Sucrose-Stimulated Release of FITC-Dextran into the Bile in the Dynamics of Its Storage and Elimination from the Liver

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Stimulation of release of FITC-dextran endocytosed in the liver by an injection of hypertonic sucrose led to the appearance of a less pronounced and prolonged peak (with the maximum at min 15-25) at later terms after injection of the marker (30 days). Biliary release of FITC-dextran was 4-9-fold accelerated on days 1-14 in comparison with the constitutive release, and after 30 days it was accelerated 19-fold, which reflects high capacity of the long storage compartment to stimulated release of FITC-dextran into the bile.

Key Words: *liver lysosomes; vesicular transport; biliary excretion; FITC-dextran; sucrose*

Vesicular transport into the bile is a constitutive process [10,13]. It consists of lysosomes unloading, including heterolysosomes and residual bodies, and the release of endosome compartment (transcytosis). Activity of this process is 3-5% liver lysosome contents per day [5,10].

Some compounds stimulate lysosome unloading into the bile, *e.g.* sucrose [6], cAMP [2], sodium taurocholate [7], *etc.* Markers of pinocytosis, the best known of which is FITC-dextran, are released into the bile via all these routes [8]. However, it remains unclear how the dynamics and duration of intravesicular storage of FITC-dextran in liver cells affect the capacity of accumulating vesicles to release it into the bile under the effect of a standard stimulus.

We studied stimulated release of endocytosed FITC-dextran from hepatocytes into the bile in the course of its intracellular storage. It should be noted that we studied the release of the marker accumulated in hepatocytes, but not its transport from the blood into the bile, and therefore all measurements were carried out 1-30 days after marker injection, when blood concentration of FITC-dextran became negligible.

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MATERIALS AND METHODS

The study was carried out on male Wistar rats (200-300 g). FITC-dextran (molecular weight 70 kDa, Sigma) was kindly provided by Prof. B. Wiederanders (Jena University). FITC-dextran was intravenously injected to rats in a dose of 20 mg/kg 1, 3, 7, 14, and 30 days before sacrifice. The animals were deprived of food on the day of the experiment. The bile duct was cannulated under ether narcosis on a thermocontrolled operation stand. Control portion of the bile was collected before sucrose injection for evaluation of constitutive excretion. Hypertonic (50%) sucrose and 0.9% NaCl (both heated to 37°C) were injected into the caudal vein in a dose of 2 ml/kg [6]. Five-minute portions of the bile were collected for 60-90 min post-injection. The animals were decapitated and the blood was collected for obtaining plasma or serum. The liver was perfused with cold isotonic sucrose and a 20% homogenate was prepared on 0.25 M sucrose and 1 mM EDTA (pH 7.4).

The content of FITC-dextran in preparations was measured by fluorescence at excitation and emission wavelengths of 493 and 524 nm, respectively, using FITC-dextran solution as the standard. The content of FITC-dextran in the liver was evaluated after treat-

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ment of liver homogenate with 0.2% Triton X-100 and removal of membrane fragments by centrifugation at 34,000*g* for 30 min. The content of FITC-dextran in the cytosol was estimated as the percentage of the marker in the supernatant of liver homogenate (34,000*g*, 30 min), which was equivalent to the classical De Duve unsedimented fraction (105,000*g*) by activity of lysosomal enzymes. The rate of constitutive (spontaneous) release of FITC-dextran into the bile was evaluated by the ratio of marker content in the first bile portion (before injection of the inductor) to its content in the liver (after the end of the experiment) with consideration for the duration of the bile collection (5 min).

For evaluation of stimulation of FITC-dextran release the control (spontaneous) level of the marker was subtracted from all subsequent values. The total label content in all fractions constituting the peak of FITC-dextran release into the bile expressed in percent of its content in the liver was taken as the measure of stimulation. Plasma content in the body was taken as 3.13% body weight (volume/weight). The content of perfusate in routinely perfused liver was experimentally evaluated as 23.6%.

Activity of N-acetyl-glucosaminidase was evaluated with 4-methylumbellylferyl-N-acetyl-β,D-glucopyranoside as the substrate and 4-methylumbellyferone as the reference substance.

Each experimental series was carried out on 8-10 animals (some experiments on 3-5 animals).

The results are presented as mean±standard error. The significance of differences was evaluated using Student's *t* test at $p<0.05$.

