# Biodegradable Microspheres. 17. Lysosomal Degradation of Primaquine–Peptide Spacer Arms

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**Abstract** □ The pharmacological activity of drugs bound to lysosomotropic drug carriers will depend on the rate of release of the drugs from the drug–carrier complex. We have now studied the enzymatic release of primaquine (PQ) from two groups of peptide–PQ derivatives by their incubation with rat liver lysosomal fraction. The derivatives have the general structure $NH_2$-X-Leu-Ala-Y-PQ and are intended to be coupled via their free α-amino group to starch microparticles. In the first group, Y was varied, being Leu, Tyr, Lys, or Asp, while X was Ala. In the second one, X was varied, being Ala, Tyr, Lys, or Asp, while Y was Leu. Thus, a systematic study of the significance of the varying amino acid composition of the tetrapeptides, which can serve as spacer arms in the microparticle–drug complexes, for the lysosomal release of PQ was possible. In addition, some ε-aminocaproic acid–PQ derivatives, which lack a free α-amino group, were incubated. This was done to study the importance of enzymes, other than aminopeptidases, during lysosomal degradation of these derivatives. The pattern and rate of degradation of all PQ derivatives was followed by HPLC analysis. The results obtained show that endopeptidases, as well as mono- and diaminopeptidases, degrade the derivatives. PQ cannot be cleaved directly from the derivatives by any carboxypeptidase-like enzyme. Asp peptides are digested slowly in the lysosomal fraction. The temporal aspects of reactions were quantitated using a kinetic model, in which first-order rate constants of all the steps of each peptide degradation sequence were estimated simultaneously.

## Introduction

The leishmaniases are a group of diseases caused by different species of protozoan parasites of the genus *Leishmania*.[1] In the visceral form, the parasites are, after infecting the host, phagocytosed into the lysosomes of the liver Kupffer cells, belonging to the reticuloendothelial system (RES). A rational approach to treat leishmaniasis would therefore be to target antileishmanial drugs directly to the same lysosomes. This can be achieved by coupling of the drugs to lysosomotropic carriers.

The polyacryl starch microparticles are lysosomotropic drug carriers developed in our laboratory.[2] It has been previously shown that an enzymatically cleavable peptide spacer arm between the drug and the carrier is an absolute requirement to get a release of free drug in a lysosomal milieu.[3,4] In our earlier studies,[5] the low molecular weight anti-leishmanial drug primaquine (PQ) was covalently coupled to microparticles (Mp) via tri-, tetra-, and pentapeptides of Ala and Leu. Mp-Ala-Leu-Ala-Leu-PQ was found to give the fastest release of free drug *in vitro* during incubations in a rat liver lysosomal fraction. The same conjugate significantly improved the effect of PQ in the treatment of leishmaniasis in mice.[6]

The liver lysosomes contain at least 50 acid hydrolases,[7] among them both endo- and exopeptidases. In order to study the specificity of these enzymes and their significance for the degradation of peptide spacer arms, via which PQ can be coupled to the microparticles, several peptide–PQ derivatives have been synthesized by us as described in the article preceeding this one.[8] Two groups of tetrapeptide-PQ derivatives, having a free α-amino-group, with general formula $NH_2$-X-Leu-Ala-Y-PQ, have in this study been incubated in a rat liver lysosomal fraction and the release of PQ quantified. The structure of the derivatives in the first group was systematically varied, so that Y was Leu, Tyr, Lys, or Asp, while X was Ala. In the second group, X was Ala, Tyr, Lys, or Asp, while Y was Leu. Leu-Ala-Leu-PQ and four amino acid–PQ derivatives (Leu-PQ, Tyr-PQ, Lys-PQ, and Asp-PQ) were separately incubated to confirm the degradation pattern of some of the PQ–tetrapeptides.

In addition, different ε-aminocaproic acid (EACA)–PQ derivatives, lacking an α-amino group, were studied to investigate the significance of a free α-amino group for the degradation of the peptides.

The digestion has been followed by reversed phase HPLC. A method was developed for each of the EACA–PQ and tetrapeptide–PQ derivatives to identify and quantify every of their possible PQ-containing cleavage products, obtained during the incubations.

Rate constants, for the separate degradation steps, have been calculated by applying a first-order chemical reaction (kinetic) model.

## Experimental Section

**Materials**—Acetonitrile was of HPLC grade and bought from Merck (Darmstadt, Germany) and Fisons Scientific Equipment (Loughborough, England). *N,N*-Dimethyloctylamine (DMOA)-pro analysi was from Janssen Chimica (Beerse, Belgium). The crystalline reduced glutathione (GSH) was from Sigma (St. Louis, MO), and the trichloroacetic acid (TCA)-pro analysi was from Merck. The following groups of peptide derivatives of PQ were incubated in a rat liver lysosomal fraction:

$P_1$ = Ala-Leu-Ala-**Leu**-PQ  $P_5$ = **Ala**-Leu-Ala-Leu-PQ

$P_2$ = Ala-Leu-Ala-**Tyr**-PQ  $P_6$ = **Tyr**-Leu-Ala-Leu-PQ

$P_3$ = Ala-Leu-Ala-**Lys**-PQ  $P_7$ = **Lys**-Leu-Ala-Leu-PQ

$P_4$ = Ala-Leu-Ala-**Asp**-PQ  $P_8$ = **Asp**-Leu-Ala-Leu-PQ

$P_9$ = Leu-Ala-Leu-PQ

In addition, EACA-Ala-Leu-PQ, EACA-Leu-PQ, and EACA-PQ were also incubated. References of all the possible PQ-containing cleavage products were used for identification purposes during the HPLC analyses.

