Estimation of the Functional Reserve of Human Liver

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Functional hepatic reserve was determined in 32 patients with known liver or biliary tract disease employing kinetic analysis of hepatic removal of indocyanine green (ICG). The initial removal rates of incremental doses of ICG (0.5, 1.0 and 5.0 mg/kg body weight) were plotted as a reciprocal against the inverse of dose (Lineweaver-Burk plot) to provide a means of determining maximal removal rate from submaximal doses (Rmax). This function equalled 3.40 mg/kg/min in ten patients with normal livers, but was only .24 mg/kg/min in eight patients with alcoholic cirrhosis. Portasystemic shunting did not further influence Rmax. Infiltrative liver disease had only a mild depressive effect on this function. The results show that hepatic function can be precisely quantitated by classical enzyme kinetics (Michaelis-Menten). If Rmax is an estimate of protein receptor mass for organic anions, then the technique may allow an indirect means for quantitating hepatocytes even in the presence of changes in blood flow or hepatic function. The profound depression in R_{max} observed in patients with alcoholic cirrhosis is consistent with the progressive loss in hepatic mass associated with this disease.

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THE LIVER has a remarkable capacity to carry out a wide variety of synthetic, storage, excretory, and metabolic activities. In fact, approximately half of its mass can be excised with only transient changes in contemporary liver function tests.[1] An infinite capacity for regeneration further complicates quantitative assessment of hepatic functional reserve. For these reasons, advanced liver disease may avoid detection, and terminal phases of hepatic dysfunction are predicted with great uncertainty.

The excretion of organic anions such as bilirubin and bromsulfalein (BSP) have been useful indicators of liver disease. Analysis of BSP retention at a specified period of time (usually 45 minutes) following its administration into a peripheral vein, has been a most sensitive index of hepatocyte function. Local toxicity and tendency towards extrahepatic removal as liver disease progresses has detracted from its usefulness. Indocyanine green (ICG), a tricarbocyanine dye, has been employed with increasing frequency in its place since ICG is without reported toxicity and undergoes neither intrahepatic conjugation nor enterohepatic circulation, and is removed from the blood stream exclusively by the liver.[4,6,8,9] Furthermore, its presence in the blood can be accurately assessed even in the presence of hyperbilirubinemia.

Paumgartner and his colleagues[12] have demonstrated that removal of incremental doses of ICG obeys classical Michaelis-Menten kinetics. Kinetic analysis in rat and man confirmed the earlier work of Hunton *et al*[5] who demonstrated in dog that hepatic removal of ICG was a saturable process. Rikkers and I[14] have recently shown that maximal removal rate of ICG provides a quantitative estimate of hepatic mass, and that this estimate is valid for normal as well as regenerating rat liver. We further demonstrated that the technique could be used in the dog before and following 70% hepatectomy but not during the early and most rapid phase of regeneration.[13] The present communication relates the results of assessment of hepatic functional mass by ICG uptake in the normal and in patients with liver or biliary tract disease.

Materials and Methods

Thirty-two patients with known liver disease and ten patients without demonstrable hepatic dysfunction were

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selected for study. The latter were considered controls who represented the expected results from this test. Included in the group were three patients, one with chronic lymphocytic leukemia with normal liver biopsy, one with morbid obesity who had moderate fatty infiltration on liver biopsy, and a third with bleeding esophagitis with normal hepatic function. The remainder of the controls were volunteers from personnel working on the project. The abnormal group consisted of eight patients with alcoholic cirrhosis studied pre-shunt, five patients with biliary or post-necrotic cirrhosis studied pre-shunt, five patients studied at various intervals following portal decompression, and 14 patients with a variety of pathological disturbances of the liver or biliary tree. Eight patients were studied before, and one or more times following, portacaval shunt (five had two post-shunt studies). Fourteen patients underwent 18 studies at an interval of one week to sixty months following portal decompression.