RESULTS

FITC-dextran was rapidly eliminated from the circulation: serum content of the marker did not surpass 0.06% of the injected dose 24 h after administration and decreased to 0.02% by day 30. The concentration of FITC-dextran in the serum was 70% lower than in the plasma, that is, was of the same order of magnitude as the plasma concentration. Hence, plasma concentration of FITC-dextran did not surpass 0.1% at all terms of the experiment.

Accumulation of FITC-dextran in the liver peaked 24 h postinjection (15% of the injected dose) and decreased to 1% by day 30 of the experiment. The total content of the marker in the liver 125-250-fold surpassed its blood (serum) concentration throughout the experiment except the last time point (30 days).

The ratio of FITC-dextran concentrations in the bile/serum system was within 3-8 (on days 1-14), that is, marker concentration in the bile was definitely higher than in the blood. This probably reflects concentration of the marker in the liver associated with its intracellular transport.

Hence, the concentration of FITC-dextran in the serum (plasma) was very low in comparison with its concentration in the liver, and the level of the marker in the serum was significantly lower than in the bile. Therefore, the contribution of transcytosis to biliary excretion of the marker becomes negligible as early as 24 h postinjection.

Evaluation of the subcellular location of the marker accumulated in the liver showed that it was present in granular fractions. The content of FITC-dextran in non-precipitated fraction of the liver homogenate was about 17% of its total content in the liver 1-7 days postinjection. This value was close to the level of free activities of lysosomal enzymes in intact liver, which indicated vesicular location of the marker. At later terms (14 and 30 days) the percentage of FITC-dextran in non-precipitated fraction increased to 20 and 24%, respectively, which probably reflects slow intralysosomal degradation of dextran and the release of the products from lysosomes.

The rate of constitutive release of FITC-dextran into the bile was the same at all terms of investigation, except day 30 (0.0035-0.0037%/min or 2% liver content of the label per day, which agrees with published data [10,13]. After 30 days the rate of constitutive release of the marker decreased and varied within a wide range.

Biliary excretion of FITC-dextran was stimulated by intravenous injection of sucrose (0.5-0.6 ml of 50% solution). Rats injected with the same volume of normal saline and receiving no FITC-dextran served as the controls. The bile contains some components, derivatives of lipofuscin (residual body marker), fluorescing at the wavelengths characteristic of FITC. In light of this the release of these components after sucrose injection into the bile was evaluated. This reaction was characterized by low amplitude and long time (Fig. 1, *a*). Kinetically it was similar to N-acetyl-glucosaminidase release. Injection of an equal volume of 0.9% NaCl also modulated biliary excretion of FITC-dextran, and the reaction of N-acetyl-glucosaminidase could sometimes be discriminated from the baseline values.

Sucrose-stimulated biliary release of FITC-dextran was observed mainly 10-50 min after the effector injection, the maximum was observed on minutes 15-25 (Fig. 1). This kinetics of the response is in line with published data on the release of liquid endocytosis substrates by [^{14}C]-sucrose [6] and horseradish peroxidase [7].

Thirty days after injection of the marker the kinetics of its release was different. The early peak of excretion was absent and the maximum was shifted to 50 min postinjection. However, the amplitude of the response was comparable with the background

