negative; 4 = maximum effect) of the amount of Maltese cross inclusions. The Table demonstrates a clear dose-effect relationship; at maximum manifestation, virtually each cell contained extremely large amounts of birefringent bodies in its cytoplasm. These presented either a clear-cut simple Maltese cross, or were of composite structure with the Maltese cross distorted to various degrees (Figure 1). The latter apparently represent multicentric MB which were frequently observed with electron microscopy[1-3,5]. In particularly clear cases, a limiting membrane surrounding the birefringent inclusions was discernible.

Two lines of evidence indicated that these birefringent inclusions represent MB and contain large amounts of phospholipids: 1. After standing for several hours, tube-like extrusions emerged from the inclusions of fresh tissue preparations which, in suitable cases, eventually gained the extracellular space from injured cells and formed tortuous birefringent 'myelin tubes' showing slow movements. Their formation strongly suggests that MB inclusions contain large amounts of polar lipids. 2. In small pieces of liver stained in an alcoholic solution of Sudan black B and embedded in glycerine jelly, the inclusions were light- to dark-blue stained, showed bronze-coloured birefringence with Maltese cross, and dichroism in linearly polarized light. These optical features have been found to be typical for phospholipids[8]. Birefringent buds or tubes with the same optical properties may emerge from such fragments.

Of particular interest are the MB induced by mepacrine, which has a marked affinity to lysosomes[7]. It was found that the Maltese cross inclusions presented the typical green fluorescence of mepacrine (Figure 2). Chloroquine-induced MB were also fluorescent. These facts support the view that MB are secondary lysosomes, and also suggest that the inducing drug is accumulated in these bodies.

Prolonged treatment of rats with the drugs used elicited also Maltese cross inclusions in a large number of other organs. This agrees with electron microscopic findings of several authors[2,3,6,9] and further confirms the identity of MB with Maltese cross inclusions. Several reports recently described the drug-induced formation of foam cells in the lungs of laboratory animals[10-12]; ultrastructurally, foam cells are characterized by the occurrence of MB in their cytoplasm[10,12]. The three drugs mentioned and several other compounds eliciting MB in the liver also induced foam cells in the lung of rats[13]. It thus appears that MB formation in various organs represents manifestations of the same generalized cytopathological mechanism which results in a 'drug-induced lipidosis'[6,10]. The findings reported here show that this pathological condition can be visualized very simply and rapidly by light-microscopy.

Semiquantitative evaluation of the induction of MB in the rat liver by the three drugs

1	2				3				4			
Single dose (mg/kg) (dosage group)	Estimation of the incidence of hepatic MB											
	Drug and No. of doses											
	Triparanol (4×)				Chloroquine (4×)				Mepacrine (2×)			
1600					–	–	–	–	–	4	4	4
800	–	–	3	3	–	4	4	4	3	4	4	4
400	–	3	4	4	3	4	4	4	3	4	4	3
200	2	3	4	2	1	2	3	2	2	2	3	3
100	2	2	1	2	2	1	0	0	2	0	0	1
50	1	0	0	1					0	0	0	0
Controls	0	0	0	0	0	0	0	0	0	0	0	0

4 doses (triparanol, chloroquine) or 2 doses (mepacrine) were given orally to 4 rats in each dosage group (column 1) at daily intervals. 24 h after the last application, the effect in the liver cells (= frequency of inclusions with Maltese cross birefringence) was evaluated semiquantitatively for each rat (0 = negative; 4 = maximum observed effect) (columns 2, 3, and 4). A dash indicates that the animal died before the experiment was terminated.

Zusammenfassung. Sekundäre Lysosomen mit multilamellärer Myelinkörper-Struktur, die in der Rattenleber nach Behandlung mit verschiedenen Agenzien (z.B. Triparanol, Chloroquin, Mepacrin) induziert werden können, sind aufgrund ihrer Malteserkreuz-Konfiguration im polarisierten Licht lichtmikroskopisch nachweisbar. Mit fluoreszierenden Substanzen (Chloroquin, Mepacrin) zeigen die Malteserkreuz-Einschlüsse im Zytoplasma starke Fluoreszenz, was auf ihre Akkumulation in den Myelinkörpern hinweist. Nach längerer Behandlung treten Myelinkörper auch in zahlreichen andern Organen auf und induzieren unter anderem die Bildung von Schaumzellen in der Lunge.

