215	2513	6824	2821	2420	6451
71	1795	2083	2214	2431	6090	-	-	-
86	1683	1870	2049	2293	5620	-	-	-
100	1478	1856	1902	2112	5408	-	-	-
173	1147	1368	1417	1563	3722	-	-	-
237	767	1091	1103	1249	2966	-	-	-
292	757	861	916	928	2432	1116	959	2324
348	567	717	745	865	1900	894	574	1699
366	552	600	712	841	1819	-	-	-
381	505	596	699	787	1662	800	608	1720
398	465	545	625	736	1657	-	-	-
415	-	539	-	-	-	678	536	1474

- all values are expressed in units per litre (u/l).

- P1-P8 : correspond to numbers of the preparations obtained from heart tissue.

Table II *In vitro* thermal inactivation of different CK preparations at 53° C.

Time minutes	P1	P2	P3	P4	P5	P6	P7	P8
0	9550	11810	10900	12650	1344	12100	10660	12060
0	9550	11800	11000	12220	1367	11893	10373	11540
0	9776	11580	10700	12380	1354	11786	10060	11560
0	9700	11700	10670	12570	1349	11980	10733	12100
0	9595	11680	10890	12130	1350	12086	10747	12480
5	9130	10620	9990	11340	1226	10747	9520	11243
10	8130	9250	8720	9860	1083	9227	8320	9670
15	6510	7470	7125	7885	819	-	-	-
20	5255	6435	6100	6655	685	6283	5660	6517
25	4440	5470	5250	5625	553	-	-	-
30	3680	4580	4470	4593	473	4637	4087	4713
40	2610	3338	3198	3438	341	-	-	-
50	1993	2300	2355	2335	247	-	-	-
60	1478	1763	1860	1970	201	-	-	-
70	1185	1335	1473	1468	154	1335	1217	1420
91	629	796	938	723	101	-	-	-
114	381	495	558	523	57	420	401	451
139	241	272	328	248	37	254	236	274
158	182	233	268	238	31	-	-	-
175	156	163	216	136	27	143	147	171
210	139	120	166	103	23	-	-	-

Table III *In vitro* thermal inactivation of different GPI preparations at 53° C.

Time minutes	P1	P2	P3	P4	P5	P6	P7	P8	P11	P21	P31
0	292	437	426	432	401	500	404	452	365	355	345
0	281	440	431	430	398	499	405	458	361	353	336
0	285	445	433	435	397	494	408	450	356	354	340
0	284	446	438	437	399	497	404	461	358	355	339
20	253	389	377	391	354	440	358	404	326	315	304
50	188	302	298	303	282	348	283	318	253	260	242
90	135	218	212	224	207	260	210	233	190	197	182
120	104	170	167	172	160	200	162	181	155	162	148
150	82	127	127	131	127	157	124	140	127	133	124
192	58	91	89	97	92	117	96	105	99	104	93
212	48	77	75	82	76	96	80	90	84	92	82
227	40	67	66	69	67	87	70	79	75	84	76

Table V *In vitro* thermal inactivation of different AST preparations at 62° C.

Time minutes	P1	P2	P3	P4	P5	P6	P7	P8	P11	P21
0	250	229	199	215	200	143	217	218	185	270
0	256	234	193	211	191	147	214	219	181	278
0	260	229	191	211	190	145	221	218	180	273
0	255	232	191	208	195	146	211	219	179	277
16	220	195	166	175	161	116	162	184	148	229
46	170	181	144	149	154	92	142	165	127	204
91	129	156	124	119	134	70	119	145	100	182
151	90	133	96	87	109	48	94	116	74	138
204	68	112	82	71	93	39	77	100	59	115
244	58	96	70	59	82	32	67	85	50	100
321	42	75	53	47	64	25	51	66	37	73
360	37	65	46	38	55	21	47	58	32	62

Table IV *In vitro* thermal inactivation of different LD preparations at 62° C.

Time minutes	P1	P2	P3	P4	P5	P6	P7	P8
0	946	796	442	785	738	632	778	768
0	907	816	437	818	706	664	788	743
0	955	789	463	805	727	650	758	785
0	918	817	434	797	690	677	760	757
16	650	575	270	519	485	400	508	583
46	490	449	199	388	379	322	413	423
91	478	425	173	380	356	304	367	405
151	416	398	160	338	308	285	353	357
204	404	348	155	300	297	233	318	332
244	376	335	134	301	285	232	308	320
321	335	295	133	260	247	210	263	273
360	330	290	130	254	233	198	257	257

- Enzyme activities are expressed in units per litre (u/l)
 - P1- P8 enzyme preparations obtained from heart tissue.
 - P11-P61 enzyme preparations obtained from liver tissue.