Protocol For Measurement of Maximal ICG Uptake

Indocyanine green (Hynson, Westcott and Dunning, Baltimore, Maryland) was dissolved in 20 ml of distilled water and 5 ml of 5% human albumin to provide a 2 mg/ml solution prepared fresh each day. Fasting subjects were provided doses of 0.5, 1.0, or 5.0 mg/kg body weight on alternate days by an intravenous route. Blood was withdrawn before and at 5, 10, and 15 minute intervals following ICG administration. Upjohn heparin which contains benzyl alcohol as a preservative, was used to wet the surface of syringes to prevent clotting. Following centrifugation, serum was analyzed for ICG by determination of its optical density in a Spectronic 70 spectrophotometer (Bausch and Lomb, Rochester, New York) at 800 μ. Concentration was determined by comparison to appropriate standards. Plasma disappearance rate of ICG was ascertained by regression analysis of the natural logarithm of optical density or ICG concentration of recovered samples as a function of time. Multiplying the plasma disappearance rate (PDR) which is equal to slope of regression times dose yields initial removal rate of dye (R). Maximal removal rate (Rmax) was calculated by regression analysis of the reciprocal of initial removal rate (R) against the reciprocal of dose (D).

Kinetics of ICG Removal

The conceptual basis for the present analysis of hepatic function rests on an analogy to classical enzyme kinetics as described by Michaelis and Menten.[11] In the analogy, ICG (substrate) interacts with a theoretical hepatic receptor (enzyme) on the sinuoidal side of the hepatocyte. Removal of ICG from the blood by this mechanism is depicted by the following equation:

$$(ICG) + (H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ICG - H) \xrightarrow{k_3} (ICG') + (H)$$

where,
(ICG) = ICG dose
(H) = hepatic receptor
(ICG-H) = ICG-receptor complex
(ICG') = ICG released from the receptor into the hepatocyte k_1, k_2, and k_3 = association and dissociation constants of the complex
When hepatic removal rate of ICG is proportional to concentration of its complex with hepatocyte receptors, instantaneous removal rate is described by the following equation (Michaelis-Menten):

$$R = \frac{Rmax \times (ICG)}{Km + (ICG)}$$

where,
R = PDR × dose = initial removal rate in mg per kg per min of an individual ICG dose Rmax = initial maximal removal rate in mg per kg per min or the rate of hepatic ICG uptake when all receptors are complexed with ICG

$$Km = \frac{k_2 + k_3}{k_1} = \text{Michaelis constant}$$

Figure 1 (top) is a schematic representation of the Michaelis-Menten equation which results in a rectangular hyperbola with R asymptotically approaching Rmax at high doses. Km represents the affinity of hepatic receptors for ICG and is equal to the dose which provides for half-maximal removal rate. Linear transformation of the Michaelis plot by the method of Lineweaver-Burk, as shown in Fig. 1 (bottom), provides a convenient way to calculate Rmax from submaximal doses of dye.[10] This is fortunate since receptor-ICG interaction may be more accurate at lower doses if indeed the analogy to substrate enzyme interaction is correct. In practice, linearity of the Lineweaver-Burk plots were determined by regression analyses and Rmax was calculated from the Y intercept by division to unity. Barber analysis[2] was employed to compare Rmax's and Km's of controls and alcoholic cirrhosis. Grouped data was also subjected to unpaired t analysis. Liver function tests were obtained by multi-channel analyzer in the clinical laboratories of the respective institutions where patients were studied.

Results

The characteristics of the plasma disappearance of ICG over a fifteen-minute interval is shown in Fig. 2 for a control and a patient with alcoholic cirrhosis. In each instance the plasma disappearance curve is linear. The