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Enhancement and Deactivation of Some Microsomal Glycosyl Transferases[1]

The activity of uridine diphosphoglucuronyl transferase and numerous other hepatic microsomal enzymes is enhanced by pretreatment of animals with phenobarbital or with other microsomal enzyme inducers[2]. More recently mammalian liver microsomes have been found to contain also uridine diphosphoglucosyl transferase capable of transferring the glucose moiety to such endogenous substrates as estrogens[3], or bilirubin[4-6] or an exogenous substrate such as *p*-nitrophenol[7,8]. Additionally, liver microsomal preparations also contain glycogen synthetase which sediments together with particulate glycogen and glucuronyl transferase[9,10]. Glucosyl transferase activity in microsomes is much lower than that of the latter enzyme[5,7]. In this study

consideration was given to the possibility that *p*-nitrophenol glucosylation is mediated by either *p*-nitrophenyl glucuronyl transferase or glycogen synthetase. In fact LAYNE[11] had speculated about possible involvement of glycogen synthetase in the glucosylation of steroids. If this were the case for *p*-nitrophenol glucosylation, parallel changes in the activites of microsomal glycogen synthetase and glucosyl transferase would be observed following phenobarbital treatment, alternatively glucuronyl and glucosyl transferases should be affected in a parallel manner. Microsomal glucuronyl transferase is known to undergo spontaneous activation on storage[12] while glycogen synthetase activity is known to deteriorate under such circumstances[9, 13]; therefore, the behavior of the three enzyme activities on storage was examined. To our knowledge, neither the stability of glucosyl transferase to storage nor the effects of phenobarbital treatment have been reported prior to this study, preliminary report of which was given[1].

Materials and methods. The following were purchased from Sigma Chemical Company: *p*-nitrophenyl-β-D-glucoside; *p*-nitroplenyl-β-D-glucuronide; uridine-5′-diphosphoglucose, (UDP-glucose); uridine-5′-diphosphoglucuronic acid (ammonium salt), (UDP-glucuronic acid); 17-β-estradiol-3-methyl ether, ($\Delta^{1,3,5}$-estratien-3-17-β-diol-3-methylether[11]); Trizma Base (*tris*). *p*-Nitrophenol was from Mann Research (Schwartz-Mann). The following were from New England Nuclear Corporation: uridinediphosphate-^{14}C-glucuronic acid, (D-glucuronic-^{14}C, uniformly labeled, > 200 mC/m*M*); uridinediphosphate-^{14}C-glucose, (D-glucose-C^{14}, uniformly labeled), 200 mC/m*M*. Bovine serum albumin was from Pierce Chemical Company.

Animal treatment and preparation of microsomes. Adult male mice (Ha:ICR) 30–35 g from Blue Spruce Farms, Altamont, New York, were maintained on Purina Chow diet and water ad libitum. All mice were sacrified at the same time of day and 18 h after the last injection; induction was achieved in 5 days by daily i.p. injections of 50 mg/kg of phenobarbital sodium in isotonic saline, the controls receiving an equivalent volume (10 ml/kg) of the saline. After cervical dislocation livers were quickly removed, gall bladders excised and the tissue (pooled livers from 5 animals) was weighed, rinsed with ice cold 0.25 *M* sucrose, blotted and homogenized in 9 volumes of 0.25 *M* sucrose using a Potter-Elvejhem homogenizer with a teflon pestle (2 passes at 1000 rpm). The homogenate was centrifuged at 12,100 *g* for 20 min, the pellet discarded and the centrifugation repeated; the supernatant was then centrifuged at 105,000 *g* for 60 min; the microsomal pellet was washed twice by resuspending it in sucrose (by mashing with a teflon pestle) and centrifuging for 60 min at 105,000 *g*. The microsomes were then suspended

