

Hepatic function in rats with dietary-induced fatty liver, as measured by the uptake of indocyanine green

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(Received 21 October 1981 – Accepted 24 November 1981)

1. The hepatic uptake of indocyanine green (ICG) has been measured in rats receiving a 50 g protein/kg diet for 6, 12 or 20 d or a choline-deficient diet for 2 or 6 d.
2. There was no effect on ICG uptake on the choline-deficient diet, although all the rats developed an intense fatty infiltration of the liver by 6 d.
3. The rats on the 50 g protein/kg diet showed impaired uptake of ICG at 6, 12 and 20 d, which appeared to be related to the extent of fatty infiltration.
4. It is concluded that ICG uptake is predominantly a function of the periportal zone of the liver lobule, and therefore likely to be sensitive to insults that exert their predominant effect in this zone.

One of the most serious complications of severe protein-energy malnutrition is hepatic failure (McLean, 1962; Garrow & Pike, 1967). There is an associated fatty infiltration of the liver, although the extent of hepatic dysfunction is not necessarily related to the severity of the infiltration (Waterlow, 1975). Two important considerations have hampered progress being made in the clinical management of this condition.

Those patients with established decompensation of liver function can be identified by the use of liver function tests. Thus there may be an increase in the plasma concentration of hepatic enzymes, such as aminotransferases, or the concentration of bilirubin (Waterlow *et al.* 1960; Garrow & Pike, 1967) or an impaired clearance of an exogenous dose of bromosulphthalein (Waterlow, 1948). Unfortunately, none of the biochemical tests that have been used is sensitive enough to identify the earlier stages of impaired liver function, when appropriate intervention may have more chance of success.

The second difficulty is that it has not been easy to develop a suitable animal model in which the condition can be investigated. The classical model of fatty liver in rat is produced by a choline-deficient diet (Best & Huntsman, 1932). Animals given this diet develop an intense fatty infiltration commencing in the centrilobular zone (Mookerjee, 1965). This contrasts with the pattern of fat deposition seen in kwashiorkor, which starts in the periportal zone and only spreads to other areas of the lobule at a later stage (Trowell *et al.* 1954). The fatty liver of kwashiorkor is unresponsive to large dietary supplements of choline, methionine or inositol (Waterlow, 1948). It is possible to produce periportal fatty infiltration in rats if they are given a low-protein diet, but this is of a relatively mild extent and takes a long period of time to develop (Waterlow & Bras, 1957; Seakins, 1971).

The uptake of indocyanine green (ICG) by the liver is considered to be a more discriminating test of liver function than the uptake of bromosulphthalein (Hunton *et al.* 1960, 1961; Leevy *et al.* 1967; Paumgartner *et al.* 1970). When the dose of ICG is increased the test becomes more sensitive, so that lesser extents of hepatic dysfunction can be identified. One advantage of the use of ICG in clinical practice is that by using dichromatic ear densitometry to measure the concentration of ICG in blood it is possible to avoid taking multiple samples of blood (Leevy *et al.* 1967). To date ICG has not been used to assess liver function in protein-energy malnutrition.

Table 1. *The energy and protein contents of the diets*

Diet	Energy (KJ/kg)	Protein (g/kg)
Laboratory chow	14090	230
Low-protein	18850	60
Choline-deficient	18100	200

This study was designed to see if ICG could be used to assess liver impairment in rats given a low-protein diet. Furthermore we determined whether ICG uptake was altered in rats in which a fatty liver had been produced by feeding a choline-deficient diet. Our results show that ICG uptake is impaired in rats on the low-protein diet, but not on the choline-deficient diet.

METHODS

Male albino rats, weighing 150–300 g, from the colony of the Tropical Metabolism Research Unit, were housed in groups in wire-bottomed cages. After weaning they were given a stock diet of Purina Laboratory Chow, then groups of rats were given either a low-protein diet for 6 (LP₆), 12 (LP₁₂), or 20 (LP₂₀) d, or a choline-deficient diet for 2 (CD₂) or 6 (CD₆) d.

