On the Nature of the Hemoglobin-Haptoglobin Interaction

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The interactions between human haptoglobin, Hp II (genetic types 2—1 and 2—2), and bovine hemoglobin, Hb, were investigated taking inhibition of complex formation and complex dissociation in various solvent media as criteria.

As shown by relative peroxidase activity and gel chromatography, complex dissociation occurs at high concentrations of guanidine·HCl, urea, sodium chloride, dioxane, and formaldehyde, while in case of sodium dodecylsulfate a low molar ratio (SDS/Hb—Hp<5) is sufficient to split the complex. In general the formation of the complex stabilizes the structure of its constituents.

Excluding solvent conditions which lead to denaturation (as measured by optical rotation), ion-pairs and H-bonds seem to prevail in the stabilization of the complex, while hydrophobic interactions should be of minor importance. Chemical modification of histidine and tyrosine with diazonium-1-*H*-tetrazole and *N*-acetylimidazole, respectively, prove histidyl-groups in Hb and tyrosyl-groups in Hp to participate in the Hb—Hp contact, thus confirming earlier results.

Hemoglobin and haptoglobin form complexes of various composition [1, 2]. Several attempts have been made to elucidate the type of interactions between both components. Evidence gained from reactions involving SH groups [3–5] and the iron atom of the heme group [6] shows that neither disulfide bridges nor heme-protein interactions can be responsible for the complex formation. On the other hand, HAMAGOUCHI et al. [7] found differences in the Soret region of the circular dichroism spectra comparing the complex and its components, which point to differences in the environment or in the attachment of the hemegroup. From the high stability of the complex in a broad range of pH one might expect hydrophobic interactions to be important [8] in addition to electrostatic interactions proposed by VAN ROYEN [9].

A discussion on the basis of the theory of NÉMETHY and SCHERAGA [10–12] and a model for the formation of the complex have been reported previously [13]. Both were based on titration and photooxidation experiments which prove histidyl groups of Hb and tyrosol groups of Hp to play a decisive role in the Hb-Hp interaction.

The present experiments give additional evidence for this hypothesis using different methods of complex inhibition and complex dissociation. They are in general concerned with the equimolar complex Hb : Hp = 1 : 1 which is supposed to show the maximum of peroxidase activity.

Materials and Methods

Blood haptoglobin Hp II (genetic types 2—1 and 2—2, c. f. ref. [14]) was isolated from the Cohn fraction IV (method 6) as described previously [15]. Bovine hemoglobin was purchased from Serva (Heidelberg), all other reagents were products of highest purity (Merck, Darmstadt; Schuchardt, München; EGA-Chemie, Steinheim).

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Abbreviations: Hb, hemoglobin; Hp, haptoglobin; Hb-Hp, hemoglobin-haptoglobin complex; DHT, diazonium-1-*H*-tetrazole; NAI, *N*-acetylimidazole; SDS, sodiumdodecylsulfate; ORD, optical rotatory dispersion.

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Bidistilled water was used throughout.

Peroxidase activity: Relative peroxidase activity of the Hb-Hp complex in various solvent media was determined according to CONNELL and SMITHIES [16]. In a first series of experiments Hb was added to the preincubated mixture of Hp, guaiacol reagent and solvent; in a second one the preformed complex Hb-Hp was added. In both cases water was used to provide a constant volume. After addition of 1% aqueous H_2O_2 the time dependent change of the absorbancy at 470 mμ was recorded in a time range between 15 and 40 seconds using an Optica (Milano) CF-4R recording spectrophotometer. The initial slope provides a measure of the relative peroxidase activity.

Gel chromatography: Sephadex G 150 (Pharmacia, Uppsala) was used in columns 1 × 30 cm. Before loading the column the gel was equilibrated with the respective solvents (Sörensen phosphate buffer, pH 7.0 plus additives) for 64 hours. The flow rate was maintained at 2.5 ml/hr. Transmittance at 254 mμ was measured with a Uvicord (LKB, Stockholm).