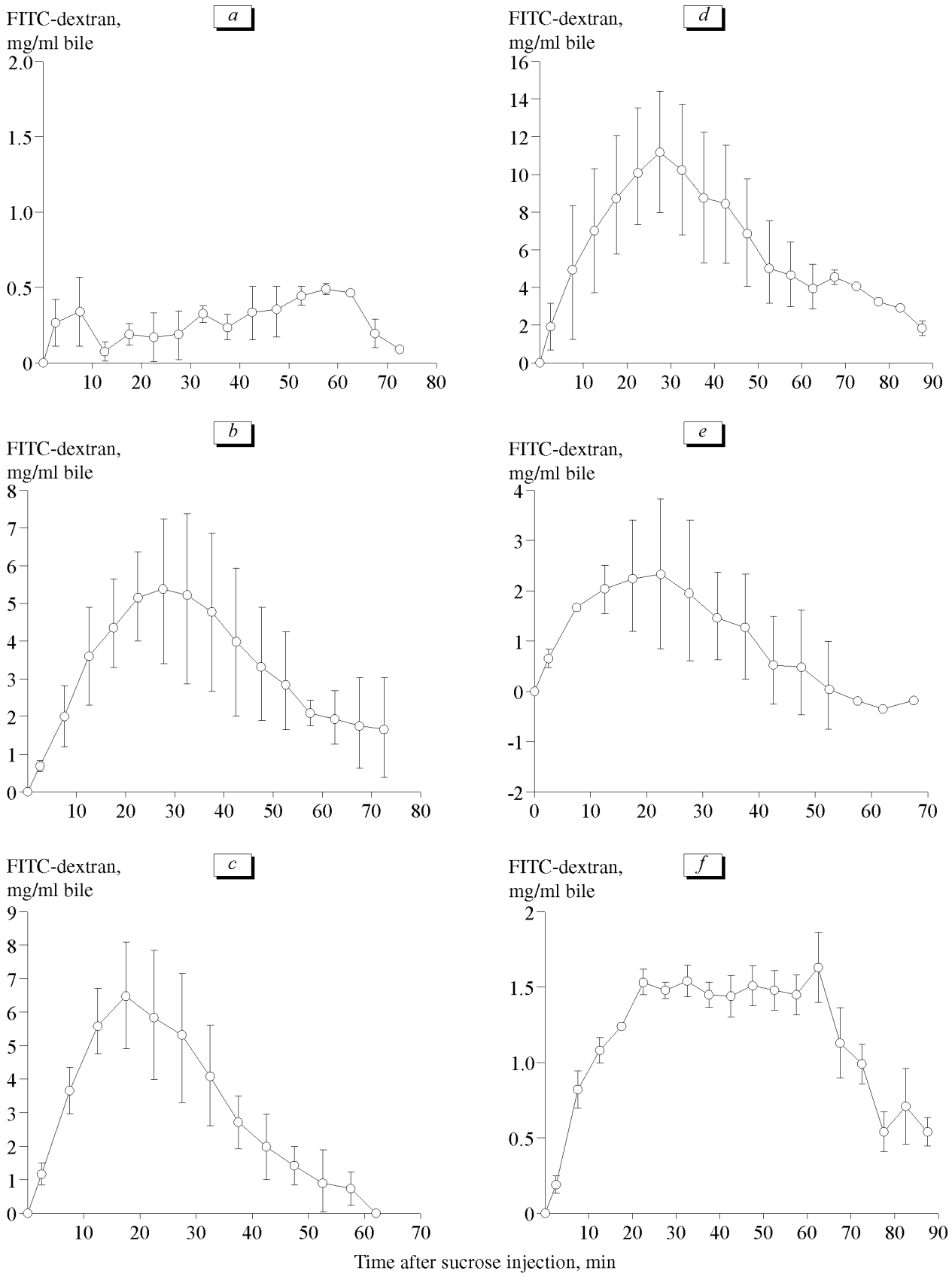

Fig. 1. Kinetics of FITC-dextran excretion into the bile under the effect of sucrose in the course of intravesicular storage of the marker in the rat liver cells. *a*) fluorescence of the bile (without FITC-dextran); *b-f*) days 1, 3, 7, 14, and 30 after injection of FITC-dextran, respectively.

because of low (1%) content of the marker in the liver at this term.

Stimulated release of N-acetyl glucosaminidase into the bile was more variable than the release of FITC-dextran, but in general it reflected the early excretion of the enzyme (15-25 min, Fig. 2). The kinetics of the release of FITC-dextran from old (30 days) vesicles was delayed compared to the release of lyso-