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Table I. Effect of phenobarbital (PB) pretreatment of mice on liver microsomal glucuronyl transferase (GAT), glucosyl transferase (GT) and glycogen synthetase (Gly.S) activities

Enzyme[a] assay	Animal group[b]	Ratio: liver wt body wt	A[c]		B[d]		C[e]	
			Enzyme act. per mg protein	Activity untreat. (%)	Enzyme act. g liver	Activity untreat. (%)	Enzyme act. g body	Activity untreat. (%)
GAT	I C	0.063	0.238	100	3.13	100	0.197	100
	I PB	0.074	0.225	95	3.65	117	0.270	137
Gly.S	I C	0.063	0.033	100	0.44	100	0.028	100
	I PB	0.074	0.032	95	0.51	117	0.038	137
GAT	II C	0.068	0.166[f]	100	2.60[f]	100	0.176[f]	100
	II PB	0.076	0.209[f]	126	3.68[f]	142	0.290[f]	165
GT	II C	0.068	0.044[f]	100	0.69[f]	100	0.047[f]	100
	II PB	0.076	0.071[f]	161	1.25[f]	181	0.096[f]	204
GT	III C	0.058	0.049	100	0.79	100	0.046	100
	III PB	0.074	0.063	128	1.32	168	0.097	212
GAT	III C	0.058	0.219	100	3.50	100	0.202	100
	III PB	0.074	0.188	86	3.95	113	0.291	144
Gly.S	IV C	0.061	0.032	100	0.38	100	0.022	100
	IV PB	0.069	0.024	75	0.35	91	0.025	116
GT	IV C	0.061	0.068[f]	100	0.80[f]	100	0.049[f]	100
	IV PB	0.069	0.080[f]	118	1.17[f]	146	0.081[f]	165

[a] Each value is an average of duplicate assays performed using microsomal preparations from 5 pooled livers; the duplicates differed from each other by no more than 10%. [b] C refers to untreated controls, and PB to phenobarbital treated animals. [c] Enzyme activity under A) is in μmoles of product formed per mg of microsomal protein per 30 min. [d] Enzyme activity under B) is in μmoles of product formed per 30 min by microsomes equivalent to 1 g liver. [e] Enzyme activity under C) is in μmoles of product formed by hepatic microsomes per 30 min per 1 g body weight. [f] These assays were carried out in the presence of propylene glycol.

in sucrose by homogenization for 30 sec at 1000 rpm; 1 ml of suspension contained microsomes equivalent to 1 g of liver. Protein was determined by the method of LOWRY et al.[14], using bovine serum albumin as the standard. All assays, except where otherwise stated, were carried out using freshly prepared microsomes; where stored microsomes were used, these were kept frozen for 7 days at −20 °C, thawed at room temperature and homogenized at 1000 rpm as above.

UDP-Glucuronyl and UDP-Glucosyl transferase assays. The assays were performed using glycosyl donors ^{14}C-labeled in the sugar moiety, and *p*-nitrophenol as the acceptor substrate. A typical incubation mixture consisted of: 5 m*M* *p*-nitrophenol, 5 m*M* UDP-^{14}-glucuronic acid or 10 m*M* UDP-^{14}C-glucose, (1 μC per ml incubation), 0.2 *M tris* buffer (for glucuronidation pH was 8.0, for glucosylation pH was 6.8) and microsomes equivalent to 0.5 g of liver per 1 ml of incubation mixture. Additionally, in inhibition experiments 10% propylene glycol was present, or 5 m*M* estradiol-3-methyl ether together with 10% propylene glycol which was used as the steroid solvent. Incubations were carried out in a Dubnoff metabolic shaker at 37 °C for 30 min in air. The reactions were stopped by the addition of 3 volumes of absolute ethanol with mixing, followed by immediate freezing on solid carbon dioxide. The samples were kept stored at −20 °C for analysis. All assays were done in duplicate.