Optical Rotation: Effects of solvent variation on the conformation of the Hb-Hp complex and its constituents were studied in a Zeiss precision polarimeter LEP 0.05, using 10 cm cells (λ = 578, 546, 436, 405, 365 mμ). In order to exclude scattering effects the solutions were clarified by filtration through millipore filters (0.45 μ). Differences in the refractive indices were corrected by the Lorentz term $(n^2_w+2)/(n^2_s+2)$ applying an Abbe refractometer (Zeiss).

Chemical modification: Histidyl residues were modified using diazonium-1-*H*-tetrazole (DHT, Schuchardt, München). The reagent was prepared according to HORINISHI et al. [17]; distilled water was added to the solution to give a final volume of 50 ml.

Tyrosyl residues were acetylated using *N*-acetylimidazole [18] (NAI, EGA-Chemie, Steinheim). The reaction was carried out for 1 hr at room temperature with an 80 fold molar excess of the reagent.

Results

Peroxidase activity of Hb-Hp in various solvent media

In order to estimate the weight of the different types of intermolecular forces in the Hb-Hp interaction the properties of the Hb-Hp complex were studied in different solvent media, varying ionic strength (+NaCl or glycine), dielectric constant ε (+dioxane, glycine etc.), H-bond capacity (+urea, guanidine·HCl, formaldehyde) and the solubilizing properties of the solvent (+SDS, urea, guanidine·HCl). The relative peroxidase activity was taken as a measure of complex formation, comparing the primary, *inhibitory effect on the formation* of the Hb-Hp complex to the secondary, *dissociating* effect of the various solvents on the preformed Hb-Hp complex [19].

Except the case of formaldehyde where both curves show identical profiles (Fig. 1), the two effects differ from each other in a characteristic manner.

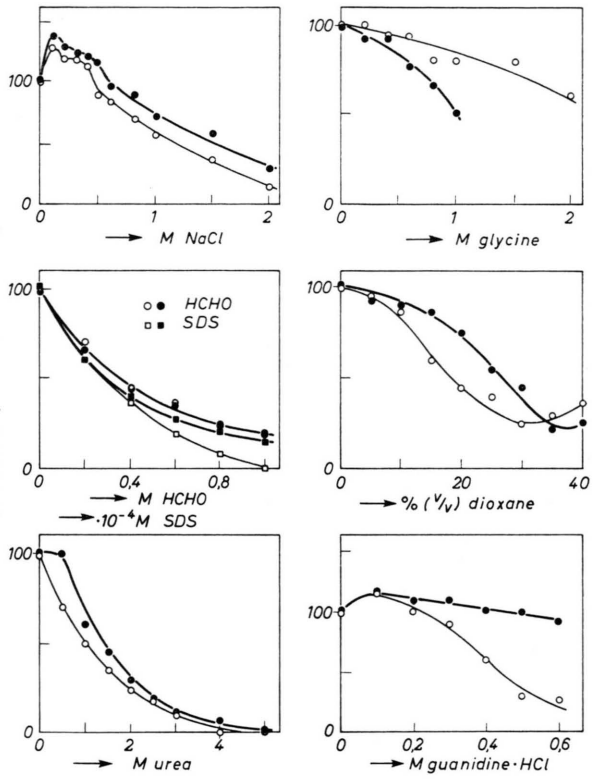

Fig. 1. Influence of additives on the peroxidase activity of the Hb-Hp complex. o—o Addition of 0.1 ml 0.1% Hb in phosphate buffer to 0.1 ml Hp solution (0.3%) in the respective media; ●—● Addition of the preformed Hb-Hp complex (0.1 ml 0.1% Hb + 0.1 ml 0.3% Hp) to the respective solvent media.

In the presence of increasing amounts of glycine the relative peroxidase activity of the preformed Hb-Hp complex decreases more rapidly than the complex under conditions where the complex is formed in the modified solvent. All the other sol-

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vents show the opposite effect, the preformed complex exhibiting higher relative peroxidase activity than the mixture Hb plus Hp under identical conditions.

It cannot be decided from these experiments whether the change of peroxidase activity is due to solvent effects on the conformation of the complex, or to a shift of the equilibrium of complex formation

$$Hb + Hp \rightleftarrows Hb - Hp .$$

Therefore, the stability of the complex in the different solvents was investigated by optical rotation and by gel chromatography using Sephadex G 150 equilibrated with the respective solvents.

