

Alteration of drug metabolism in Gilbert's syndrome

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SUMMARY The pathophysiology of Gilbert's syndrome was studied by investigating the metabolism of the drug tolbutamide, which is metabolised by the liver but does not undergo glucuronidation. Using rat liver cell supernatant, tolbutamide was shown to bind to the hepatic cytoplasmic Y protein in a manner similar to other organic anions, but not to Z protein. In 31 patients with Gilbert's syndrome the plasma disappearance (plasma half-life, mean \pm SD : 628 \pm 84 min) and metabolic clearance (7.9 \pm 1.8 ml/min) were significantly ($P < 0.0005$) altered compared with the 13 controls (mean half-life 393 \pm 26 and mean clearance 13.4 \pm 1.5). The eight patients with hyperbilirubinaemia due to haemolytic disease showed no difference from the normal control subjects. In three patients with Gilbert's syndrome the cumulative urinary excretion of tolbutamide metabolites, 24 hours after the administration of the drug, was 30% lower than in the controls. In the five patients with Gilbert's syndrome, phenobarbital administration (100 mg/day) produced a significant increase in clearance of the drug from 8.8 \pm 0.8 to 13.4 \pm 1.9 ml/min; this was paralleled by a fall in serum bilirubin concentration. The plasma half-life of tolbutamide was similar in Gunn rats and Wistar rats. The results suggest that the metabolic defect(s) of Gilbert's syndrome affects compounds other than bilirubin and that defective uptake is probably the major factor.

The chronic low grade unconjugated hyperbilirubinemia known as Gilbert's syndrome (constitutional hepatic dysfunction) is thought to result from a selective defect involving one or more steps in the handling of bilirubin by the liver (Arias, 1966).

Most of the information on the pathophysiology of the syndrome has been obtained by investigating the kinetics of an exogenous bilirubin load (Billing *et al.*, 1964; Berk *et al.*, 1970) or by the direct assay of the hepatic glucuronyl transferase activity (Black and Billing 1969; Felsher *et al.*, 1973). Defective uptake of bilirubin by the liver cell and/or defective conjugation of the pigment are considered to be responsible for the hyperbilirubinaemia observed in these patients.

If one assumes that the transfer of bilirubin from plasma to bile is a linked process embracing many steps, then a defect in one step will probably affect the overall process. On the basis of kinetic studies of plasma bilirubin it will not, however, be possible to define exactly which is the defective step. Additional

information could be obtained by investigating the behaviour of other organic anions the hepatic metabolism of which is similar in some respects to bilirubin.

Marginal abnormalities in BSP plasma disappearance curves have recently been reported in some patients with Gilbert's syndrome (Berk *et al.*, 1972). These imply that there is defective liver metabolism of organic anions other than bilirubin. To study this aspect we investigated the *in vivo* metabolism of tolbutamide in Gilbert's syndrome. This drug appears to be actively taken up by the liver (Nistrup Madsen *et al.*, 1971) and metabolised by the microsomal drug metabolising system (Darby *et al.*, 1972). An average of 85-92% of the drug is recovered in the urines as carboxy- and hydroxytolbutamide metabolites (Nelson and Reilly 1961; Thomas and Ikeda, 1966).

The similarity of tolbutamide to bilirubin, where uptake and transport mechanisms are concerned, was checked by studying the binding of the drug to the liver cell cytoplasmic proteins Y and Z.

The possibility that unconjugated hyperbilirubinaemia might *per se* influence tolbutamide metabolism was eliminated by investigating the plasma clearance of the drug in patients with haemolytic hyperbilirubinaemia.

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Tolbutamide plasma half-life was also estimated in Gunn rats in order to evaluate any possible influence of deficient hepatic conjugation on the drug's metabolism.

Finally, in some patients with Gilbert's syndrome we studied the effect of treatment with phenobarbital on the disposal of tolbutamide in order to see if tolbutamide clearance was affected by such treatment in a manner similar to bilirubin.

Methods

PATIENTS

Group I In 31 patients referred to the Medical Clinic of the University of Modena for a chronic unconjugated hyperbilirubinaemia, the diagnosis of Gilbert's syndrome was made by exclusion. None of the patients had a history of liver disease or haematological disorder and physical examination was negative. The results of liver function tests were within the normal range except for the serum total bilirubin which ranged from 20.5 to 53.0 $\mu\text{mol/l}$ and was mainly in the unconjugated form (14.5 to 46.0 $\mu\text{mol/l}$). Normal RBC and reticulocyte counts and RBC osmotic fragility indicated that there was no sign of gross haemolysis in any patient; red blood cell survival was not, however, evaluated.