All PQ derivatives used were synthesized by us as described in the article preceeding this one.[8]

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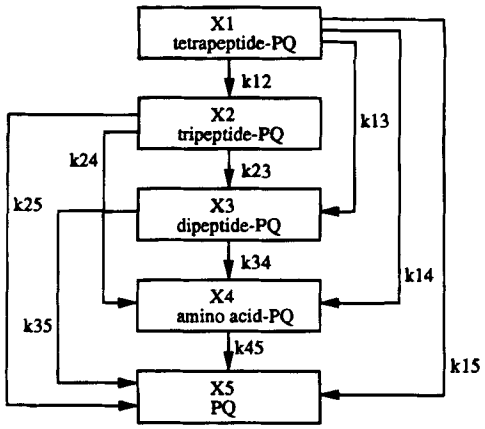


**Scheme 1**—Pattern of lysosomal degradation of the tetrapeptide–PQ derivatives ($X_1$), including all the possible PQ-containing cleavage products ($X_2$, $X_3$, $X_4$, and $X_5$), and the respective rate constants ($k_{12}$, $k_{13}$, etc.).

**Isolation of a Lysosomal Fraction from Rat Liver**—The lysosomal fraction was prepared by differential centrifugation and characterized according to a reported method.[9] The same batch of lysosomal fraction was used in all degradation experiments.

**Lysosomal Incubations**—PQ–peptides were incubated in the rat liver lysosomal fraction in order to study *in vitro* the kinetics of their degradation by the lysosomal enzymes. The PQ–peptides (400 μg/mL) were dissolved in 0.2 M sodium phosphate buffer, pH 5.5, 25% (v/v) methanol, 5 mM reduced glutathione and mixed with an equal volume of 10% (or 100% in case of the EACA derivatives) lysosomal fraction in the same buffer. The samples were incubated with shaking in a water bath at 37 °C. At certain incubation times an aliquot was taken from the incubation mixture and the action of the lysosomal enzymes was stopped by adding an equal volume of 10% trichloroacetic acid. The mixture was allowed to stay for 1 h in the dark. After centrifugation, the clear supernatants, containing ca. 100 μg/mL of PQ–peptides, were filtered through Millipore (Millex-$HV_4$-SJHVLO4NS) filter units for HPLC samples with 0.45 μm pore size and used for RP-HPLC.

**Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)**—The supernatants were run on a HP-1090 chromatograph (Hewlett-Packard) with a Spherisorb column 25 cm × 4.6 mm i.d., $S_5$ $ODS_2$ (Phase Separations Inc.) and Lichrosorb RP-18 precolumn (Merck) and 3392 A integrator (Hewlett-Packard). The injection volume was 20 μL. The flow rate was 1 mL/min and the absorbance was followed at 254 nm. The following mobile phases were used to study the lysosomal degradation of the respective compounds: I, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3 (600 mL), and acetonitrile (400 mL) (Ala-Leu-Ala-Leu-PQ, Ala-Leu-Ala-Tyr-PQ, Tyr-Leu-Ala-Leu-PQ, Lys-Leu-Ala-Leu-PQ, Leu-Ala-Leu-PQ); III, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 4 (600 mL), and acetonitrile (400 mL) (Asp-Leu-Ala-Leu-PQ); IV, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3 (750 mL), and acetonitrile (250 mL) (Ala-Leu-Ala-Lys-PQ); V, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3.6 (700 mL), and acetonitrile (300 mL) (Ala-Leu-Ala-Asp-PQ). All mobile phases contained 0.6 mM dimethyloctylamine (DMOA).

The mobile phases used for the EACA-peptides were VI, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3 (400 mL), and acetonitrile (600 mL) (EACA-PQ); VII, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3 (500 mL), and acetonitrile (500 mL), 0.75 mM DMOA (EACA-Ala-Leu-PQ); VIII, 0.025 M $NaH_2PO_4{\cdot}H_2O$, pH 3 (500 mL), and acetonitrile (500 mL), 0.25 mM DMOA (EACA-Leu-PQ).

The HPLC systems were able to separate in each case the tetrapeptide–PQ derivative, its PQ-containing cleavage products, and free PQ, obtained during the lysosomal incubations. This was proven with references of all the possible PQ-containing cleavage products. The lowest detectable concentration of PQ was 0.8 μg/mL.

**Kinetic Mathematical Modeling**—The lysosomal degradation of the PQ derivatives was analyzed by HPLC, whereby the PQ-containing cleavage products were identified and quantified. The kinetics of the degradation of the two groups of tetrapeptide–PQ derivatives, $P_1$–$P_4$ and $P_5$–$P_8$, and $P_9$ was further analyzed by applying a mathematical model of differential equations using nonlinear regression.

*Mathematical Model*—The lysosomal cleavage rate of each tetrapeptide–PQ derivative was assumed to be proportional to its concentration (or amount) in the incubation mixture (i.e. first-order kinetics). Moreover, the calculations were based on the initial assumption that every theoretical PQ-containing cleavage product could be formed during the lysosomal incubations with the rate constants specified in Scheme 1. Thus, the mathematical model, used to specify the concentration (or more practically the amount) of each component, can be described by the following set of differential equations:

$$\frac{dX_1}{dt} = -(k_{12} + k_{13} + k_{14} + k_{15})X_1$$

$$\frac{dX_2}{dt} = k_{12}X_1 - (k_{23} + k_{24} + k_{25})X_2$$

$$\frac{dX_3}{dt} = k_{13}X_1 + k_{23}X_2 - (k_{34} + k_{35})X_3$$

$$\frac{dX_4}{dt} = k_{14}X_1 + k_{24}X_2 + k_{34}X_3 - k_{45}X_4$$

$$\frac{dX_5}{dt} = k_{15}X_1 + k_{25}X_2 + k_{35}X_3 + k_{45}X_4$$

In these equations, $k_{12}$, $k_{13}$, $k_{14}$, etc., are first-order rate constants (in $h^{-1}$), $X_1$ is the amount of each tetrapeptide–PQ derivative, subjected to lysosomal incubation, and $X_2$, $X_3$, $X_4$, $X_5$ are the amounts of its respective PQ-containing cleavage products, i.e. tripeptide–PQ, dipeptide–PQ, amino acid–PQ, and PQ itself.