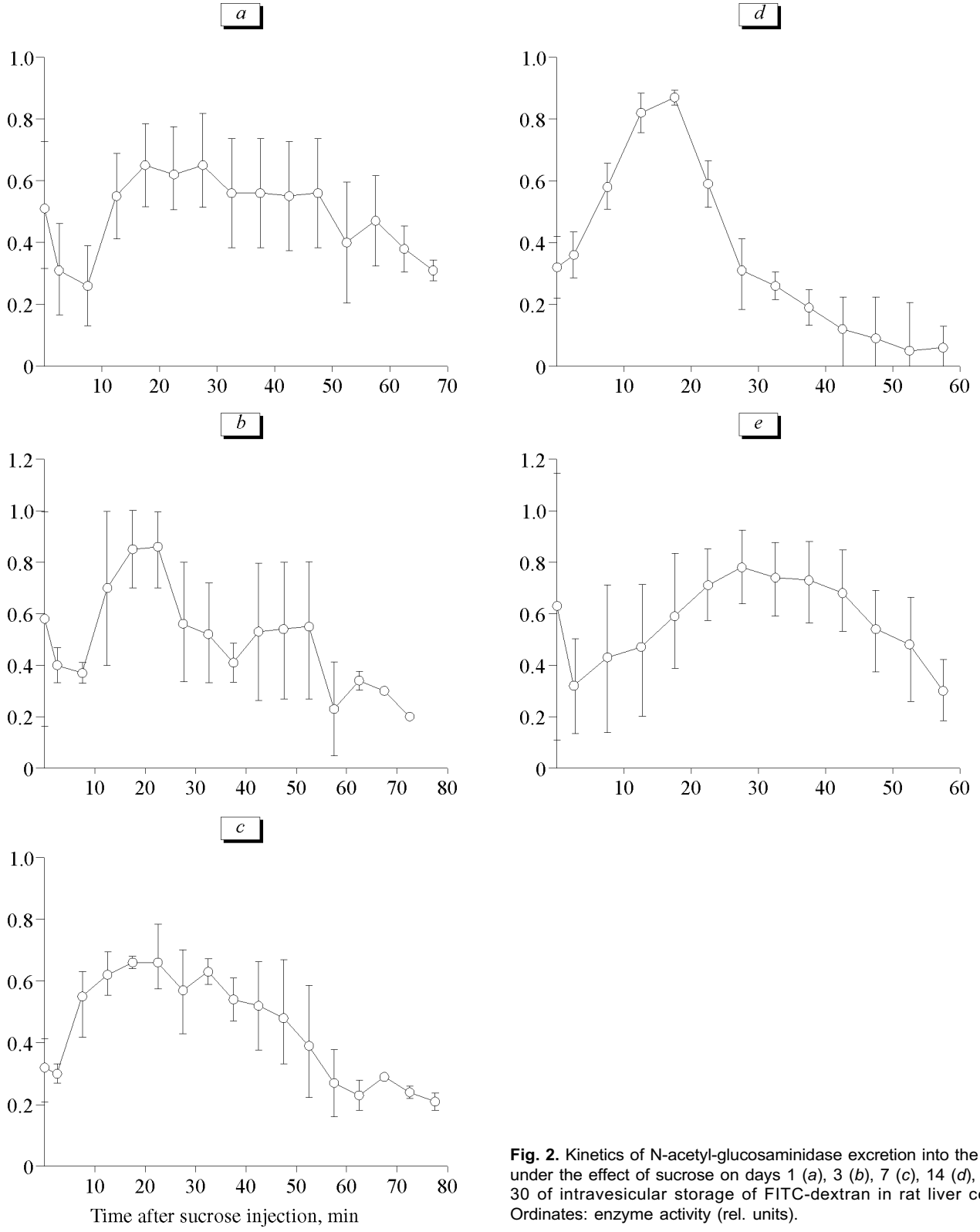

Fig. 2. Kinetics of N-acetyl-glucosaminidase excretion into the bile under the effect of sucrose on days 1 (*a*), 3 (*b*), 7 (*c*), 14 (*d*), and 30 of intravesicular storage of FITC-dextran in rat liver cells. Ordinates: enzyme activity (rel. units).

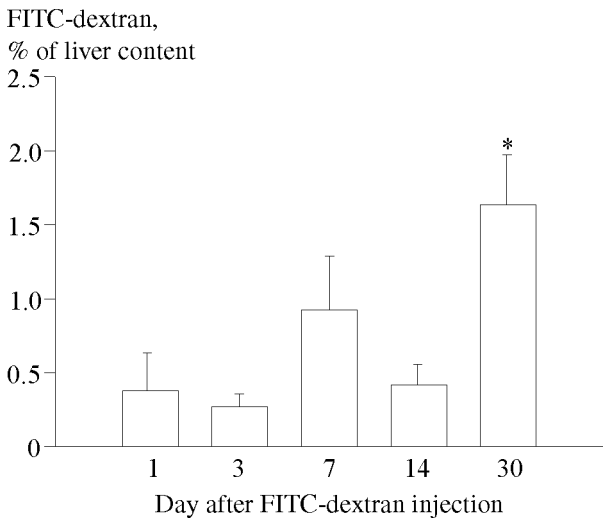

Fig. 3. Total sucrose-stimulated release of FITC-dextran into the bile in the course of intravesicular storage of the marker. *$p<0.02$ compared to days 1 and 3.

somal marker N-acetyl glucosaminidase. Thus, the reaction of old vesicles containing FITC-dextran to sucrose differed from that of the early vesicles and lysosomes.