Glycogen synthetase assay. A typical incubation mixture consisted of: 10 m*M* UDP-^{14}C-glucose (1 μC/ml of incubation), 10 m*M* glucose-6-phosphate, 0.2 *M tris* buffer at pH 8.0, 5 mg of rabbit liver glycogen and microsomal preparation equivalent to 0.5 g liver per ml incubation. The incubations were carried out for 30 min at 37 °C as before. All assays were done in duplicate.

Estimation of metabolites. The amount of ^{14}C-glucose or ^{14}C-glucuronic acid incorporated into *p*-nitrophenyl glucoside or *p*-nitrophenyl glucuronide from the nucleotide precursors was determined following chromatographic separation of the metabolites on paper developed in *n*-butanol:acetic acid:5% aq. ammonia (7:5:3:3). Strips containing metabolite peaks were counted in scintillation vials as described earlier[15].

Newly synthetized glycogen was estimated by determining the amount of ^{14}C-glucose incorporated from UDP-^{14}C-glucose. The method of THOMAS et al.[16] was used.

Results and discussion. In order to test whether synthesis of *p*-nitrophenyl glucoside could be attributed to catalysis by either glycogen synthetase of glucuronyl transferase present in the microsomal preparations, the three enzyme activities were assayed concurrently in pairs; drug induced parallel fluctuations in specific activities were sought. Results in Table I show that 5 day treatment of animals with phenobarbital consistently produced a marked increase in the specific activity (μmoles product/mg protein/min) of microsomal glucosyl transferase only (see Table I, column A); fluctuations in glycogen synthetase and glucuronyl transferase activities were similar to each other but did not parallel the increase associated with glucosyl transferase activity. This provided a strong indication that microsomal *p*-nitrophenolglucosyl transferase activity was not merely a result of catalysis by one of the other two enzymes.

Since hepatic microsomal enzyme activities are frequently expressed per g. of wet weight of liver[17] and sometimes per g of body weight[18], such values are also given in Table I (columns B and C, respectively). Comparison of the three indices of activity (A, B and C) in Table I illustrates that different conclusions can be reached regarding the magnitude of effects produced by phenobarbital treatment. In terms of enzyme activity per g of tissue, both glucosyl transferase and glucuronyl transferase activities appear substantially increased by phenobarbital treatment; and when calculated as per g body weight, increases are seen in all three enzyme

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Table II. Effect of storage of microsomes on activities of glucuronyl transferase (GAT), glucosyl transferase (GT) and glycogen synthetase (Gly.S)

Enzyme assay	Animal treatment	Quality of microsomes	Enzyme activity[a]	Effect of storage (% activity of fresh prep.)
GT	None	Fresh	0.044	100
		Stored	0.028	63.6
Gly.S	None	Fresh	0.032	100
		Stored	0.004	12.5
GAT	None	Fresh	0.238	100
		Stored	0.481	202
GT	Phenobarb.	Fresh	0.074[b]	100
		Stored	0.034[b]	46.5
Gly.S	Phenobarb.	Fresh	0.024	100
		Stored	0.003	12.5
GAT	Phenobarb.	Fresh	0.225	100
		Stored	0.390	173

[a] Enzyme activity is in μmoles of product formed per mg of microsomal protein per 30 min. Each value is an average of duplicate assays performed using microsomal preparations from 5 pooled livers; the duplicates differed from each other by no more than 10%. [b] These assays were carried out in the presence of propylene glycol.

activities (Table I, column C). These differences occur because phenobarbital produces alterations in the composition of hepatic microsomes[19,20], changes in protein concentration[18,20], and increases in the liver to body weight ratios[17,18,21,22]. Thus for instance, due the enlarged liver size of a phenobarbital treated animal (see liver wt/body wt, Table I) in vivo the treated animal is capable of substantially more conjugation and more glycogen synthesis than the corresponding control animal, hence the largest drug induced increases are seen in column C.