Such a set of differential equations was written for each of the ($P_1$–$P_9$) derivatives. The integrated, closed-form solutions of the above equations were fitted to all data in a degradation sequence, simultaneously.

*Data Acquisition*—During the lysosomal incubation of each PQ–peptide, supernatants were taken out at certain incubation times and analyzed by HPLC. The different cleavage products, yielding separate peaks, were identified on the chromatograms with known reference substances. The AUC (area under curve) values of the peaks, from one set of data for every PQ–peptide, were obtained by integration. Mass balances of PQ were performed during the degradation studies to check for closure in the product assays. This was made by comparing the AUC of the peak of PQ–tetrapeptides at $t = 0$ and the sums of AUC of the peaks of the PQ-containing cleavage products at each incubation time. Small deviations between them, due sometimes to slight volume decreases of the incubation mixture with time, were compensated by percentage normalization of the absolute AUC. The normalized values ($X_1$, $X_2$, etc.) were further used for the computer calculation of $k_{12}$, $k_{13}$, etc.

*Estimation of Degradation Rate Constants*—The rate constants were estimated by nonlinear iteratively reweighted least squares regression of the model to data, using the program PCNONLIN.[10] As a dependent variable, each individual measurement was used and all rate constants from each sequence were estimated in a single fit. Uncertainty in parameter estimates are expressed as relative standard error provided by the program divided by the parameter estimate. The data were weighted by the reciprocal of the prediction, as suggested by residual analyses of preliminary fits of the model to the data.[11]

Peptides $P_2$–$P_4$ differ only in their C-terminal amino acid, and during the lysosomal incubations each of them will give three different PQ-containing cleavage products ($X_2$, $X_3$, $X_4$) and PQ ($X_5$). Due to this fact, the data obtained from the lysosomal cleavage of $P_2$, $P_3$, and $P_4$ derivatives were fitted separately.

Peptides $P_5$(=$P_1$)–$P_8$ differ only in their N-terminal amino acid, and during the lysosomal incubations they will give identical PQ-containing cleavage products ($X_2$, $X_3$, $X_4$, $X_5$), meaning that some of the constants ($k_{23}$, $k_{24}$, $k_{25}$, $k_{34}$, $k_{35}$, and $k_{45}$) can be assumed to be the same. In addition, the tripeptide–PQ derivative ($P_9$), having the same structure as the cleavage product ($X_2$), will itself also give the same cleavage products ($X_3$, $X_4$, $X_5$). Thus, the data obtained from the degradation of $P_5$(=$P_1$)–$P_8$ and $P_9$ can be combined. A simultaneous fit of $P_5$, $P_7$, $P_8$, and $P_9$ was performed (the program could not analyze more data at the same time). In the same manner $P_5$, $P_6$, $P_8$, and $P_9$

were fitted. The two sets of data gave coinciding results. It should be observed that $P_1 = P_5$.

## Results

**Lysosomal Incubations of PQ–Peptides**—To study the pattern and rate of degradation by the rat liver lysosomal enzymes, all EACA-peptides, as well as the tetrapeptide–PQ ($P_1$–$P_8$), tripeptide–PQ ($P_9$), and the amino acid–PQ derivatives were incubated in a rat liver lysosomal fraction, containing reduced glutathione. The incubations were stopped after different incubation times and the supernatants were analyzed on HPLC systems, where the different PQ-containing cleavage products, as well as the free drug (PQ), could be separated and identified. A typical example is shown in Figure 1.

*EACA-Peptides*—Three different derivatives were studied, EACA-PQ, EACA-Leu-PQ, and EACA-Ala-Leu-PQ. Only the last one was cleaved by the lysosomal enzymes, as shown in Figure 2. One of the possible cleavage products, Ala-Leu-PQ, was not detected in the chromatogram, while the other two, Leu-PQ and PQ, were. The amounts of the incubated peptide EACA-Ala-Leu-PQ and the cleavage product Leu-PQ progressively decrease, while the amount of the released free PQ increases with time.

*Derivatives ($P_1$–$P_4$)*—All three derivatives $P_1$–$P_3$ were completely degraded and released free PQ (ca. 35–90%) within 4 h, while PQ was not at all released from the Asp-containing derivative ($P_4$) as shown in Figure 3. This was confirmed in another experiment where Asp-PQ was separately incubated. No free PQ was detected after 96 h of incubation. With $P_1$ and $P_2$ all the possible PQ-containing cleavage products ($X_2$, $X_3$, $X_4$, $X_5$) were detected, while in the case of $P_3$ and $P_4$, the respective $X_2$ and $X_5$ were not. The peptide with Lys in the C-terminal position ($P_3$), unlike the others, was immediately cleaved as shown in Figure 3 and not even traces of it were detected after 10 min of incubation.

*Derivatives ($P_5$–$P_8$)*—All three derivatives $P_5$–$P_7$ were completely degraded and released free PQ (ca. 95–98%) within 4 h, while PQ was only slowly released from the Asp-containing derivative ($P_8$), as shown in Figure 4. With $P_5$–$P_7$, all the possible PQ-containing cleavage products ($X_2$, $X_3$, $X_4$, $X_5$) were detected, while in the case of $P_8$, the respective $X_2$ and $X_3$ were not.

*Derivative ($P_9$)*—The tripeptide–PQ ($P_9$) derivative, having the same structure as one of the possible cleavage products ($X_2$) and obtainable from peptides $P_1$–$P_4$, was completely degraded and released free PQ (ca. 78%) within 4 h. In this case all the possible PQ-containing cleavage products ($X_3$, $X_4$, $X_5$) were detected, as shown in Figure 5.