Table VI *In vitro* thermal inactivation of different ALT preparations at 62° C.

Time min.	P4L	P5L	Time min.	P1L	P2L	P3L	P6L
0	271	533	0	318	215	466	417
0	275	561	0	330	215	495	411
0	267	558	0	324	218	495	432
0	272	556	0	323	227	483	429
0	264	554	15	292	200	456	403
11	249	521	46	245	183	412	355
26	227	-	100	184	150	348	293
34	222	496	165	132	125	284	236
45	209	-	233	97	98	230	189
61	158	437	30	83	86	193	153
75	177	-	341	67	70	165	126
118	139	369	375	55	64	140	121
153	123	-	-	-	-	-	-
189	100	275	-	-	-	-	-
221	89	-	-	-	-	-	-
260	66	205	-	-	-	-	-
307	60	-	-	-	-	-	-
325	51	164	-	-	-	-	-

Table VII. Temperature dependence of CK thermal inactivation rate.

Temperature °C	C/C ₀ (p1)	C/C ₀ (p3)
46	0.96 ± 0.01	0.8 ± 0.1
47	0.87 ± 0.05	0.81 ± 0.06
48	0.88 ± 0.05	0.84 ± 0.07
49	0.75 ± 0.09	0.81 ± 0.07
50	0.68 ± 0.09	0.77 ± 0.06
51	0.56 ± 0.04	0.69 ± 0.05
52	0.49 ± 0.09	0.50 ± 0.05
53	0.37 ± 0.07	0.45 ± 0.04
54	0.23 ± 0.03	0.23 ± 0.02
55	0.19 ± 0.01	0.190 ± 0.006
56	0.164 ± 0.01	0.168 ± 0.003
57	0.054 ± 0.006	0.058 ± 0.001
58	0.0034 ± 0.0006	0.0036 ± 0.0009

Table VIII. Temperature dependence of GPI thermal inactivation rate.

Temperature °C	C/C ₀ (p1)	C/C ₀ (p3)	C/C ₀ (p1L)	C/C ₀ (p2L)
48.8	0.99 ± 0.02	1.01 ± 0.02	1.01 ± 0.01	1.00 ± 0.01
49.8	0.99 ± 0.01	0.99 ± 0.02	1.00 ± 0.01	0.98 ± 0.02
51.2	1.00 ± 0.04	0.96 ± 0.01	0.98 ± 0.01	0.96 ± 0.02
52.0	0.94 ± 0.03	0.92 ± 0.01	0.93 ± 0.02	0.91 ± 0.02
52.9	0.84 ± 0.02	0.82 ± 0.01	0.85 ± 0.01	0.83 ± 0.02
54.1	0.64 ± 0.02	0.66 ± 0.01	0.68 ± 0.02	0.67 ± 0.01
55.1	0.41 ± 0.01	0.43 ± 0.007	0.47 ± 0.010	0.46 ± 0.010
56.2	0.20 ± 0.005	0.22 ± 0.005	0.27 ± 0.006	0.26 ± 0.007
57.2	0.051 ± 0.0040	0.070 ± 0.0015	0.12 ± 0.0056	0.11 ± 0.0037

Table IX. Temperature dependence of AST thermal inactivation rate.

Temperature °C	C/C ₀ (p1)	C/C ₀ (p3)	C/C ₀ (p1L)	C/C ₀ (p2L)
58.3	0.85 ± 0.02	0.73 ± 0.01	0.81 ± 0.02	0.85 ± 0.01
59.2	0.83 ± 0.01	0.74 ± 0.01	0.81 ± 0.03	0.88 ± 0.02
60.1	0.80 ± 0.01	0.70 ± 0.01	0.79 ± 0.02	0.85 ± 0.02
61.4	0.80 ± 0.02	0.70 ± 0.01	0.79 ± 0.02	0.85 ± 0.02
62.5	0.76 ± 0.01	0.65 ± 0.01	0.76 ± 0.02	0.83 ± 0.02
63.4	0.75 ± 0.01	0.63 ± 0.01	0.73 ± 0.02	0.83 ± 0.02
64.5	0.71 ± 0.02	0.61 ± 0.01	0.71 ± 0.02	0.79 ± 0.01
65.5	0.66 ± 0.008	0.58 ± 0.02	0.70 ± 0.02	0.79 ± 0.02
66.8	0.59 ± 0.009	0.53 ± 0.01	0.65 ± 0.03	0.72 ± 0.02
67.7	0.54 ± 0.02	0.48 ± 0.01	0.61 ± 0.02	0.66 ± 0.005
68.7	0.44 ± 0.005	0.42 ± 0.005	0.53 ± 0.02	0.56 ± 0.012

Table X.