Liver biopsies performed in six subjects of this group did not reveal any abnormal findings.

Group II This comprised eight thalassaemic subjects who had hyperbilirubinaemia (20.0 to 39.3 $\mu\text{mol/l}$) mostly unconjugated (16.0 to 32.0 $\mu\text{mol/l}$). A constant finding in this group of subjects was abnormal RBC osmotic fragility, but in four patients only an increased reticulocyte count was found. In three patients in whom the test was performed a decreased RBC survival was demonstrated.

Group III This consisted of 14 healthy volunteers whose history and clinical and laboratory findings were negative for liver, blood, and metabolic disorders. The age range and sex distribution of the subjects in this group was comparable with group I.

None of the subjects entering the study was receiving or had received drug treatment during the three weeks before the test.

All the subjects were fully informed of the study and procedures and gave their consent.

EXPERIMENTAL PROCEDURE

Tolbutamide binding to hepatic cytoplasmic proteins

The procedure followed was that originally described by Levi *et al.* (1968). Male Wistar rats weighing 180–200 g were used. The animals were killed after an overnight fast and the liver immediately homogenised with 0.25 M sucrose (1:4, w/v); the homogenate was centrifuged at $100\,000 \times g$ for two hours. To 3

ml of the supernatant, corresponding to 1 g of liver, was added a trace amount of S-35 labelled tolbutamide (specific activity: 36 mCi/mmol, Amersham, England) and the mixture was placed on a Sephadex G-75 column (3 \times 45 cm). Elution was performed with 0.1 M K-phosphate buffer pH 7.4, using a pump driven upward flow system at a rate of 20 ml/h. The collected fractions were analysed for protein concentration by absorbance at 280 nm and for tolbutamide by counting aliquots in a liquid scintillation spectrometer (Packard Tri-Carb mod 3320) using Bray's (1960) scintillation mixture. The protein chromatogram was checked by a parallel run of the same supernatant fraction to which BSP was added instead of tolbutamide.

Tolbutamide metabolism in vivo

All subjects received a light breakfast in the morning and 30 minutes later 15 mg/kg bodyweight of tolbutamide (Rastinon, Hoechst) was administered intravenously. Blood samples were drawn hourly for nine to 10 hours after the administration of the drug. Plasma tolbutamide concentration was estimated using Spingler's procedure (Spingler, 1957).

The concentrations of the drug were plotted against time on semilog paper and the best linear fit was drawn using the least squares method. The extrapolated concentration at the time of the injection (C_0) and the half-life ($T/2$) were read from the graph. The clearance (CL) of tolbutamide was obtained using the following equation:

$$CL = aVd \times \frac{0.693}{T/2}$$

where aVd is the apparent volume of distribution, obtained by dividing the injected dose by C_0 , and $0.693/T/2$ is the elimination rate constant.

In five subjects with Gilbert's syndrome tolbutamide clearance was assessed before and after 15 days of treatment with phenobarbital (100 mg/kg).

Urinary excretion of tolbutamide metabolites

In three subjects with Gilbert's syndrome and three normal controls the urinary excretion of tolbutamide metabolites was evaluated. After the oral administration of 1.5 g tolbutamide, containing 15 μCi S-35 labelled drug, urine samples were collected every two hours then, after collecting the urine samples of the night in a single sample, two more samples were taken at 22 and 24 hours.

According to the method of Tagg *et al.* (1967) a known volume of the urine samples was extracted with ether, evaporated to dryness, and then taken up with absolute ethanol. Aliquots of the extracts were counted in a liquid scintillation spectrometer using Bray's solution.

Tolbutamide plasma half-life in Gunn rats

Three male Wistar rats and three male Gunn rats were used. The animals (180 g bodyweight) were fed a standard laboratory diet and water *ad libitum* and were kept in an environment free from chlorinated insecticides. Five milligrams of tolbutamide containing trace amounts of S-35 labelled drug were injected into the tail vein. Under a light ether anaesthesia blood samples were drawn from the retrobulbar space at 45 minute intervals for six hours after the administration of the drug. The samples were extracted with amyl acetate (Spingler, 1957) and the extracts counted directly in a liquid scintillation spectrometer using Bray's scintillation mixture.

Statistical analysis.

Differences in drug pharmacokinetic parameters between the groups studied were assessed for statistical significance by Student's *t* test.