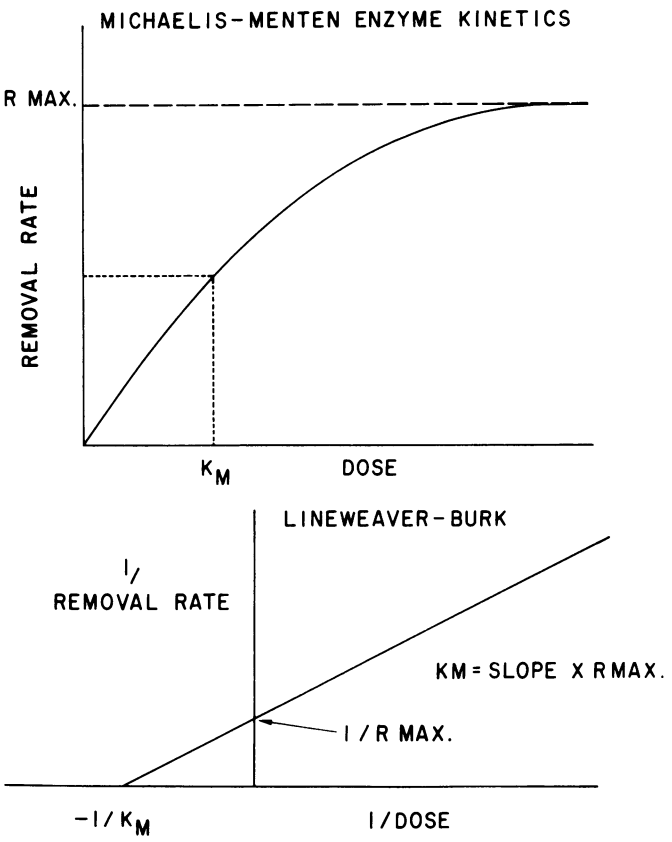

FIG. 1. (Top) Schematic representation of a Michaelis plot. Initial removal rate is plotted as a function of dose. As dose increases, removal rate reaches an asymptote which represents maximal removal rate (Rmax). (Bottom) The Michaelis plot has been transformed to a straight line by relating the reciprocal of removal rate to reciprocal of dose. The Y intercept represents reciprocal of (Rmax. Km can be calculated by multiplying slope times Rmax.

slower removal rate (shallower slope) seen in the alcoholic cirrhotic was characteristic of this group.

Maximal removal rate of ICG was ascertained 63 times in 42 individuals thereby providing 189 plasma disappearance curves. Nearly two-thirds (62%) of PDR's demonstrated a correlation coefficient equal to or greater than .997, the value which provides a p value of less than .05 for 3 points. Eighty-six per cent provided a correlation greater than .98. In five instances, only two of the three plasma concentrations were used to obtain a PDR since best fit calculations revealed an obvious analytical discrepancy in the values obtained with the three-point analysis.

Forty-four Lineweaver-Burk regressions demonstrated correlation coefficients equal to or greater than .997 ($p < .05$). Eighty-one per cent were greater than .98. Inspection of those values which fell below .98 did not reveal a consistent source for error. In two instances, PDR's with low correlation may have contributed to discrepant results. In most cases, however, poor correlations on the Lineweaver-Burk plot did not relate to erratic PDR's, but rather appeared to be a reflection of a true change in hepatic function. For example, a patient recovering from halothane jaundice (A.M., 53-year-old female, Table 1) gave an Rmax of .41 with an R of .96, but all PDR correlations were above .998. Controls gave relatively uniform values, with six of ten having correlations greater than .997. In one of four in which the correlation was less than .997, an extension to five-dose analysis to yield a highly significant correlation only provided a minor change in Rmax (an increase from 1.0 to 1.7 mg/kg/min). Failing to find a consistent way to exclude Rmax's with a regression coefficient less than .997, all values obtained were used in the following analyses.

Figure 3 compares a Michaelis plot of controls to that of alcoholic cirrhotics before portal decompression. The Rmax's represented by the horizontal dashed lines were calculated by regression analysis of the reciprocal of removal rate and dose. The points inscribe the actual values observed and the solid line represents the Michaelis plot calculated from the Michaelis-Menten equation. Vertical broken lines identify the respective Km's. It can readily be appreciated that the observed removal rates for controls is well below the maximal removal rate calculated from the observed values. In fact, the 5 mg/kg dose is significantly less than that required to