Temperature dependence of ALT thermal inactivation rate.

Temperature °C	C/C ₀ (p1L)	C/C ₀ (p2L)
58.3	0.97 ± 0.03	0.97 ± 0.02
59.2	0.95 ± 0.02	0.96 ± 0.02
60.1	0.93 ± 0.03	0.94 ± 0.02
61.4	0.92 ± 0.02	0.92 ± 0.02
62.5	0.88 ± 0.03	0.88 ± 0.01
63.4	0.80 ± 0.02	0.82 ± 0.01
64.5	0.71 ± 0.01	0.73 ± 0.02
65.5	0.53 ± 0.01	0.60 ± 0.01
66.8	0.35 ± 0.007	0.43 ± 0.004
67.7	0.19 ± 0.0006	0.27 ± 0.006
68.7	0.09 ± 0.0081	0.13 ± 0.0080

Table XI.

Temperature dependence of LD thermal inactivation rate.

Temperature °C	C/C ₀ (p1)	C/C ₀ (p3)
58.3	0.76 ± 0.03	0.81 ± 0.04
59.2	0.68 ± 0.02	0.76 ± 0.02
60.1	0.60 ± 0.02	0.67 ± 0.02
61.4	0.52 ± 0.02	0.58 ± 0.03
62.5	0.45 ± 0.02	0.51 ± 0.01
63.4	0.39 ± 0.01	0.47 ± 0.02
64.5	0.37 ± 0.02	0.44 ± 0.01
65.5	0.31 ± 0.01	0.36 ± 0.02
66.8	0.21 ± 0.003	0.24 ± 0.012
67.7	0.08 ± 0.0084	0.12 ± 0.012
68.7	0.02 ± 0.0060	0.03 ± 0.0082

- Thermal inactivation time: 1500 sec.

- C : concentration in U/l after 1500 sec thermal inactivation

- C₀ : concentration in U/l before thermal inactivation was started

- p1, p3: enzyme preparations obtained from heart tissue.

- p1L, p2L enzyme preparations obtained from liver tissue.

COMPLETE DATA CHAPTER VII.

Table XIII, 2,3,4 In vivo clearance of four different CK and AST preparations in four different dogs.

Dog 1						
Enzyme preparation	K_d CK (h^{-1})	dK (%)	Res (%)	K_d AST (h^{-1})	dK (%)	Res (%)
1	0.26	9.3	1.1	0.23	18.6	1.5
2	0.24	8.0	1.1	0.16	10.0	0.9
3	0.24	14.6	2.1	0.13	13.5	1.0
4	0.26	10.5	1.5	0.19	13.8	1.3
mean	0.26	9.8	1.4	0.22	14.3	1.6
CV	5.9	45	48	34	27	47

Dog 2						
Enzyme preparation	K_d CK (h^{-1})	dK (%)	Res (%)	K_d AST (h^{-1})	dK (%)	Res (%)
1	0.37	6.8	1.5	0.24	7.0	0.9
2	0.39	6.9	2.1	0.21	13.7	2.1
3	0.41	12.2	2.4	0.17	10.0	0.7
4	0.37	10.4	1.6	0.19	10.3	1.1
mean	0.37	7.9	1.7	0.27	16.5	1.1
CV	8.5	41	35	16.5	29	61

Table XIII2

Table XIII3

Dog 3						
Enzyme preparation	K_d CK (h^{-1})	dK (%)	Res (%)	K_d AST (h^{-1})	dK (%)	Res (%)
1	0.32	3.9	1.0	0.19	8.4	1.2
2	0.31	11.3	1.8	0.17	18.5	1.2
3	0.39	12.2	2.2	0.19	11.0	2.6
4	0.36	4.3	4.6	0.22	1.9	1.7
mean	0.37	8.3	3.8	0.24	12.1	3.3
CV	0.31	8.5	2.0	0.28	7.2	1.4