*Amino Acid–PQ derivatives Leu-PQ, Tyr-PQ, Lys-PQ, Asp-PQ*—The first three derivatives were degraded and released free PQ to different extent within 4 h, while PQ was not at all released from the Asp-PQ (results not shown).

**Calculation of the Rate Constants of the Lysosomal Degradation of all Tetrapeptide–PQ Derivatives ($P_1$–$P_8$) and the Tripeptide–PQ ($P_9$)**—For this purpose a mathematical model of differential equations, using nonlinear regression, was applied as described above. The results are summarized in Table 1.

When $P_3$, $P_4$, and $P_8$ were studied, some of the PQ-containing cleavage products could not be detected in the chromatographic analyses. The reason can be that either these products are not obtained or they are obtained, but are very quickly further cleaved. In these cases, the rate constants for their production and elimination were a priori set to zero. In Table 1, these constants are denoted with an asterisk. In the same way, parameters that were found to be

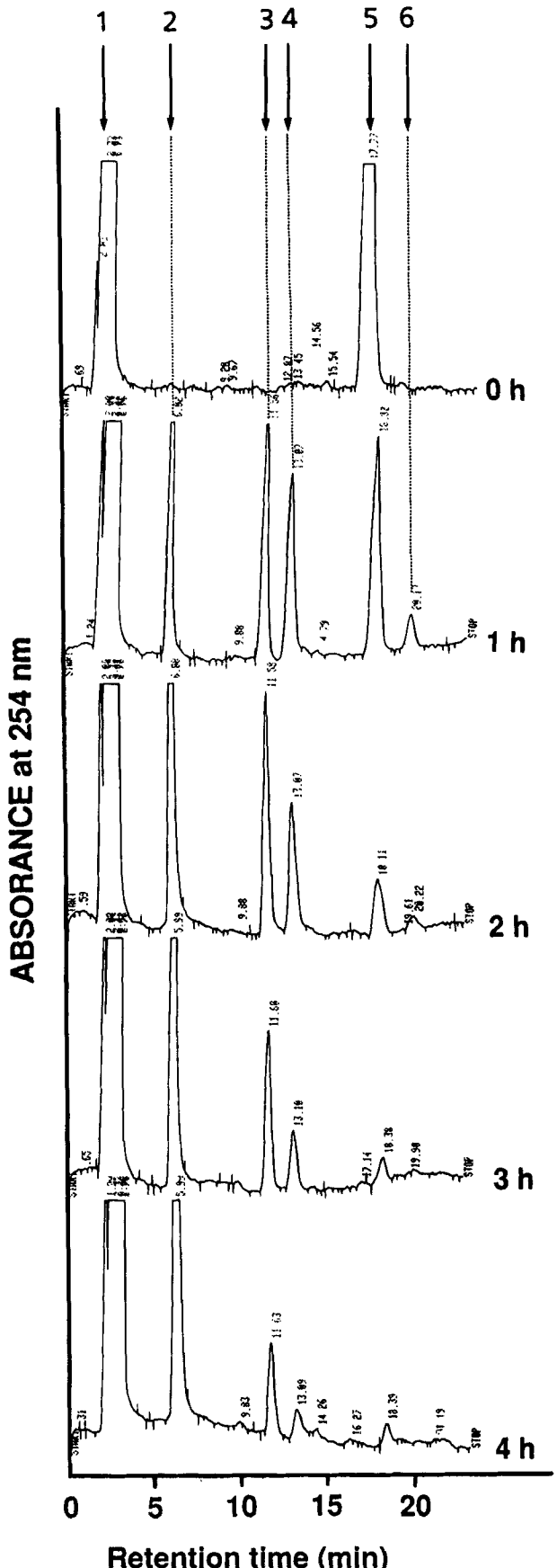


**Figure 1**—HPLC chromatograms of the lysosomal digests obtained at specified times (0–4 h) during the incubation of Ala-Leu-Ala-Leu-PQ ($P_1$). Arrows: (1) solvent front; (2) PQ; (3) Ala-Leu-PQ; (4) Leu-PQ; (5) Ala-Leu-Ala-Leu-PQ, and (6) Leu-Ala-Leu-PQ.

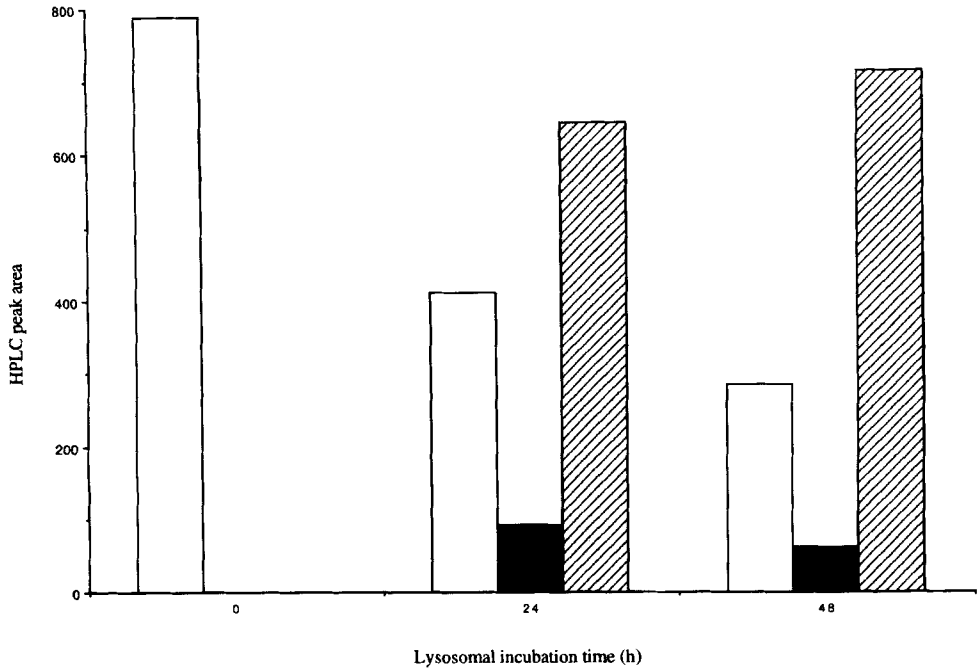


**Figure 2**—Degradation kinetics of EACA-Ala-Leu-PQ in a rat liver lysosomal fraction. Key: (empty) EACA-Ala-Leu-PQ; (filled) Leu-PQ; (hatched) PQ.