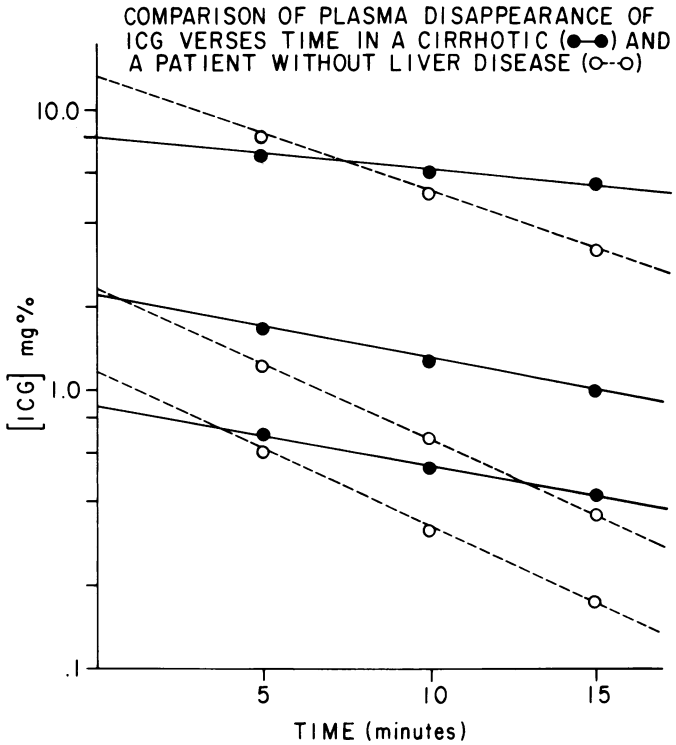

FIG. 2. This represents a semilogarithmic plot of serum concentration of ICG as a function of time. The broken line represents the plasma disappearance curve for a normal subject; the solid line represents this function for an alcoholic cirrhotic.

TABLE 1. *Clinical Correlation Between Rmax and Hepatic Function in Patients With Liver or Biliary Tract Disease*

Patient	Age/Sex	R_{max}	r	Total Bilirubin	Alk. Phos.	Albumin	Diagnosis
F. M.	62 F	3.36	.998	0.5	78	3.2	Methotrexate fibrosis
E. M.	50 F	1.84	1.000	2.0	474		Bile duct stricture, pericholangitis
A. M.	53 M	1.20	1.000	0.6	133	3.0	Caroli's disease with hepatic stones
E. D.	36 F	1.56	.998	1.1	55	3.9	Early cirrhosis, fatty infiltration
H. M.	41 M	1.61	.995	10.0	340	3.2	Sclerosing cholangitis, post colectomy ulcerative colitis
D. W.	51 M	1.08	.979	0.6	50	4.1	Massive hemangioma
S. R.	28 M	.91	.985	0.9	58	4.8	Acute cholecystitis, post-op
R. D.	61 F	.88	1.000	1.7	199	2.6	Metastatic liver carcinoma, extensive
S. T.	30 F	.87	.996	1.5	228	4.1	Pericholangitis, post colectomy ulcerative colitis
J. B.	52 M	.78	1.000	1.6	98	2.8	Sarcoidosis with liver involvement
H. L.	30 M	.42	.999	1.2	93	3.5	Renal transplant—one year
A. M.	57 F	.47	.960	15.0	560	1.8	Halothane hepatitis
R. C.	40 M	.34	.981	8.6	320	3.0	Alcoholic hepatitis, bile duct stricture
F. F.	40 M	.18	.924	6.3	720	3.3	Pericholangitis, post colectomy ulcerative colitis

give a half-maximal response. On the contrary, a 5 mg/kg dose in the alcoholic cirrhotic group elicits a near maximal response. The Lineweaver-Burk plot for these data are shown in Fig. 4. Regression lines are distinctively different as are the respective reciprocals of Rmax. The Y intercept yields an Rmax of 2.11 mg/kg/min for controls and .19 mg/kg/min for cirrhotics. Because of the sizable variance in the removal rates of the cirrhotic population, it was necessary to calculate confidence limits by regression analysis as described by Barber. This analytical method provided an Rmax of 3.40 mg/kg/min with 95% confidence limits of 3.11 to 3.72 for controls. The cirrhotic group gave an Rmax of .24 mg/kg/min with limits of .18 to .31. As can be seen, there was no overlap between groups providing a high level of statistical significance. The Km's were also derived by Barber analysis yielding a value of 8.22 and 4.14 respectively for controls and alcoholic cirrhotics without overlap in their 95% confidence limits. This re-