Additive S	C_S [M]	Dissociation[a]	α_{rel}[b]
NaCl	2[c]	++	1.01
Glycine	2	+	1.00
SDS	$2 \cdot 10^{-4}$[d]	++++	1.00
HCHO	1.5[e]	+++	1.18
Urea	6	+++	1.45
Guanidine · HCl	0.6	+	1.04
	6[c]	++++	1.50

Table I. *Dissociation and Conformational Stability of Hb-Hp in various solvent media.* Common solvent for all additives S: phosphate buffer pH 7.0, $I = 0.15$; 20 °C. [a] Dissociation of Hb-Hp after incubation in S at room temperature, measured on Sephadex G 150 columns (30 × 1), equilibrated with S; full separation of Hb and Hp is achieved in 6 M guanidine · HCl. 10 mg of the Hb-Hp complex were applied in each experiment. [b] α_{rel}, optical rotation of Hb-Hp in solvent medium S, corrected for the refractive index $(\alpha_S \cdot \frac{n^2_w+2}{n^2_s+2})$, relative to optical rotation in pure buffer (w). Concentration of Hb-Hp: 2.75 mg/ml. [c] Preliminary experiments in the ultracentrifuge prove Hp to dissociate into smaller subunits. [d] molar ratio (S)/(Hb-Hp) ~ 3. [e] molar ratio (S)/(Hb-Hp) $\sim 2.5 \cdot 10^4$.

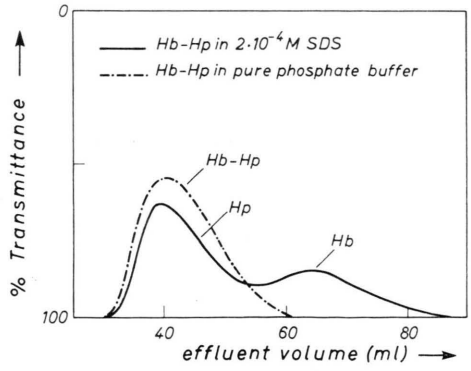

Fig. 2. Separation of the Hb-Hp complex in $2 \cdot 10^{-4}$ M SDS (phosphate buffer pH 7, $I = 0.15$) on Sephadex G 150. 10 mg of the complex were subjected to gel filtration on a 30 × 1 column, equilibrated with the same solvent. Flow rate 2.5 ml/hr.

The composition of the solvents was chosen in the region of minimum peroxidase activity. Results are given in Table 1; as an example, Fig. 2 gives the elution pattern for the separation of Hb-Hp in $2 \cdot 10^{-4}$ M SDS (phosphate buffer, pH 7).