very low ($<0.1$) and to not significantly ($p < 0.05$) affect other values, when tested, were also taken to be zero and are denoted as such in Table 1. Rate constants close to zero were then fixed to zero in a repeated run. Statistical comparison was made between full and reduced models, showing that no differences could be ascertained.

The constants $k_{15}$, $k_{25}$, and $k_{35}$ were low or zero for most of the peptides. Thus, enzymes directly releasing PQ from the C-terminal end of the tetra-, tri-, and dipeptides are shown not to be present in the lysosomal vacuome, as was also found when the EACA-peptides were studied. Only aminopeptidases can hydrolyze the bond between PQ and the C-terminal amino acid, as defined by $k_{45}$, and in some cases a low activity of diaminopeptidases ($k_{35}$) was detected. One exception was Ala-Leu-Ala-Lys-PQ, where the analysis indicated that PQ was released with a relatively large $k_{15}$ rate constant.

## Discussion

Our earlier studies have clearly shown that the pharmacological activity of PQ *in vivo* is significantly improved when bound to polyacryl starch microparticles.[6] The activity, estimated as the killing capacity in mice infected by *Leishmania donovani*, was improved more than 100 times, apparently due to the lysosomal targeting achieved with the starch particles as carrier. The results indicate that PQ can be released by the lysosomal enzymes in the Kupffer cells of the liver, where also the *L. donovani* parasites are phagocytosed. PQ was in this case bound to the carrier via an Ala-Leu-Ala-Leu spacer arm, which was enzymatically cleaved in the lysosomal vacuome. A similar approach was used by Trouet et al.,[12] who found that PQ, bound to asialofetuin via the same spacer arm, was active in the treatment of malaria in mice.

Drugs, when bound directly to carriers, cannot be released by lysosomal enzymes.[4,5] Thus, it is necessary to introduce a spacer arm between the drug and the carrier,[3,4] and its length and composition determine the enzymatic release of the drugs. When the significance of the length of the peptide spacer arms consisting of Ala and Leu was investigated, it was found that PQ was most rapidly released from the tetrapeptide–PQ derivatives when incubated in a rat liver lysosomal fraction.[5,13]

In a series of papers, Kopeček and co-workers[14,16] and Duncan et al.[15] synthesized water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing different oligopeptide side chains with *p*-nitroaniline. They studied the release of *p*-nitroaniline by individual enzymes or a mixture of lysosomal enzymes. For instance, they have shown[14] that a substantial part of the hydrolyzing activity in the liver lysosomes is related to SH-dependent enzymes. *p*-Nitroaniline was released from a larger number of the above conjugates when reduced glutathione was added to the incubation mixture. It was found, as well, that leupeptin, known to inhibit SH enzymes, completely inhibited the degradation of some of the conjugates. Consequently, reduced glutathione has been added to the lysosomal fraction in our studies.

Duncan and co-workers[17–19] have also shown that drugs (daunomycin, adriamycin, puromycin, doxorubicin) were released from soluble HPMA copolymers, when linked to the polymer via different oligopeptides. In some cases, they could show that endopeptidases first cleaved the spacer arms and, subsequently, aminopeptidases released the free active drugs.

From the present study, some firm conclusions can be drawn on the specificity of the rat liver lysosomal enzymes as far as the capacity to release PQ from some tetrapeptide derivatives and EACA-peptide derivatives is concerned. In the latter group, $\epsilon$-aminocaproic acid (EACA) missing a free $\alpha$-amino group, but having a free $\epsilon$-amino group, was introduced as the N-terminal amino acid. Due to the absence of the free $\alpha$-amino group, EACA cannot be released from such a position by any known aminopeptidase.

PQ was not released from EACA-PQ and EACA-Leu-PQ. This shows that hydrolytic activity, cleaving any of the bonds in these two derivatives, is not present in the lysosomal vacuome. On the other hand, the release of PQ from EACA-Ala-Leu-PQ indicates that PQ can be released after a primary cleavage of the Ala-Leu peptide bond by some endopeptidase-like activity. This hypothesis is supported by the fact that no Ala-Leu-PQ is detected in the incubation mixture but only Leu-PQ, which subsequently is cleaved by an aminopeptidase attack on the Leu-PQ bond.