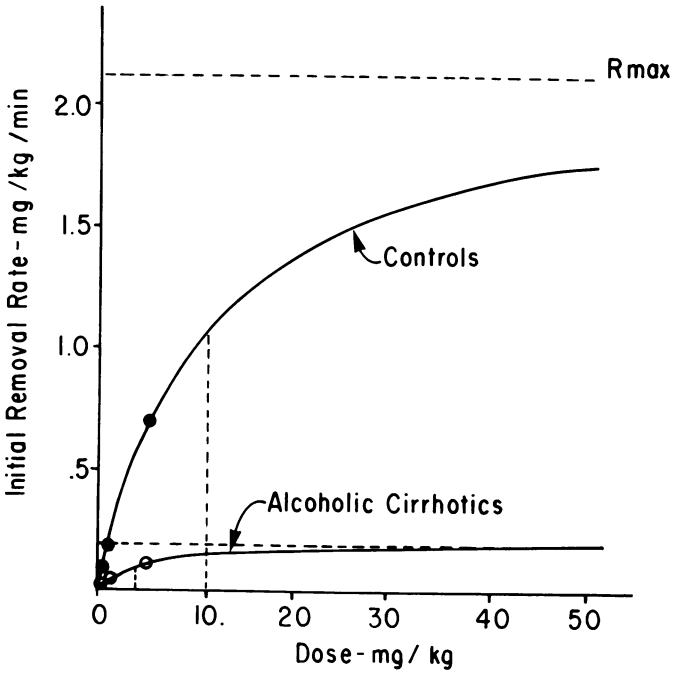

FIG. 3. The upper line represents the Michaelis plot for 10 controls, as compared to that obtained for eight alcoholic cirrhotics (lower line). The horizontal dashed lines represent Rmax's calculated from the Lineweaver-Burk regression obtained from the data points for removal rates at the three doses studied. The vertical dashed lines identify the respective Km's.

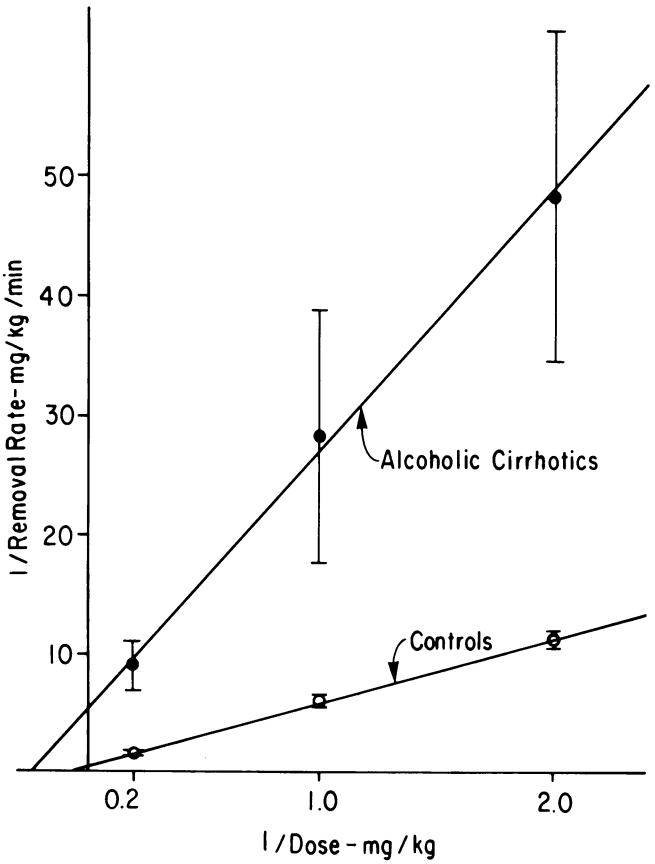

FIG. 4. These Lineweaver-Burk plots for controls and alcoholic cirrhotics demonstrate a high degree of linearity for grouped data (r = .9999 and .9985 respectively for controls and cirrhotics). The points represent the means bracketed by their standard errors.