Table XIII4

Dog 4						
Enzyme preparation	K_d CK (h^{-1})	dK (%)	Res (%)	K_d AST (h^{-1})	dK (%)	Res (%)
1	0.30	3.2	0.9	0.21	7.4	1.3
2	0.31	8.2	2.2	0.18	6.5	1.2
3	0.30	12.8	1.9	0.16	12.9	1.8
4	0.39	7.1	3.3	0.14	7.1	1.1
mean	0.33	14.4	2.2	0.18	11.5	1.2
CV	0.29	12.8	2.0	0.23	7.0	0.8

K_d : apparent clearance constant (h^{-1})

dK: 95% confidence limit expressed as a percentage of K_d
 residue: quality of fit expressed as mean residual deviation
 per data point expressed as a percentage of measured parameter (%).

APPENDIX II

Mathematical models used

The response on a known input in plasma

In this section we derive some formulas commonly used for the description of plasma activity (or concentration) curves as observed upon infusion or bolus injection of protein into the circulation. As discussed in chapter II.2. the dynamics of the plasma and extravascular concentration are given by (cf. equation (12) and (13), chapter II.2.).

$$\frac{d}{dt} C_i = -kC_i + P/V_i C_e - P/V_i C_i + f(t) \quad (A_1)$$

$$\frac{d}{dt} C_e = P/V_e C_i - P/V_e C_e$$

- with C_i - the plasma concentration (mol/l) or (U/l)
 C_e - the concentration in the extravascular pool
 k - the fraction of protein catabolized per hour in plasma;
 k is the rate constant of the first order degradation reaction (h^{-1})
 P/V_i - the transcapillary escape rate (= TER), i.e. the fraction of protein escaping per hour from the vascular pool through the capillary wall to the interstitium (h^{-1})
 P/V_e - the fraction of the extravascular pool of protein returning to the circulation (h^{-1}).

Solutions of A_1 are in general combinations of exponential terms. The solution $C_i(t)$ and $C_e(t)$ in case of a bolus injection, i.e. $f(t) = 0$ $t > 0$; $C_i(0) = f_0$ and $C_e(0) = 0$ is given by:

$$C_i(t) = Ae^{-at} + Be^{-bt} \quad (A_2)$$

$$C_e(t) = \frac{P/V_e}{b-a} (e^{-at} - e^{-bt})$$

$$\text{with } A = \frac{P/V_e - a}{b - a} f_0, \quad B = \frac{b - P/V_e}{b - a} f_0; \quad A + B = f_0$$

f_0 = amount injected per litre of plasma

$$a = \frac{(k + P/V_i + P/V_e) - \sqrt{((k + P/V_i + P/V_e)^2 - 4kP/V_e)}}{2}$$

$$b = \frac{(k + P/V_i + P/V_e) + \sqrt{((k + P/V_i + P/V_e)^2 - 4 kP/V_e)}}{2}$$

The responses $C_i(t)$ and $C_e(t)$ for general $f(t)$ may be constructed from (A₂) by conceiving the input $f(t)$ as a series of bolus injections f_i on $t = t_i$. Due to the linearity of equation (A₁) the sum of a series inputs results in the sum of the corresponding responses:

$$C_i(t) = \sum_{i=1}^n f_i (Ae^{-a(t-t_i)} + Be^{-b(t-t_i)})$$

and

$$C_e(t) = \sum_{i=1}^n f_i \frac{P/V_e}{b-a} (e^{-a(t-t_i)} - e^{-b(t-t_i)})$$

In the limit of very many bolus injections on a dense set of times t_i the above equations transform to

$$\begin{aligned} C_i(t) &= \int_0^t f(s) (Ae^{-a(t-s)} + Be^{-b(t-s)}) ds \\ C_e(t) &= \int_0^t f(s) \left(\frac{P/V_e}{b-a} \right) (e^{-a(t-s)} - e^{-b(t-s)}) ds \end{aligned} \quad (A_3)$$