The mathematical model describing the lysosomal degradation of the tetrapeptide–PQ derivatives was constructed in

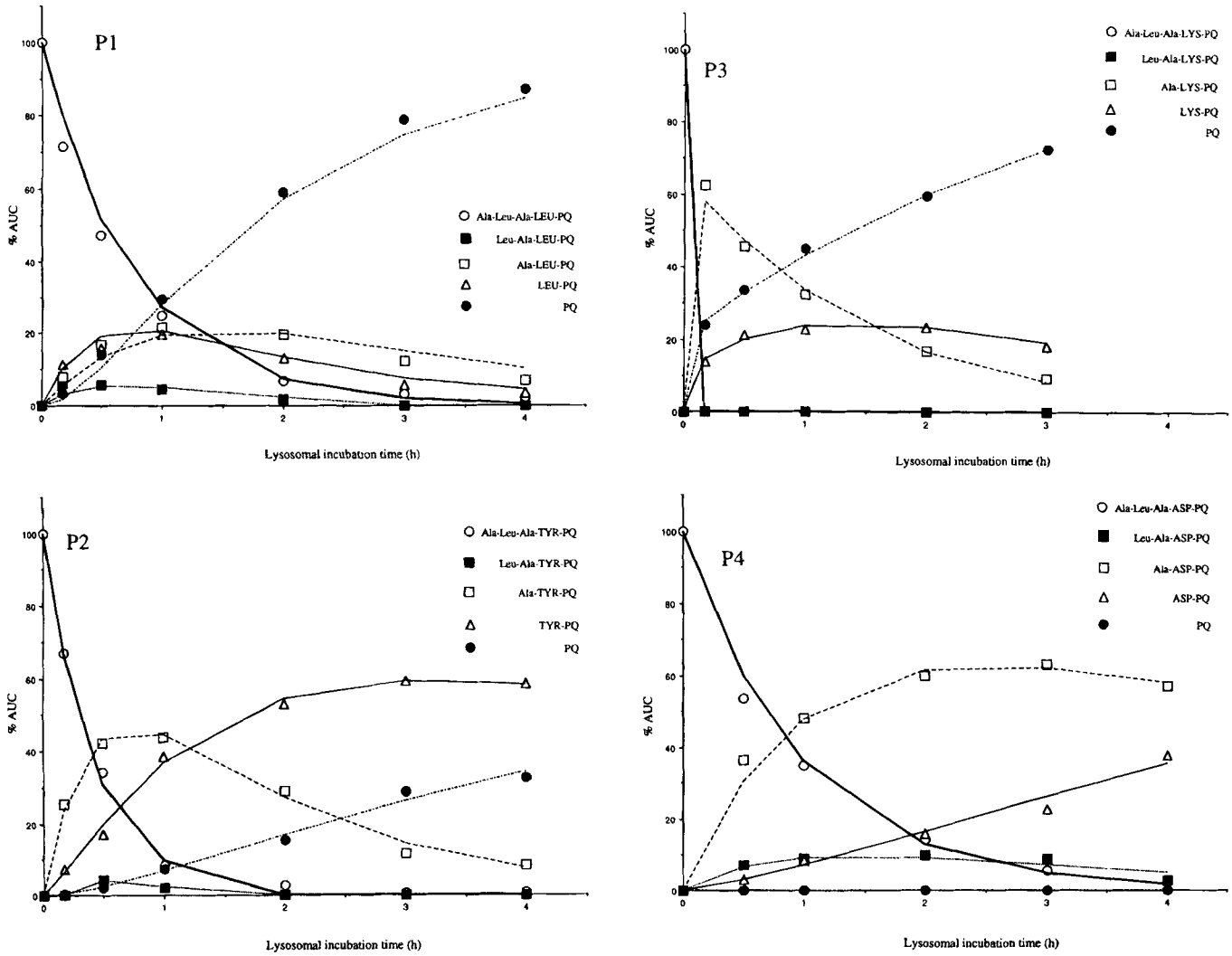


**Figure 3**—Degradation kinetics of tetrapeptide–PQ derivatives ($P_1$–$P_4$) in a rat liver lysosomal fraction. The results are expressed as percent AUC of the HPLC peaks. Symbols not shown, are zero or close to zero. The symbols represent the experimentally obtained values, while the lines are drawn from the data calculated by nonlinear regression analysis.

such a way that all the possible cleavages forming PQ-containing peptides are included. It should be noted that several enzymes may contribute to each degradation step. That means that a $k$ value obtained may be a summation constant with contributions from several enzymes. The rate of formation of a PQ derivative—assuming first order kinetics—is then $k_i[X_i]$. The model and the obtained constants gave a good fit to the experimental data found for the lysosomal degradation of the PQ derivatives.

In the series of four tetrapeptide–PQ derivatives ($P_1$–$P_4$), in which the C-terminal amino acid was varied between Leu, Tyr, Lys, and Asp, the pattern of cleavage of the Asp derivative ($P_4$) was significantly different from the pattern of the other three derivatives. PQ was not at all released from the Asp derivative, and the main product formed was Asp-PQ. This product was not further cleaved to PQ, and that is why $k_{45}$ was a priori set to zero, indicated in Table 1 as an asterisk. Knowing that the aminopeptidase activity is a major enzyme activity for the degradation of peptides in the lysosomes,[20] it is interesting to note that the Asp component obviously influenced the activity of these peptidases negatively. The effect of the different C-terminally placed amino acids was also obvious, when the degradation of the three other tetrapeptide-PQ derivatives of this group was studied. Thus, for example, for the Lys-containing tetrapeptide PQ derivative ($P_3$), $k_{13}$ and $k_{14}$ were relatively high. This means that Ala-Lys-PQ and Lys-PQ were formed relatively rapidly. Also, $k_{15}$ was large, describing a direct formation of primaquine from the tetrapeptide by an endopeptidase with a high affinity for Lys. From the Tyr-containing tetrapeptide PQ derivative ($P_2$), Ala-Tyr-PQ and Tyr-PQ were likewise formed in high concentrations and later relatively rapidly degraded further, either directly through a diaminopeptidase activity or stepwise by aminopeptidases to release free PQ.

From the studies on the other series of tetrapeptide-PQ derivatives ($P_5$–$P_8$), where the N-terminal amino acid was varied, it is immediately seen that the Asp-containing derivative ($P_8$) also here was relatively more slowly degraded in the lysosomes than the other derivatives. No di- and tripeptide–PQ could be detected, which made it impossible to determine the $k_{13}$ and $k_{12}$ constants for the hydrolysis. However, Leu-PQ was detected and this derivative was further digested to PQ.