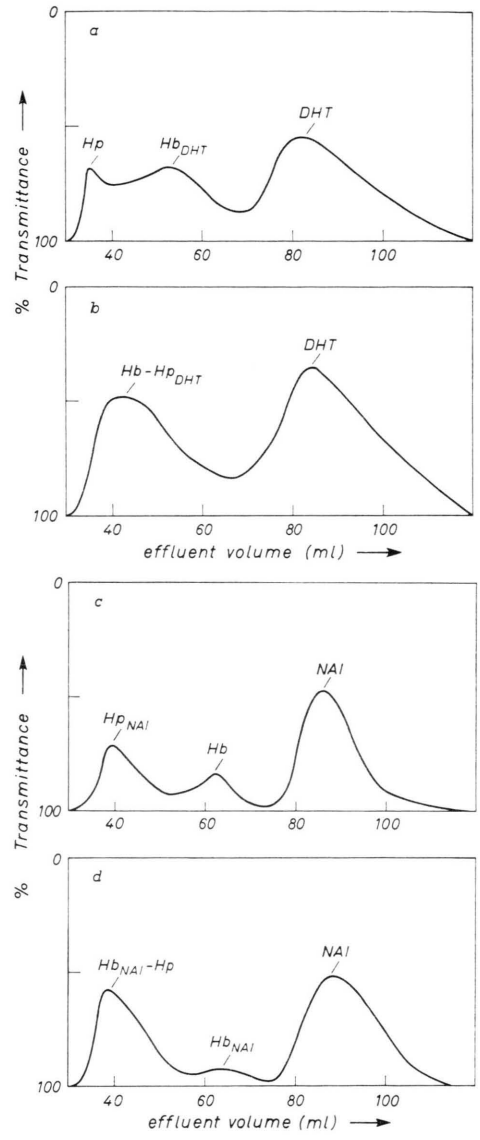

Fig. 3. Gel chromatography of the Hb-Hp complex after chemical modification with DHT and NAI (Sephadex G 150, phosphate buffer pH 7, $I = 0.15$, flow rate 2.5 ml/hr). a. 0.1 ml DHT solution was added to 2 mg Hb in 0.1 ml 0.67 M bicarbonate buffer (pH 8.8); after 20 min, 6 mg Hp in 0.4 ml bicarbonate buffer were added, and the mixture subjected to gel filtration. b. 6 mg Hp in 0.1 ml bicarbonate buffer plus 0.1 ml DHT solution; after 20 min, addition of 2 mg Hb in 0.4 ml bicarbonate buffer. c. 0.4 ml 0.8 M NAI were added to 6 mg Hp in 0.1 ml 0.01 M veronal/HCl buffer (pH 7.5); after 60 min, 2 mg Hb in 0.1 ml veronal buffer were added, and the mixture subjected to gel filtration. d. 2 mg Hb in 0.3 ml veronal buffer plus 0.2 ml NAI solution; after 60 min, addition of 6 mg Hp in 0.1 ml veronal buffer.

Complex formation after chemical modification of the components

Indirect evidence for the participation of histidyl- and tyrosyl-groups of Hb and Hp in the formation of the Hb-Hp complex was obtained from acidimetric and spectrophotometric experiments [13]. A direct verification may come from chemical modification, using DHT for the his-residues of Hb, and NAI for the tyr-residues of Hp. After reacting the respective component, complex formation with the unmodified partner was tested by gel chromatography on Sephadex G 150. As shown in Fig. 3, no complex formation was observed after combination of native Hp with DHT reacted Hb, as well as native Hb with NAI reacted Hp. On the other hand, modification of his-residues of Hp (with DHT) does not affect the formation of the Hb-Hp complex, while treatment of Hb with NAI leads to a certain decrease of Hb-Hp complex formation.

Discussion

There is clear evidence from the foregoing dissociation experiments, e. g. in guanidine·HCl, that covalent bonds cannot be involved in the formation of the Hb-Hp complex. Spectroscopic [20], potentiometric [13], and calorimetric [21] data, as well as chemical modification [13] rather prove multicenter interactions of high specificity to be involved.

The discussion of the potential participants in these interactions, which include ion-pairs, hydrophobic interactions, and H-bonds, may be based on the systematic modification of the solvent medium, using KAUZMANN's criteria [22] in connection with more recent findings on water-solute interactions [23].

Evidently, unambiguous conclusions can only be drawn from this approach if the modification of the medium does not automatically cause structural changes of the proteins under concern. Under the conditions of the foregoing experiments this assumption is clearly not justified in case of guanidine·HCl, urea, and dioxane, since in these media the structure of the complex and its constituents is modified drastically (c. f. ORD data in Table 1). On the other hand, 2 M NaCl or glycine, 10^{-4} M SDS (and 1.5 M formaldehyde) do not show measurable effects of the optical rotation of the complex. Therefore, the discussion of the interactions stabilizing the complex may be based on these solvent media.

With respect to *ion-pairs*, which are suggested by the dissociation of the complex under conditions of high electrolyte concentrations, the stability of the complex in the pH-range $4.5 < pH < 9$ points to carboxyl-, histidyl-, and lysyl-residues [24]. As shown by ANTONINI et al. [25] and KIRSHNER and TANFORD [26], bovine Hb dissociates into half-molecules with increasing NaCl concentration, the product being the $\alpha\beta$-dimer. There is clear evidence from titration experiments of Hp with small amounts of Hb (PAVLÍČEK and JAENICKE, in preparation) that this dissociation in itself does not interfere with complex formation nor peroxidase activity. On the contrary, fluorescence studies [27], and calorimetry [21] suggest the $\alpha\beta$-dimer to represent the unit of maximum reactivity in the formation of the Hb-Hp complex. Therefore, the decrease of peroxidase activity at high levels of NaCl or glycine must be due to a shift of the equilibrium towards complex dissociation, which is confirmed by gel chromatography (Table 1). Comparing this result to the fluorescence quenching at high and low salt concentrations (ref. [27], Fig. 7, p. 3431), differences in the kinetics and equilibria are obvious, which extrapolate to different plateau values, as expected from the present experiments. A quantitative comparison is inadequate, since both, human Hb-Hp $(1-1)$ and bovine Hb-Hp $(2-1)$ or Hp $(2-2)$ might very well show different properties.