Results**BINDING OF TOLBUTAMIDE TO LIVER CELL PROTEINS**

Figure 1 shows the elution pattern of Sephadex G-75 gel filtration of the supernatant fraction from rat liver with S-35 tolbutamide added in a trace amount. The protein pattern is similar to that reported in the literature; tolbutamide binding therefore appears to be similar to that of other organic anions, being almost exclusively located in the Y fraction. A small peak of radioactivity can be seen in the region of a high molecular weight protein (X). No radioactivity was found in the Z fraction. Unbound tolbutamide was eluted in later fractions not shown in the Figure.

TOLBUTAMIDE PHARMACOKINETICS

Mean disappearance curves of tolbutamide in the group with Gilbert's syndrome and the group of normal subjects are illustrated in Fig. 2. It can be

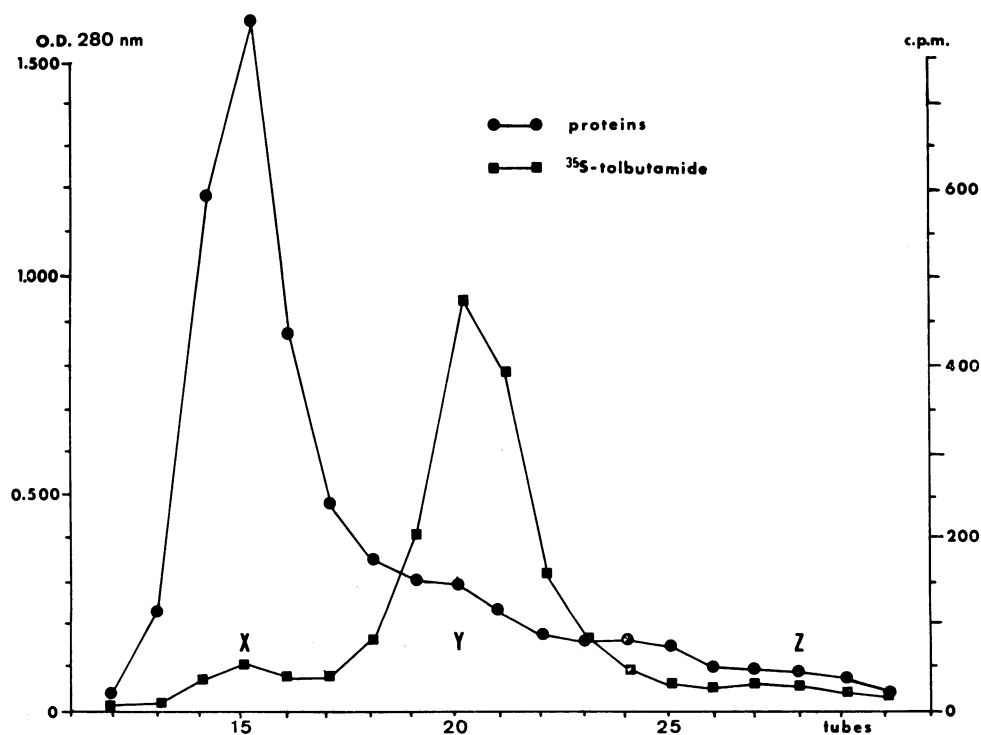


Fig. 1 Sephadex G-75 gel filtration of rats liver supernatant fraction ($100\,000 \times g$) with trace amount of S-35 labelled tolbutamide. Optical density at 280 nm indicates protein, while the tolbutamide is expressed as counts per minute in each tube.