Quantitation

Often one encounters the situation that one wants to infer from the measured plasma activity curve $C_i(t)$ the amount of protein released into the plasma. *I.e.* disposing of $C_i(t)$, given in formula (A₃) one must calculate $f(t)$ or preferably the integral $F(t)$:

$$F(t) = \int_0^t f(s) ds \quad (A_4)$$

$F(t)$ is the cumulative input in plasma. An expression for $F(t)$ is obtained by interpretation of equation (A₁): The amount of protein released into the circulation must equal the sum of the quantity catabolized and the quantity still present the body *i.e.* $V_i C_i + V_e C_e$. Per hour there is catabolized $kC_i(t)$ from the plasma pool; hence the quantity catabolized up to time t equals

$$k \int_0^t C_i(s) ds$$

and thus

$$V_i F(t) = V_i C_i(t) + V_e C_e(t) + V_i k \int_0^t C_i(s) ds \quad (A_5)$$

Now we should express $C_e(t)$ in terms of $C_i(t)$. This is done by solving equation (cf. A_1)

$$\frac{d}{dt} C_e = -P/V_e C_e + P/V_e C_i(t) \quad (A_6)$$

in an analogous way as we solved equation (A_1).

The special exponential solution of (A_6) with $C_i(t) = 0$ is

$$C_e(t) = e^{-(P/V_e)t}$$

This special solution satisfies

$$\frac{d}{dt} C_e(t) = -P/V_e C_e ; C_e(t=0) = 1$$

which is the solution of (A_6) in the case $P/V_e C_i(t)$ is a bolus injection at $t = 0$ in the extravascular space. Using the linearity of equation (A_6) and the superposition principle, i.e. the responses upon a sum of inputs ($P/V_e C_i(t)$) is the sum of the responses upon each of the inputs, the general solution is written as (cf. the derivation of formula A_3)

$$C_e(t) = \int_0^t P/V_e C_i(s) e^{-P/V_e(t-s)} ds$$

Insertion of this last expression for $C_e(t)$ in (A_5) results in:

$$F(t) = C_i(t) + k \int_0^t C_i(s) ds + P/V_i \int_0^t C_i(s) e^{-(P/V_e)(t-s)} ds \quad (A_7)$$

Thus we have expressed the cumulative input into plasma in terms containing only observable plasma concentrations and the values of the model parameters k , P/V_i and P/V_e .

In the special case that the time interval $(0, t)$ is so short that the exponential term $e^{-(P/V_e)(t-s)}$ shows little variation on this time interval, we may in good approximation substitute $e^{-(P/V_e)(t-s)}$ in the third term of (A_7) by a constant c , close to unity. Thus we obtain the approximation

$$F(t) = C_i(t) + k_d \int_0^t C_i(s) ds \quad (A_8)$$

of (A_7) , with $k_d = k + cP/V_i$.

APPENDIX III

Thermal inactivation of enzymes

S. Arrhenius, studying the influence of temperature on the rate of inversion of sucrose in 1889, was the first to give a physical interpretation of the observed relation between reaction rates and temperature. He established the relation

$$k = A.e^{-E_A/RT} \quad (1)$$

k = rate of inversion

R = gas constant

A = constant (so called "frequency factor")

E_A = constant (so called "Arrhenius activation energy")

T = absolute temperature.

In analogy with the classical (Boltzmann) theory which states that the number of particles with energy E (in a system with particles of different energies) depends on E according to $\exp(-E/RT)$, Arrhenius postulated an equilibrium between inert and activated molecules. Only activated molecules were able to take part in the activation process. Conversion of inert to activated state requires the energy E_A . About 1930 H.Eyring succeeded in deriving expressions for the empirical quantities A and E_A . He considered the activated state as an ordinary state possessing the usual thermodynamic properties, with the exception that motion in a specific direction in phase space, i.e. along the reaction co-ordinate, leads to irreversible denaturation. For the thermal inactivation of an enzyme E this implies:

where E denotes the native enzyme, $E^{\ddagger}$ the activated state, D the denatured state and k the rate of inactivation (denaturation). The number of activated molecules depends on the equilibrium constant $K^{\ddagger} = k_{+1}/k_{-1}$ between inert and activated state, so we obtain:

$$k = A.K^{\ddagger} = A.e^{-(\Delta G^{\ddagger}/RT)} \quad (2)$$

where the fundamental thermodynamic relation $\Delta G = -RT \ln K_{eq}$ has been used for the change in free energy ΔG between inert and activated state.