Interpreting the dissociation at high salt concentration on the basis of counter-ions screening Hb . . Hp ion-pairs, the effect of glycine can only be understood assuming the decrease of the Coulombic potential at high dielectric constant ε to be less important as compared to the increase of ionization, determining the number of interacting ion-pairs [28].

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[28] The static dielectric constant ε of 2 M NaCl and 1 M glycine in water at 20 °C is 60 and 110, respectively.

This assumption fits the observation that glycine under various conditions is known to inhibit subunit dissociation [29, 30].

On the other hand, the stabilization of the complex upon solvent-Hp interaction (c. f. the inversion of the profiles of complex + solvent and solvent + Hp + Hb in Fig. 1) suggests specific interactions (ion-binding) to play a role in this case. This is in contrast to the other media, which evidently prove the complex to be more stable towards deactivation (dissociation) than its constituents (Fig. 1).

The *hydrogen-bond* as an alternative possibility differs from ion-pairs only in a formal way as long as no precise atomic distances are known. Depending on the more or less polar environment of the dissociable sidechains, the interacting groups represent more or less polarized mesomeric states. For the carboxyl-amino interactions, as an example, this is illustrated by the following scheme:

$$-COOH\ \ H_2N- \quad -COO\cdots H\cdots H_2N- \quad -COO^{\ominus}\ \ {}^{\oplus}H_3N-$$

← decreasing increasing →
polarity of the environment

Any change of the structure of the aqueous medium leads to changes in the net bond energy; this way the additives used in the dissociation experiments cause alterations in the protein interactions which in general favour the dissociation of the complex [31].

At the present stage no clear-cut conclusion in terms of specific intermolecular forces can be drawn on this basis. On the other hand, preferential binding of solvent components has to be considered too, which may compete with the constituents of the complex for specific sites responsible for complex formation. For example, formaldehyde reacts with the uncharged amino and imino group, but not with tyrosyl groups or the amide linkage of the peptide chain [32]; similarly the SDS-anion under the given experimental conditions most likely reacts with lysyl- and histidyl-residues of Hb and Hp, so that, considering both additives, the stabilization of the Hb-Hp complex seems to depend on amino- and imidazole groups in their dissociated or undissociated form.

With respect to *hydrophobic interactions* no unambiguous conclusions can be drawn from the present experiments: 40% dioxane ($\varepsilon \sim 45$) and 6 M urea (c. f. TANFORD [33]) lead to structural changes. On the other hand, the SDS concentrations at the given molar ratio (<5) is below the range where solubilization of the hydrophobic core of the protein or denaturation take place [19, 34]. As mentioned before, SDS most likely is bound specifically to cationic sites on Hb or Hp, thus either occupying association sites or introducing additional negatively charged groups. The fact that the Hb-Hp reaction turns out to be strongly exothermic (-70 kcal/mole [21]), clearly contradicts hydrophobic interactions as the main reason for the stability of the Hb-Hp complex. The effects of high salt point into the same direction.

Summarizing the conditions of dissociation the most likely probability for the Hb-Hp interaction turns out to be a superposition of ion-pairs and hydrogen bonds, the partners being carboxyl-lysyl and carboxyl-histidyl pairs on one hand, and histidine and tyrosine residues on the other hand. The participation of $-COO^{\ominus}$ (Hb) and $-NH_3^{\oplus}$ (Hp) was postulated earlier by VAN ROYEN [9]; the coincidence of the range of stability with the ionized state of the carboxyl, amino, and imidazole groups, and the effect of low molar ratios of SDS as well as the screening effect of high salt confirm this hypothesis. On the other hand, the effect of formaldehyde points to binding to undissociated lysyl-, histidyl-, or tyrosyl-residues, suggesting a hydrophobic environment for part of the sites. Previous experiments [13] proved titratable histidyl-groups to be buried upon complex formation; a correlation of this effect to Hb came from the photooxidation of his, which paralleled the decrease of peroxidase activity, while the corresponding effect in case of Hp was observed after photooxidation of tyr.