The hydrolytic pattern of the other three derivatives in this series is similar in a sense that they were relatively quickly degraded and released about 100% PQ. After the initial cleavage of the N-terminal amino acids from peptides $P_5$–$P_8$, identical PQ-containing cleavage products ($X_2$, $X_3$, $X_4$, $X_5$) are obtained. In addition, the tripeptide derivative ($P_9$), having the same structure as $X_2$ from all $P_5$–$P_8$ derivatives, itself gave the same cleavage products ($X_3$, $X_4$, $X_5$). Thus, it was possible to combine the kinetic analyses of $P_5$–$P_8$ and $P_9$. The analysis

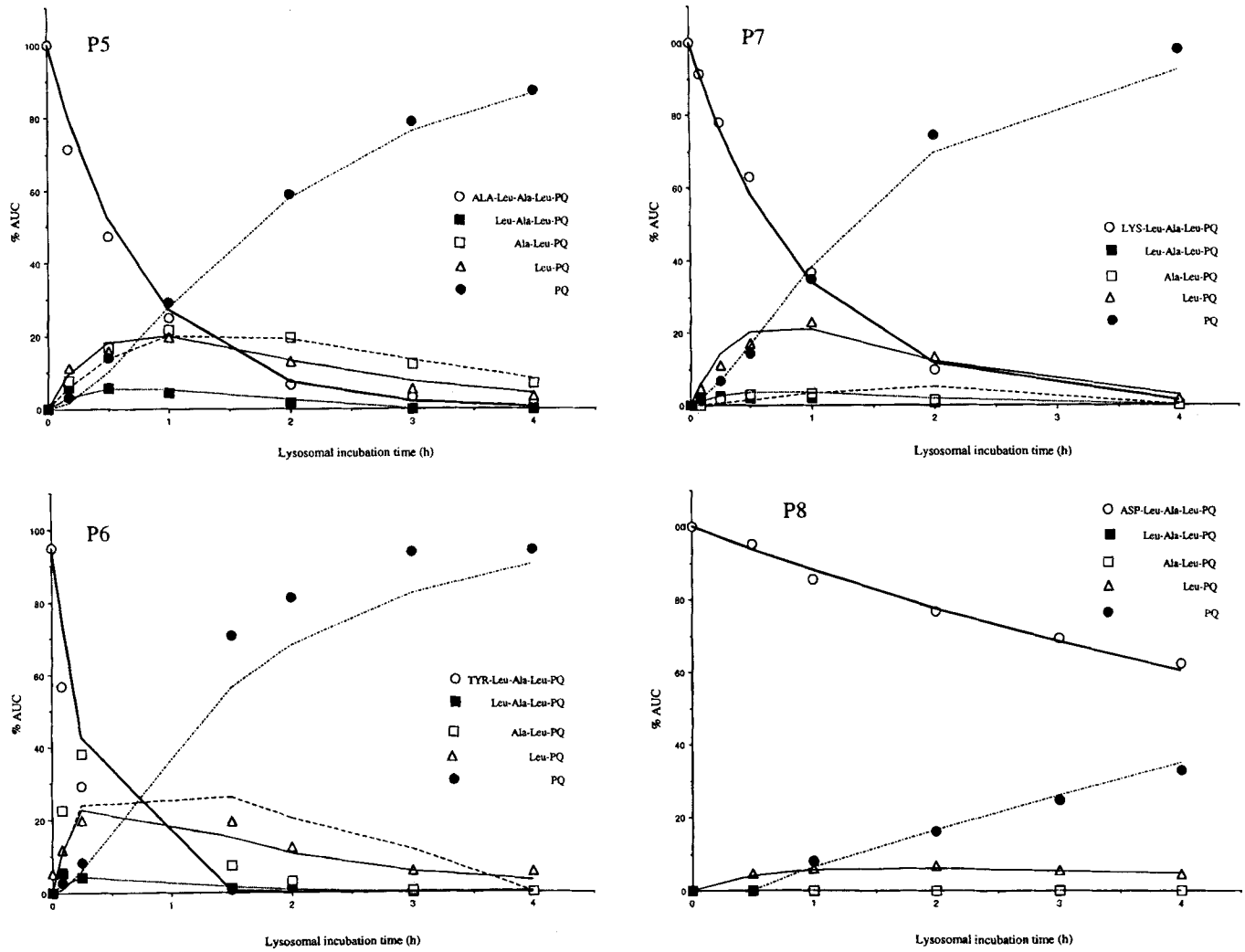


**Figure 4**—Degradation kinetics of tetrapeptide–PQ derivatives ($P_5$–$P_8$) in a rat liver lysosomal fraction. The results are expressed as percent AUC of the HPLC peaks. Symbols not shown, are zero or close to zero. The symbols represent the experimentally obtained values, while the lines are drawn from the data calculated by nonlinear regression analysis.

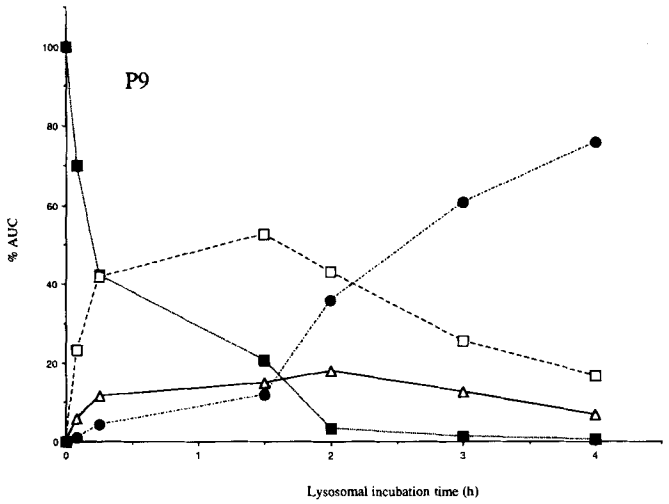


**Figure 5**—Degradation kinetics of tripeptide–PQ derivative ($P_9$) in a rat liver lysosomal fraction. The results are expressed as percent AUC of the HPLC peaks. Key: (■) Leu-Ala-Leu-PQ; (□) Ala-Leu-PQ; (▲) Leu-PQ; (●) PQ.