The frequency factor A is equated to the vibrational frequency ν of the critical vibration leading to irreversible transition. Combining the quantum mechanical expression $\epsilon = k_B T$ for the vibrational energy ϵ (h and k_B are Planck's and Boltzmann's constant) we have $\nu = \epsilon/h = k_B T/h$ and equation (2) becomes

$$k = \frac{k_B T}{h} \cdot e^{-(\Delta G^\ddagger/RT)} \quad (3)$$

or

$$\Delta G^\ddagger = RT \ln\left(\frac{k_B T}{kh}\right) \quad (4)$$

Substituting the thermodynamic relation $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ in (3), with $\Delta H^\ddagger$ and $\Delta S^\ddagger$ the change in respectively enthalpy and entropy for the transition from inert to activated state, we obtain by differentiating $\ln k$ with respect to $1/T$:

$$\Delta H^\ddagger = -R \frac{d \ln k}{d(1/T)} - RT \quad (5)$$

From (4) and (5) we obtain

$$\begin{aligned} \Delta S^\ddagger &= (\Delta H^\ddagger - \Delta G^\ddagger)/T \\ \Delta S^\ddagger &= -\frac{R}{T} \frac{d \ln k}{d(1/T)} - R - R \ln \frac{k_B T}{kh} \end{aligned} \quad (6)$$

Abbreviations

a	Slow disappearance constant in case of biphasic clearance (h^{-1}).
ADP	Adenosine 5'-diphosphate.
ATP	Adenosine 5'-triphosphate.
b	Fast disappearance constant in case of biphasic clearance (h^{-1}).
BW	Body weight.
C_e	Extravascular enzyme activity (U/l).
C_{eq}	Enzyme activity at equilibrium conditions after constant rate infusion (U/l).
C_i	Intravascular enzyme activity (U/l).
CL	Caudate lobe of the liver.
C_n	Normal steady state plasma enzyme activity (U/l).
C_o	Extrapolated activity at zero time (U/l).
C(t)	Enzyme activity in plasma at time t (U/l).
CV	Coefficient of variation, i.e. standard deviation as a percentage of the mean (%).
EIS	Enzymatic infarct size.
f	Rate of input of enzyme per litre plasma (U/l.h).
FITC-dextran	Fluorescein isothiocyanate labeled dextran.
g	Centrifugal acceleration (number of times gravity in the middle of the tube) (cm/sec^2).
$\Delta G^\ddagger$	Free energy of activation (J/mol). The difference in free energy between reactant (s) and transition state.
h	Planck's constant ($6.6256 \cdot 10^{-34} J \cdot sec$).
$\Delta H^\ddagger$	Enthalpy of activation; the difference in enthalpy between reactant (s) and transition state (J/mol).
HIS	Histologic infarct size.
Ht	Hematocrit value (%).
k	True clearance rate (h^{-1}).
K_{+1}	Reaction rate constant (h^{-1}).
$K^\ddagger$	Equilibrium constant for the formation of an activated complex.
K_B	Boltzmann's constant; $1.381 \cdot 10^{-23} J/deg$.
LL	Left lobe of the liver.
LV	Left ventricle of the heart.
NADH	Nicotinamide adenine dinucleotide.
NADPH	Nicotinamide adenine dinucleotide phosphate.
P	Permeability constant (l/h).
PV	Plasma volume (l).
QL	Quadrate lobe of the liver.
R	Gas constant ($8.3143 J/deg \cdot mol$).

res	Residue; quality of fit expressed as the mean residual deviation per data point expressed as a percentage of the measured values.
RL	Right lobe of the liver.
RV	Right ventricle of the heart.
$\Delta S^\ddagger$	Entropy of activation; the difference in entropy between reactant (s) and transition state.
SE	Septum of the heart.
T	Absolute temperature in degrees Kelvin $^\circ K = ^\circ C + 273.15$.
T-1824	Evans Blue dye.
TER	Transcapillary escape rate (%/h).
Tris	Tris (hydroxymethyl) - aminoethane.
U	International Unit. One unit of enzyme converts one micromole of substrate per minute under the given conditions.
V_e	Extravascular volume (l).
V_i	Intravascular volume (l).