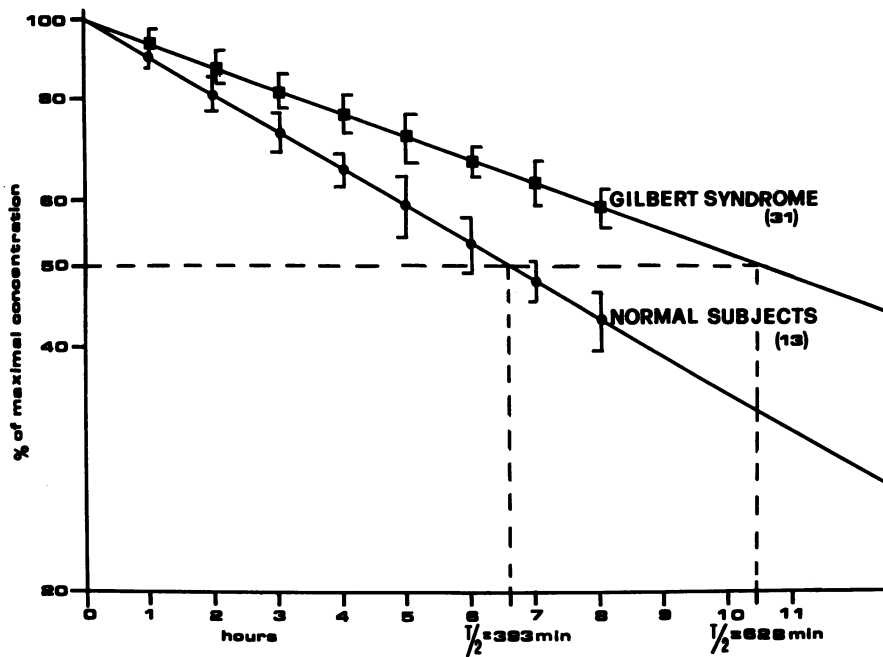


Fig. 2 Plasma disappearance curves of tolbutamide in Gilbert's syndrome and control subjects, expressed as percentage of maximal concentration (C_0). Each point represents the mean of the value at any given time. Standard deviation of the means is also indicated. Half-lives are shown by the dotted lines.

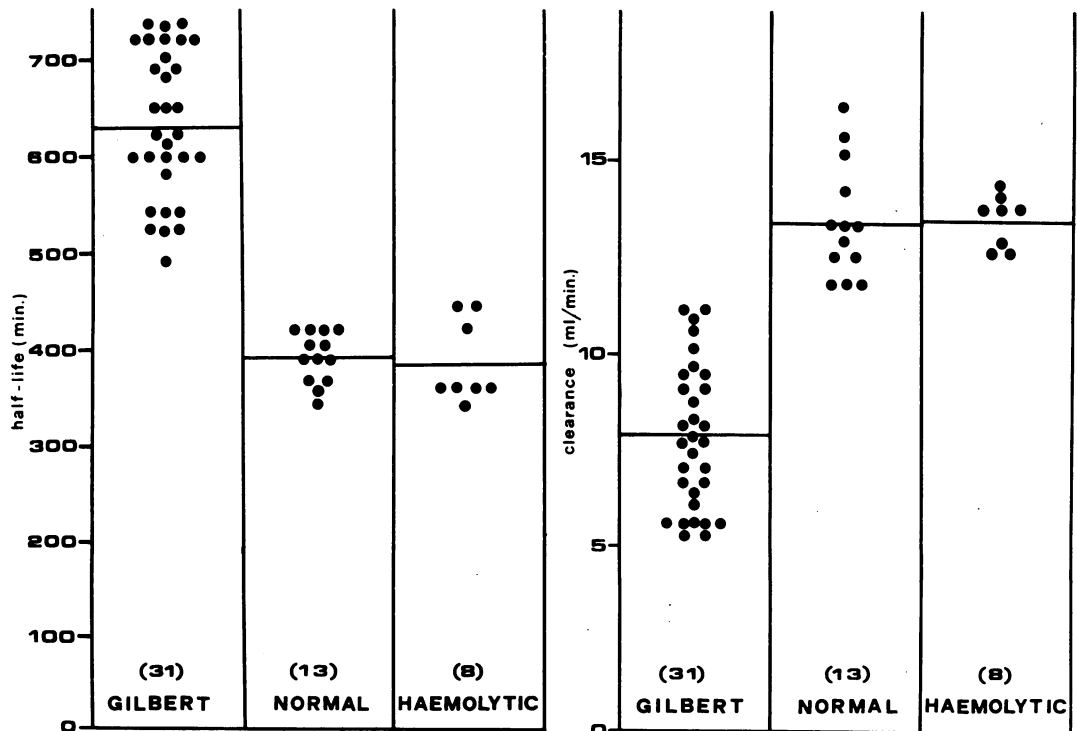


Fig. 3 Individual values of clearance and plasma half-life of tolbutamide in the three groups of subjects studied. The bars represent the means of the values.