then showed that $k_{23}$ and $k_{45}$ are larger than $k_{34}$, indicating that the monoaminopeptidase(s) has (have) higher specificity for Leu derivatives than for Ala derivatives. However, the analysis also shows that diaminopeptidase activity may be present, hydrolyzing Leu-Ala from the tripeptide derivative, as a constant $k_{24}$ could be determined. On the other hand, the dipeptidase activity releasing Ala-Leu from Ala-Leu-PQ obviously is of minor significance, as $k_{35}$ could not be separated from zero, indicating that also the diaminopeptidases have higher specificity for Leu derivatives than for Ala peptides.

Comparing the rate constants in Table 1, all the different $k_{13}$ are larger than the corresponding $k_{12}$ values (except for $P_7$). This finding shows that the diaminopeptidases are relatively more important than monoaminopeptidases. However, comparing all the $k_{23}$ values with the respective $k_{24}$ values and all the $k_{34}$ values with the respective $k_{35}$ values, one can see that $k_{23} > k_{24}$ (except for $P_2$ and $P_3$) and $k_{34} > k_{35}$. This picture indicates that in these situations the monoaminopeptidases generally are more effective, which may be due to the influence from the drug. This is accordance with the comment made by Kopeček[21] that the active site of the peptidases usually accomodates two amino acid residues of the substrate toward the C-terminal of the cleaved bond. However, in the dipeptide–PQ substrates, the drug molecule occupies one of these positions. Thus, the drug itself influences the interaction of the substrate with the active site of the enzyme. Obviously, in our case PQ did not fit into the active site of the diaminopeptidases, but PQ dipeptides fitted

**Table 1—Summary of the Rate Constants for the Lysosomal Degradation of Tetrapeptide–PQ Derivatives[a]**

| Rate Constant[b] ($h^{-1}$) | Tetrapeptide–PQ Derivatives[c] | | | | (X-Leu-Ala-Y-PQ) | | |
|---|---|---|---|---|---|---|---|
| | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_6$ | $P_7$ | $P_8$ |
| X = | Ala | Ala | Ala | Ala | Tyr | Lys | Asp |
| Y = | Leu | Tyr | Lys | Asp | Leu | Leu | Leu |
| $k_{12}$ | 0.22 | 0.26 | *[d] | 0.19 | 0.31 | 0.14 | * |
| $k_{13}$ | 0.34 | 1.77 | 14.1 | 0.79 | 1.48 | 0 | * |
| $k_{14}$ | 0.74 | 0.32 | 2.7 | 0.04 | 1.6 | 0.79 | 0.13 |
| $k_{15}$ | 0[d] | 0 | 4.9 | * | 0 | 0.14 | 0 |
| $k_{23}$ | 1.36 | 0.3 | * | 0.45 | 1.36 | 1.36 | 1.36[a] |
| $k_{24}$ | 0.31 | 2.14 | * | 0 | 0.31 | 0.31 | 0.31 |
| $k_{25}$ | 0 | 0 | * | * | 0 | 0 | 0 |
| $k_{34}$ | 0.47 | 0.54 | 0.42 | 0.15 | 0.47 | 0.47 | 0.47 |
| $k_{35}$ | 0 | 0.13 | 0.27 | * | 0 | 0 | 0 |
| $k_{45}$ | 1.7 | 0.12 | 0.43 | * | 1.7 | 1.7 | 1.7 |

[a] The constants ($k_{23}$–$k_{45}$) for $P_6$–$P_8$ as well as for the tripeptide–PQ derivative ($P_9$) are the same as for $P_1$. [b] The rate constants are estimated by nonlinear regression analysis, using the model shown in Scheme 1. The relative standard error (RSE), which is obtained as the standard error provided by the program divided by the parameter estimate, for most of the constants was <25%. However, for the following rate constants it was >25% (but < 50%): $P_1 = P_5$ ($k_{12}$, $k_{24}$), $P_2$ ($k_{12}$, $k_{14}$), $P_3$ ($k_{13}$, $k_{14}$, $k_{15}$, $k_{35}$), $P_4$ ($k_{14}$), $P_6$ ($k_{13}$, $k_{24}$), $P_7$ ($k_{12}$, $k_{24}$), and $P_8$ ($k_{24}$). [c] Derivative $P_5$ is identical to $P_1$. [d] For the explanation of * and 0, see the Results Section.

well into the active site of the monoaminopeptidases. The latter was proved by the fact that $k_{34} > k_{35}$ and the relatively large $k_{45}$.

In summary, the detailed studies on the lysosomal degradation of different PQ–peptides show that they are degraded by different aminopeptidases and endopeptidases. In this study, the rate of release of PQ has essentially been determined by enzymes with mono- and diaminopeptidase activity. However, it is obvious from the studies on the EACA-PQ derivatives, missing a free α-amino group, that also endopeptidases play a decisive role for the release of PQ from such derivatives as no direct release of PQ from the C-terminal end can be expected. Moreover, when the PQ derivatives are bound to a carrier like polyacryl starch microparticles, it has been concluded[5] that the endopeptidases are important for the initial cleavage of the spacer arm. In such a situation, it is probable that changes of the C-terminal amino acid, to which PQ is bound, will have a larger influence on the rate of release than changes of the N-terminal amino acid. This problem will be subject of a later paper.

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