Abbreviations used for enzymes

		EC
ALD	Aldolase	4.1.2.13
ALT	Alanine aminotransferase	2.6.1.2
AP	Alkaline phosphatase	3.1.3.1
AST	Aspartate aminotransferase	2.6.1.1
CAST	Cytoplasmic isoenzyme of AST	2.6.1.1
mAST	Mitochondrial isoenzyme of AST	2.6.1.1
CK	Creatine kinase	2.7.3.2
CK-MM	CK isoenzyme from muscle	2.7.3.2
CK-MB	CK isoenzyme from heart	2.7.3.2
G6P-D	Glucose 6-phosphate dehydrogenase	1.1.1.49
GPI	Glucosephosphate isomerase	5.3.1.9
HBD	α -Hydroxybutyrate dehydrogenase	1.1.1.27
HK	Hexokinase	2.7.1.1
ICD	Isocitrate dehydrogenase	1.1.1.42
LD	Lactate dehydrogenase	1.1.1.27
LD-1,2,3,4,5	LD-isoenzymes	1.1.1.27
MD	Malate dehydrogenase	1.1.1.37
PK	Pyruvate kinase	2.7.1.40

NAWOORD/ACKNOWLEDGEMENTS

Bij het gereedkomen van dit proefschrift wil ik iedereen, die hieraan op enigerlei wijze heeft bijgedragen van harte bedanken. Een aantal mensen wil ik toch ook nog graag met name noemen.

Hierbij denk ik in de allereerste plaats aan Wim Hermens en aan George Willems, die door hun altijd aanwezige interesse in de proeven, de positieve kritiek, maar vooral de zeer vele suggesties het verschijnen van dit proefschrift mogelijk hebben gemaakt. Ook Marijke Krill, die een gedeelte van de proeven voor haar rekening heeft genomen mag ik zeker niet vergeten. Vic Dreessen wil ik bedanken voor alle hulp bij het computerwerk.

Mijn promotor Prof. Dr. H.C. Hemker dank ik voor zijn interesse in het werk, de stimulerende invloed en voor de geboden faciliteiten binnen de Capaciteitsgroep Biochemie.

Voor steun bij het dierexperimentele werk dank ik Ton van den Bogaard, Inger Simons-Achtenberg en Cees Verlaan, maar vooral Ruud Kruger, die gedurende de afgelopen drie jaar op ieder moment bereid was me te helpen bij de experimenten en met wie ik altijd op zeer plezierige wijze heb samengewerkt. De goede verzorging van de honden door Huub Simons is zeker ten gunste van de experimenten geweest.

Voor het vele typewerk, dat gedurende de afgelopen maanden is verricht dank ik Mevr. E.M. Lelyveld-Jansé, maar zeer in het bijzonder Rosy Borgman-Hanssen, die de uiteindelijke versie van dit manuscript op uiterst plezierige en ook professionele wijze heeft verzorgd en daarbij de nodige fantasie in de lay-out heeft gestopt.

Ook mijn ouders, die zowel tijdens mijn studie, als tijdens de voorbereidingen voor dit proefschrift altijd zeer veel interesse hebben getoond en ook de nodige hulp hebben geboden, wil ik via deze weg nog eens van harte bedanken.

Gerbrand echter, heeft op velerlei wijzen een bijdrage geleverd aan de totstandkoming van dit proefschrift. Vooral tijdens het schrijven is menig discussie thuis gevoerd over zowel de inhoud als de stijl. De laatste maanden soms misschien ook wel eens teveel.

CURRICULUM VITAE

Op verzoek van de faculteit volgen hier enkele persoonlijke gegevens. Ik ben geboren op 27 februari 1954 te Amsterdam.

Na het eindexamen HBS-B aan het Develsteincollege te Zwijndrecht in 1971, liet ik mij in hetzelfde jaar inschrijven aan de Rijksuniversiteit te Leiden.

In juni 1974 werd het kandidaatsexamen in de studierichting S_4 (Scheikunde en wiskunde met natuurkunde) afgelegd. In augustus 1977 behaalde ik het doctoraalexamen met als hoofdvak Biochemie (Dr.J.E.Mellema/Prof. Dr.A.Rörsch), bijvak Medische Informatieverwerking (Dr.Ir.C.Tuinstra/Prof.Dr.A.C.Arntzenius) en tweede bijvak Röntgendiffractie (Prof.Dr. C.Romers).

Van september 1974 tot oktober 1977 was ik student-assistent op het Laboratorium voor Fysiologie bij Dr.G.Kerkhof.

Sinds oktober 1977 ben ik verbonden aan de Capaciteitsgroep Biochemie van de Medische Faculteit van de Rijksuniversiteit Limburg te Maastricht, alwaar dit proefschrift werd bewerkt.

Het onderzoek beschreven in dit proefschrift maakt deel uit van het project quantitering van weefselnecrose en eiwitmetabolisme en is uitgevoerd onder leiding van Dr.W.Th.Hermens.