TABLE 2. *Clinical Correlation: Alcoholic Cirrhotic Group*

Patient	Age/Sex	R_{max}	r	Bilirubin	Alk. Phos.	Trans.	Alb.	P. T.	BSP
L. J.	40 M	.49	.999	.8	60	27	2.6	85%	2%
A. M.	50 F	.27	1.000	1.0	102	25	3.1	92%	
M. P.	35 F	.25	.995	.7	128	149	3.0	52%	29%
M. H.	48 F	.25	.992	1.0	46	136	3.9	100%	3%
C. H.	43 M	.21	1.000	1.2	115	75	4.3	90%	
D. B.	51 M	.12	.970	1.1	110	60	3.3	65%	
H. E.	44 M	.06	.996	3.2	107			38%	24%
T. O.	58 M	.07	.986	7.6	110	75	2.0	63%	

sult suggests that the hepatocytes of the alcoholic cirrhotic have a decreased affinity for ICG uptake, although interpretation of the true meaning of Km in this context requires further study. Inspection of the Km's for various other groups did not reveal any consistent relationships which might provide a further clue as to the meaning of this particular function.

A comparison of maximal removal rate to conventional hepatic function indices (total bilirubin, alkaline phosphatase, and serum albumin) for a variety of diseases of the liver and the biliary tree, are shown in Table 1. Inflammatory lesions of the bile duct did not appear to significantly affect Rmax except in one case (F.F.) where an extremely poor correlation was obtained for the Lineweaver-Burk transformation. Likewise, infiltrating diseases were accompanied by only a moderate depression of Rmax.

Comparative liver function studies for alcoholic cirrhotics are shown in Table 2. The random nature of changes in traditional liver function tests is well demonstrated in this group. For example, two patients had

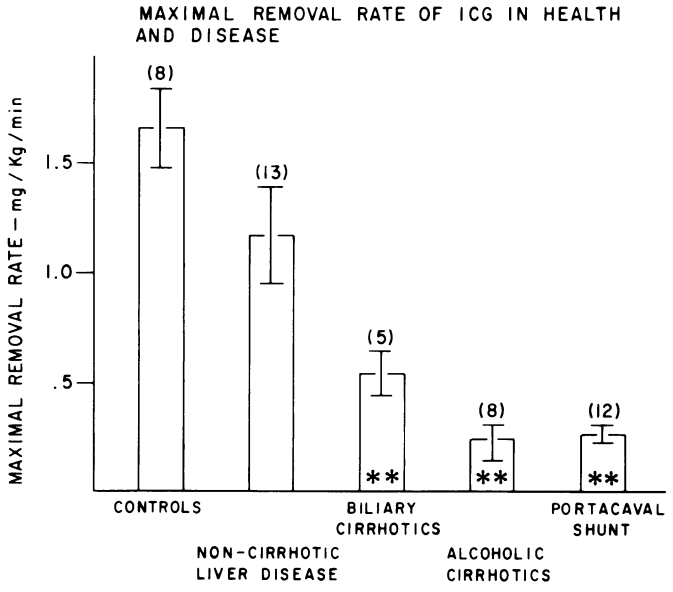

FIG. 5. This histogram represents the mean plus or minus the standard error of Rmax's for controls and for a variety of diseases of the liver and biliary tree. Numbers in parentheses represent the population size.

normal BSP retention (L.J. and M.H.) with only a single disturbance in other parameters measured. Two additional patients (A.M. and C.H.) had only minor changes in hepatic function, but profound depression in maximal removal rate of ICG. It is apparent that the two patients with the lowest Rmax's (H.E. and T.O.) also had the most marked changes in the usual parameters used to assess hepatic function. All eight patients had gross and microscopic evidence of advanced cirrhosis. Figure 5 compares the Rmax of patients with alcoholic and non-alcoholic liver disease. Two of the controls presented in Figs. 3, 4 have been omitted since their Y intercepts (1/Rmax) gave Rmax values (18.52 and 344.8) which appeared spuriously high as a consequence of the closeness of the intercept to the origin.