Table 1 Tolbutamide clearance and serum total bilirubin in patients with Gilbert's syndrome before and after phenobarbital treatment (100 mg/kg for 15 days)

Patient	Age (yr)	Sex	Tolbutamide clearance (ml/min)		Serum bilirubin ($\mu\text{mol/l}$)	
			Before	After	Before	After
O.S.	27	M	8.1	12.1	54.7	30.8
C.I.	31	M	10.1	16.5	30.8	12.0
M.F.	34	M	9.1	11.5	42.7	23.9
G.S.	25	M	9.1	13.0	39.3	25.6
C.A.	30	M	7.9	14.1	29.1	13.7
		Mean	8.8	13.4	39.3	20.5
		SD \pm	0.8	1.9	10.3	6.8

seen that the plasma disappearance of the drug in patients with Gilbert's syndrome is slower than in the controls.

Individual plasma half-life and clearance values are shown in Fig. 3. In patients with Gilbert's syndrome the mean plasma half-life mean \pm standard deviation: 628 ± 84 min) of tolbutamide is significantly ($P < 0.0005$) greater than the control value (393 ± 26); the clearance value of the drug is therefore significantly ($P < 0.0005$) less in Gilbert's

syndrome (7.9 ± 1.8 ml/min) compared with controls (13.4 ± 1.5).

In the group with haemolytic disease neither values (mean half-life 387 ± 44 ; mean clearance 15.2 ± 2.4) differed from those of the control group.

The apparent volume of distribution did not differ in the three groups studied (controls: 7.6 ± 1.0 l; Gilbert's syndrome 7.1 ± 1.8 l; and haemolytic disease 7.5 ± 0.5 l).

Phenobarbital treatment (Table 1) increased the mean clearance of tolbutamide in the patients with Gilbert's syndrome, from 8.8 ± 0.8 to 13.4 ± 1.9 ml/min. The difference attained statistical significance ($P < 0.005$) using the paired t test. The change in tolbutamide clearance was paralleled by a reduction in serum bilirubin concentration; this fell from a mean value of 39.3 ± 10.2 to 20.5 ± 6.8 $\mu\text{mol/l}$ after the treatment.

URINARY EXCRETION OF TOLBUTAMIDE METABOLITES

Figure 4 shows that the urinary excretion of tolbutamide metabolites was slower in the patients with Gilbert's syndrome than in normal controls. After 24

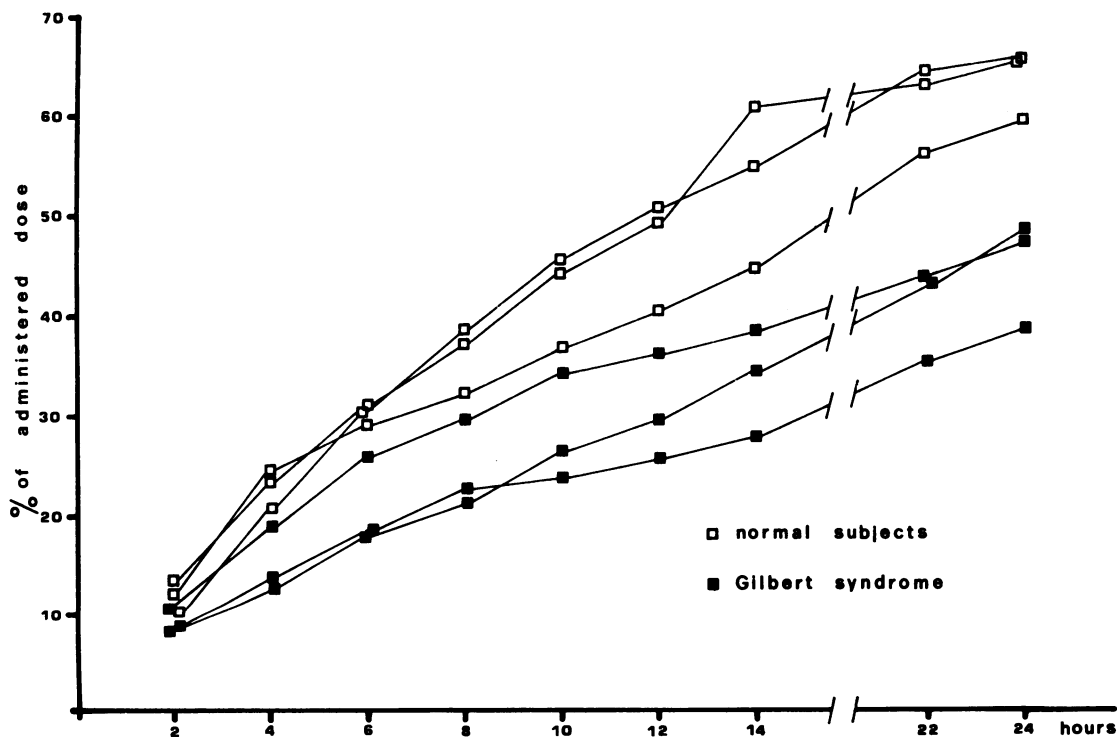


Fig. 4 Cumulative urinary excretion of tolbutamide and its metabolites in Gilbert's syndrome control subjects, expressed as percentage of administered dose.