The intensity of cell response (FITC-dextran release into the bile) was evaluated by the total content of the marker in bile fractions constituting in the peak of excretion. At the early terms after injection of the marker (days 1-3) 0.3-0.4% of the label was released with the peak (Fig. 3). At later terms a tendency to intensification of this cell response was observed. On day 30 the hypertonic stimulus caused the release of about 1.6% label accumulated in the liver into the bile, which differed significantly ($p<0.02$) from the amplitude of this cell reaction at early terms. We do not overstate this fact, because the marker content in the liver was very low at early terms. However this result can reflect enhanced release from old vesicles into the bile due to, *e.g.*, their migration into the pericanalicular area. Other possibilities are label redistribution into hepatocytes with aging of vesicles and more rapid release of the marker from the sinusoidal cells compared to its release from hepatocytes, which led to seeming intensification of cell response. Anyway, we conclude that old accumulation vesicles retain their capacity to release the marker into the bile under the effect of sucrose, though blurred peak of FITC-dextran on day 30 indicates the absence of a standard clear-cut cell response.

The stimulated release of FITC-dextran into the bile (1.6% 30 days after its injection) is close to daily constitutive excretion of the marker (2%) detected in our experiment and corresponds to more rapid (19.2 times) release of the marker (within 1 h) in comparison with the constitutive release. However, at the early terms the stimulated excretion of FITC-dextran did not surpass 0.3-0.4% (4-5-fold acceleration) and was just 15-20% of its normal daily excretion. Presumably, the cell most effectively releases accumulated matter after repeated stimulation under conditions of the absence or short refractory period.

One more step of our study was to find out from which cellular compartment FITC-dextran was released into the bile. FITC-dextran is a lysosome marker [4,9,11], but there are reports about its peculiar location. Together with other markers of endocytosis FITC-dextran it was detected in very early endosomes (osmotically resistant) after 10 min, and after 25 min was detected in lysosomes [3,12]. The marker appeared in perfused liver after 13 min in the old endocytosis compartment and was later transferred mainly into lysosomes, but not into the late transcytosis compartment [1]. However, according to I. R. Lake *et al.* [5], FITC-dextran was only partially accumulated in lysosomes of isolated hepatocytes, but mainly in acid vesicles different from lysosomes, and as early as 1-6 h after endocytosis there was no notable intracellular transport of the label. Other authors showed that after 2 h FITC-dextran was still present in early endosomes sensitive to anisotonia, but after 7 h it was transported into the next compartment, old endosomes/lysosomes [13]. At later terms of *in vivo* experiment (16 h post-injection) FITC-dextran was detected in very acid compartment (pH 4.67) characterized as lysosomes [9]. In other experiments FITC-dextran was first rapidly transported into the pericanalicular hepatocyte area and was then seen in the perinuclear region. After 17 h it was located in compact vesicles close to lysosomes by their characteristics [15]. Hence, published reports are contradictory, and in general we can speak about dual nature of the compartment of FITC-dextran storage: nonlysosomal accumulation vesicles (old endosomes) and heterolysosomes (and postlysosomes as their final stage).

In our experiments the kinetics of stimulated release of FITC-dextran into the bile in general (on days 1-14) coincided with that of lysosome unloading. Hence, FITC-dextran can be released into the bile from heterolysosomes at this term. More precise discrimination of the kinetics of the marker release is difficult, because at early terms of endocytosis stimulated release of other pinocytosis substrata (horseradish peroxidase) and lysosomal enzymes into the bile is also observed 15-20 min after stimulation [7], *i.e.* transcytosis vesicles (endosomes) react similarly.

By day 30 of the experiment FITC-dextran-containing heterolysosomes seem to loose lysosomal enzymes, become postlysosomes, and are released into the bile under the effect of the stimulus. This release was characterized by a delayed kinetics. However, the rate of this process is comparable with that of heterolyso-

somes. Presumably, some heterolysosomes migrate to the pericanalicular region of the cell, which can promote their interactions with the contractile system and enhance biliary release of FITC-dextran-accumulating vesicles.

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