Figure 5 compares the Rmax of patients with alcoholic and non-alcoholic liver disease. As can be seen, non-cirrhotic liver disease as a group, did not differ from controls. Cirrhotics, however, before and after shunting, demonstrated a highly significant decrease from normal. The effects of portacaval shunting was studied in a systematic manner in eight patients, five with alcoholic cirrhosis and three with biliary or post-necrotic cirrhosis. In the alcoholic group, three patients were studied one week and at three months following portal decompression; one additional patient was studied early (one week) and another at 2½ years. In the biliary or post-necrotic group two patients were studied at one week and again at three months and a third patient was studied three months following surgery. No significant difference was found between Rmax before or at intervals after shunting in either of these groups, confirming the impression that portal decompression did not influence this aspect of hepatic function.

An attempt was made to relate bilirubin, alkaline phosphatase, transaminase, albumin and prothrombin time to levels of Rmax. The only significant correlation occurred between bilirubin and Rmax of the post-portal decompression group. In this case, there was a negative correlation of .5656 ($p < .05$) thereby revealing an inverse relationship between bilirubin and Rmax. This was not true, however, for other groups, although correlations for alcoholic cirrhotics were close to a level of sig-

nificance with a negative correlation of .6020 ($p < .1$ but $>$ than .05). Regression analysis also revealed a highly significant correlation between PDR's at all doses and respective Rmax's, with the 5 mg/kg dose giving the highest correlation coefficient (.822, $p < .01$). This is consistent with the observation that this dose approached a near maximal level in the alcoholic cirrhotics, the population which showed the largest depression in maximal removal rate.

Discussion

Results of the present study confirm that hepatic uptake of incremental doses of ICG in human is a saturable process which can be subjected to analysis utilizing the concepts of classical enzyme kinetics. In this analogy, ICG participates as a substrate, and the hepatic receptor mechanism functions as an enzyme. The linearity of the plasma disappearance rate of individual doses of ICG in both the normal and in patients with liver disease reflects first order kinetics and allows use of Michaelis-Menten analysis and its linear transformation by Lineweaver-Burk plot. We were surprised to find such a reasonable correlation for the linear regression of the reciprocal of removal rate versus dose with only three-point analysis. Linearization of this function by Scatchard plot[15] did not appreciably change the result. Possibly four or five-point analysis would have greatly increased the confidence level of individual maximal removal rates, but expense and inconvenience to the patient precluded such an approach in this initial survey.

The Rmax obtained from controls by Barber[2] analysis of grouped data gave a value which was comparable to that observed by Paumgartner[12] and his colleagues (ca 3.5 mg/kg/min). Their alcoholic cirrhotics, however, provided an Rmax in the range of .7 mg/kg/min, a value about three-fold greater than that seen in the alcoholic cirrhotics in the present study where Rmax equalled .24 mg/kg/min with confidence limits of .18 to .31. Since comparable techniques were employed, it is likely that the latter group were more advanced in their alcoholic liver disease.

We had hoped that this test would provide a way to quantitate hepatic functional reserve of cirrhotics who were to undergo portal decompression for esophageal varices. One individual (T.O.) with a low Rmax died in the early postoperative period from progressive hepatic and renal failure, but a second (H.E.) with an equally low value has now survived for two and one half years with an Rmax that remains at a very low level (.15 mg/kg/min). The data suggest that portacaval shunting has no influence on maximal removal rate of dye. Sufficient numbers of patients have not been studied for a long enough period of time to conclude that major portal diversion does not lead to progressive loss of hepatic mass. Acute changes in hepatic blood flow which may accompany a shunt, however, do not appear to interfere with this particular function. We have found this to also be the case following performance of end to side portacaval shunt in the rat (unpublished results).