There is an apparent controversy regarding the effects of barbiturate treatment on glucuronyl transferase activity, some workers observing enhancement[21,23–28] others failing to do so [17,22,29,30]. The controversy stems in part from use of any of the three indices of activity to report the effects, and in part from differences in the method of preparation of glucuronyl transferase which is subject to spontaneous activation[12]. Considering the parameter of specific activity (column A), our results, obtained from assays of freshly prepared mouse liver microsomes (Table I), are in agreement with those of WINSNES[22] who also observed that phenobarbital pretreatment produced no significant increase in the specific activity of *p*-nitroplenol glucuronyl transferase of mouse liver homogenates; similar observations were reported by POTREPKA and SPRATT[30] about guinea-pig hepatic microsomal glucuronyl transferase. More recently MULDER[17] and WINSNES[22] demonstrated that phenobarbital induced increases (they report a factor of 1.5–2) were detectable only when activities were assayed using detergent activated liver preparations. Mulder pointed out that other workers may have obtained a detergent-like effect by repeated rehomogenization of their microsomal pellets. Phenobarbital induced increases in glucuronyl transferase activity per g of liver have been consistently observed by many workers[17,21,27,28] including us (Table I, column B).

Spontaneous activation of microsomal glucuronyl transferase is a well documented phenomenon; this can occur as a result of prolonged storage at low temperatures[12,31], detergent activation[12,17,22,31,32], or several hours of preincubation at 37 °C[32]. We have compared the effects of storage on the three enzyme activities under investigation.

Results in Table II show that there are no parallel changes in the activities of the three enzymes. Glucosyl transferase appears to be unstable and about half of the activity was lost during storage at −20 °C for 7 days; under the same conditions, glucuronyl transferase activity had increased by about a factor of 2, while glycogen synthetase activity fell to about $^1/_{10}$ of the value in fresh preparations. Instability of the latter enzyme is in agreement with the observations of LELOIR and GOLDEMBERG[9]. The microsomal enzymes from phenobarbital treated animals behaved similarly on storage; the significance of a somewhat lower activation of glucuronyl transferase cannot be assessed at this time.

Both the phenobarbital treatment of animals and the storage of microsomal preparations appeared to produce qualitative changes in the transferases, as revealed by differences in the inhibitory effects of estradiol-3-methyl ether and the steroid solvent, propylene glycol. Glucosyl transferase (GT) of control animals was sensitive to propylene glycol inhibition (19% and 6% inhibition being repeatedly observed in fresh and stored preparations, respectively); phenobarbital pretreatment of animals abolished this sensitivity of the enzyme. Glucuronyl transferase (GAT), on the other hand, was inhibited by the glycol (10–16% inhibition) only in assays of fresh preparations from either type of animals; stored preparations were insensitive to the glycol. Estradiol-3-methyl ether had a similar effect on both enzymes: it was less inhibitory in fresh than in stored preparations from control animals (inhibition of: fresh GT, 58%; stored GT, 70%; fresh GAT, 56%, stored GAT, 61%), but was equally inhibitory in preparations from phenobarbital treated animals irrespective of storage (inhibition of GT, 64%; GAT, 53.5%). It is interesting that SHOEMAN et al.[33] have observed that cold storage of microsomal preparations resulted in preferential loss of Type I binding site of the cytochrome P-450 redox system. Qualitative changes caused by storage of microsomal enzymes may be more prevalent than hitherto realized. Our studies underscore the importance of assays of glucosyl and glucuronyl transferase activities in fresh microsomal preparations, unless any changes brought about by storage or purification procedures are well understood[34].

Résumé. L'administration de phénobarbital à la souris augmente l'activité de l'UDP-glucosyltransférasique dans les microsomes hépatiques. On a remarqué que le traitement par la drogue, ou la conservation des microsomes à −20 °C, change la qualité et la quantité de cet enzyme, ainsi que de l'UDP-glucuronyltransférase.

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