Table 2 Tolbutamide plasma half-life (min) in Gunn and Wistar rats

Gunn rats		Wistar rats	
1	480	1	480
2	480	2	420
3	355	3	390

hours the metabolite excretion expressed in terms of the administered dose was about 30% less in the Gilbert's syndrome patients than in the controls.

TOLBUTAMIDE PLASMA HALF-LIFE IN GUNN AND WISTAR RATS (Table 2)

The plasma half-life of tolbutamide in the Gunn rats (mean 438 ± 72 min) did not differ from that in Wistar rats (mean 426 ± 50 min).

Discussion

Our results suggest that patients with Gilbert's syndrome metabolise tolbutamide at a slower rate than normal subjects.

The normal values of tolbutamide clearance observed in the haemolytic group indicate that the expansion of the bilirubin pool does not play a role in the alteration of tolbutamide clearance seen in the Gilbert's syndrome patients. The alteration seems to be specific for Gilbert's syndrome and possibly involves the same mechanism as that responsible for the characteristic hyperbilirubinaemia of this syndrome.

Involvement of organic anions other than bilirubin has been recently reported in patients with Gilbert's syndrome. Berk *et al.* (1972) found that the BSP plasma disappearance curve was abnormal in 10 out of 26 patients. This suggests that in some there was a defect in hepatic uptake of the dye. Similar findings were observed by Aldercreutz working with oestrogens (Aldercreutz *et al.*, 1973).

Unlike bilirubin and other organic anions tolbutamide is not significantly excreted into the bile; moreover, its metabolism does not involve conjugation with glucuronic acid. So, whatever the role played by glucuronidation and biliary excretion in the hyperbilirubinaemia of Gilbert's syndrome, these steps would not contribute to the changes in tolbutamide clearance. Indeed, as was expected, a defective conjugating capacity of the liver, such as occurs in the Gunn rat, did not influence the overall metabolism of tolbutamide. A theoretical possibility is that in Gilbert's syndrome patients a microsomal alteration might exist, involving both glucuronyl transferase and drug metabolising enzymes. To our knowledge there have been no reports concerning this specific aspect. A significant hypertrophy of the

hepatic smooth endoplasmic reticulum has, however, been recently reported (McGee *et al.*, 1975) as a distinctive feature of Gilbert's syndrome. The authors speculate that the alteration might represent an attempt to compensate for the decreased activity of microsomal glucuronyltransferase.

Phenobarbital treatment produced in our Gilbert's syndrome patients an increase in tolbutamide clearance which was paralleled by normalisation of the plasma bilirubin level. While phenobarbital induction of the microsomal drug metabolising system is a well-known phenomenon (Conney, 1967), the induction of glucuronyltransferase, at least in Gilbert's syndrome, is still questioned (Felsher *et al.*, 1973; Black *et al.*, 1974). The 'phenobarbital effect' on tolbutamide and bilirubin, however, does not shed any illumination on the defective steps as this drug has been shown to act on several sites of the process of the transport, across the liver cell, of organic anions (Berthelot *et al.*, 1970; Reyes *et al.*, 1971).

Since our results demonstrated that tolbutamide binds to the cytoplasmic Y protein of the liver cell, a similar mechanism for uptake and cytoplasmic transport for tolbutamide to that of bilirubin and other anions has to be considered. Defective uptake (at a membrane level or within the cytoplasm of the liver cell) would seem the most likely explanation for the delayed clearance of tolbutamide. In this context, Frezza and Tiribelli (1974) recently reported that in a patient with Gilbert's syndrome the liver cell cytosol binding capacity for BSP was lower than in the liver of a control subject, thus implying that a defect of the carrier proteins should also be considered in the pathophysiology of Gilbert's syndrome.

In conclusion, whatever the exact molecular mechanism responsible for the impairment of tolbutamide metabolism, our results give additional evidence that the transport of organic anions other than bilirubin may be abnormal in Gilbert's syndrome and lend support to the hypothesis that the major defect may be located in the steps before conjugation with glucuronic acid.

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