The present chemical modification experiments prove the participation of his and tyr in the formation of the complex in a more direct way, show-

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[31] Taking the viscosity increment as a measure, all additives in Table 1 produce significant alterations of water structure; this holds even for 10^{-4} M SDS, where the relative viscosity amounts to 1.023.

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ing the expected inhibition of complex formation after blocking the his residues on the surface of Hb, or the tyr residues on the surface of Hp. The fact that the inverse combination does not cause major changes in the complex reaction gives this interpretation even stronger support: In the direct inhibition (Hb + DHT, Hp + NAI), conformational changes upon chemical modification might be responsible for the inhibitory effect; in the inverse combination, however, (e. g. after the Hp-DHT reaction) this possibility is clearly ruled out.

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Der Einfluß von partiellen Hepatektomien auf die Hepatomrate nach Diäthylnitrosamin-Gaben

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Rats were hepatectomised and subsequently diethylnitrosamine was applied orally (2 mg DENA/kg/d). Compared with a non-treated control group the mean survival time was lowered from 225 to 158 days. In a second experiment repeated hepatectomies were performed. All these rats were killed after 40 weeks and after a total DENA dose of 351 mg/kg. By histological examination of the livers a higher rate of hepatomas was found in the hepatectomised group.

Thus the proliferating processes during liver regeneration seem to favour the hepatocarcinogenesis under DENA treatment.

Wir untersuchten in zwei Versuchsserien, ob die Regenerationsvorgänge nach partieller Hepatektomie zu einer Erhöhung der Hepatomrate nach Nitrosamingaben führen. Einmal wurde nach einer $^2/_3$ Resektion der Rattenleber bei kontinuierlicher Nitrosamingabe die Überlebenszeit beobachtet, zum anderen wurde 5-mal eine Teilresektion der Leber durchgeführt, die Tiere nach diskontinuierlicher Nitrosaminfütterung nach 40 Wochen getötet und die Leber histologisch und enzymatisch untersucht.

Material und Methode

1. Versuchsserie: 30 ca. 200 g schwere weibliche Wistar-Ratten aus eigener Zucht erhielten bis zu ihrem Tode pro Tag 2 mg Diäthylnitrosamin (DENA) pro kg Körpergewicht im Trinkwasser gelöst. Bei einem Drittel der Tiere (Gruppe A) wurde zu Beginn der Nitrosaminfütterung eine typische $^2/_3$ Resektion der Leber (HIGGINS und ANDERSON)[6] durchgeführt, bei 10 weiteren Tieren wurde auf gleiche Weise 86 Tage nach Nitrosamingabe operiert (Gruppe B), das restliche Drittel erhielt nur Nitrosamin im Trinkwasser (Gruppe C). Nach dem Tode der Tiere wurde die Leber nach Färbung mit Hämatoxylin-Eosin und Toluidinblau histologisch untersucht.

2. Versuchsserie: 60 weibliche Wistar-AF-Ratten (Zentral-Institut für Versuchstierzucht, Hannover), ca. 200 g schwer, wurden in zwei Gruppen D und E von je 24 Tieren und in eine Kontrollgruppe F von 12 Tieren eingeteilt. Den Tieren der Gruppe D wurde sofort nach einer partiellen Hepatektomie, denen der Gruppe E eine Woche später 3 Tage lang 9 mg DENA pro kg Körpergewicht pro Tag im Trinkwasser gegeben. Während dieser Zeit wurden die Tiere einzeln im Käfig gehalten. Die Trinkmenge wurde anfangs auf 5%, als die Tiere weniger tranken, auf 3% des Körpergewichtes festgelegt. Operiert wurde im Abstand von 14 Tagen, nach der dritten Operation wurde jedoch eine Pause von 4 Wochen eingelegt. Die erste Hepatektomie betraf den vorderen linken, die zweite den mittleren cranialen, die dritte den vorderen rechten, die vierte und fünfte Hepatektomie den hinteren Leberlappen. Die excidier-

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