There is controversy as to whether single dose analysis of ICG is a more useful or sensitive test than BSP for identifying liver disease.[3] Comparison of Rmax with plasma disappearance rates of individual doses of ICG in the present study did establish a high level of correlation with all doses, but especially the largest (5 mg/kg). A single large dose may be an accurate way to follow progression or resolution of liver disease if the dose is near maximal such as was seen for the 5 mg/kg dose in the alcoholic cirrhotic. A 10 mg/kg dose may even have provided a better index of maximal removal rate. We found this to be the case during the rapid phase of regeneration following two-thirds hepatectomy in the dog.[13] This dose, however, was accompanied by emesis in these studies. Furthermore, high doses are known to cause cholestasis in experimental animals.[7] If this is also true for the human, then retention of bilirubin and bile salts could be accentuated by such a test. We did not observe an adverse effect on serum bilirubin levels at doses employed in the present study.

A statement as to false positives and false negatives cannot be made with such a small number of studies. In fact, the measurement of Rmax is viewed by us as a potential tool for attempting to quantitate hepatic function and not as a practical clinical means for the differential diagnosis of liver disease. Refinement and perfection of this approach, however, may provide a means for identification of occult liver disease or end-stage hepatic dysfunction. The development of a quantitative prognostic index for acute or chronic liver disease will require larger clinical experience that relates Rmax to ultimate outcome.

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Discussion

Dr. Robert Zeppa (Miami): I have just a few questions. Is it possible that the test is just too sensitive for the information that we seek concerning individuals with cirrhosis?

For example, if the protein receptor mass, whether this is in that group of protein receptors that have been labeled X and Y proteins, if this protein mass is not synthesized at the same rate as other proteins, as, for example, albumin, fibrinogen, prothombin, then perhaps it will indicate a very severe problem, as he has suggested with his data, but may not be functionally relevant to prognosis for the patient.

Taking it the other way, in a regenerating liver where normal cellular function appears to be returning, because the cells themselves are normal, one can utilize the technique to correlate well with the regenerating mass. In a liver that has hepatocellular dysfunction, if the dysfunction is spotty in terms of all of the functions of a liver cell, it may be possible, or even likely that this technique may be just too sensitive.

Dr. Harry H. LeVeen (Brooklyn): We are interested in whether absorbed ammonia might be toxic to the liver cell and impedes its regeneration. I would greatly like to hear Dr. Moody's comments as to whether his model might be applied to studying the effect of diet, drugs, poisons and ammonia on liver regeneration.

Dr. Frank G. Moody (Closing discussion): We're just beginning to study hepatic reserve in surgical patients. Paumgartner and his colleagues have explored this technique in medical patients, and we have tried to carry it a bit further, relating it to hepatic mass, protein receptors, and possibly the function of Y and Z proteins. The manner whereby organic anions gain entrance into the hepatocyte is unknown. We have hopes that R max is a measurement of the number of hepatocytes present, as well as an indicator of a disturbance in organic anion transport.

In answer to Dr. Zeppa's question, I would look at it in quite the reverse, that probably this is a more stable function than looking, for example, at something that has to be metabolized in the hepatocyte, or has to be secreted by the cannuliculus. We assume that we are looking at a function on the sinusoidal side of the hepatocyte and, therefore, are observing membrane function of the transport process to get these organic anions into the cell.

In answer to Dr. LeVeen, we haven't tried to hold back regeneration. We have learned several things about this particular function, however. One is that if you do an acute portacaval shunt in the rat, it does not influence this particular parameter. Although at low doses blood flow influences the PDR, in high doses the PDR is primarily a reflection of hepatocellular function. If you use the whole dose range, then you eliminate these variables, which is an important consideration, because the ICG just has to get there; it doesn't make any difference how long it takes it to get there (within reason) or how it gets there. I think that's the value of the test.

We have done only one other study, and that's trying to enhance regeneration, initially in normal rat liver, and now with regenerating rat liver. Our next approach is to see if we can enhance regeneration possibly with some of the nonspecific mitogens, but we haven't done anything to try to hold back regeneration, and I think it might be an interesting model to try it in.