The low-protein diet was made in batches and 100 g contained (g); maize starch 44, D-glucose 15, wheat flour 15, vegetable shortening 15, casein 5, vitamin mix 1 (Tagle & Donoso, 1965), mineral mix 5 (Glaxo Laboratories Ltd, Greenford, Middlesex). The choline-deficient diet was obtained from ICN Life Science Group. The energy and protein contents of the diets are shown in Table 1.

Experimental procedure

Animals. Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbitone, 50 mg/kg body-weight, and secured to a dissecting board in the supine position. The left jugular vein and the left femoral artery were cannulated with polyethylene tubing (PP25, Portex; Hythe, Kent). A sample of blood, 0.5 ml, was withdrawn from the femoral cannula. The dose of ICG was then injected through the jugular cannula, and the cannula was quickly flushed with 0.2 ml of a solution of heparin in normal saline (9 g sodium chloride/1; 1 ml/4 ml). Blood was withdrawn from the femoral cannula at timed 1–3 min intervals over a period of up to 15 min, being replaced by an equal volume of heparin in saline solution on each occasion.

The animals were killed by inhalation of chloroform. The livers were removed, lightly blotted, weighed and stored at -20° .

ICG solutions. A solution of human serum albumin (50 g/l; Sigma Chemical Co., St Louis) in sterile water (1:4, v/v) was added to vials containing 25 mg ICG (Hynson, Westcott and Dunning, Baltimore). Standard curves for each individual solution were constructed by measuring the optical density at 805 nm. Maximum absorption was only obtained when all dilutions were carried out with a solution of human serum albumin in normal saline (1:4, v/v). All ICG solutions were kept at 4° and used within 8 h of preparation. Three dose levels, 5, 10 and 20 mg ICG/kg body-weight were given in a constant volume, 2 ml/kg body-weight.

Blood samples. At the end of the sampling period duplicate specimens of blood were diluted appropriately, mixed, centrifuged, and the absorption of the supernant fraction measured against a blank at 805 nm.

Tissue analysis. Fat-free dry liver weight was measured by extracting thin slices of liver in ethanol-diethyl ether (3:1, v/v) overnight, then drying to constant weight at 90° .

The method of Fleck & Munro (1966) was used to measure DNA.

The total fat content was measured by the method of Stern & Shapiro (1953), and triglycerides by the method described by Kessler & Lederer (1965) and Fletcher (1968). Glycerol trioleate was used as a standard.

Theoretical considerations (log concentration ICG in blood v. time). Linear regression analysis was used to calculate the half-life for ICG disappearance, $t_{\frac{1}{2}}$, (Riggs, 1970). The percentage disappearance rate, PDR, was calculated as:

$$\text{PDR} = \frac{0.693}{t_{\frac{1}{2}}} \times 100.$$

The initial removal rate of ICG, R , was then given by

$$R = \text{PDR} \times D,$$

where D was the dose of ICG administered. As the dose of ICG was increased R increased asymptotically to approach a maximum value, R_{max} . It was assumed that the rate at which ICG was removed from the blood by the liver was proportional to the rate at which ICG reacted with the hepatic receptor (Paumgartner *et al.* 1970) and so Michaelis-Menten kinetics could be applied:

$$R = \frac{R_{\text{max}} \times D}{K_m + D},$$

where K_m is the Michaelis constant, or the affinity of the hepatic receptor for ICG. Conformity of the values to a linear reciprocal transformation (Lineweaver & Burke, 1934) was used as the criterion that the values from any group were in agreement with these principles.

Statistical handling. The method of Barber *et al.* (1967) was used to derive the values and 95% confidence limits for R_{max} and K_m .

The Student's t test was used to determine differences between any two groups of values.

RESULTS

A total of ninety rats was studied, five in each dietary group at each of the three dose levels.

The composition of the livers is shown in Table 2, and it can be seen that on the protein-deficient diet there was a progressive fall in wet liver weight/kg body-weight, the difference reaching significance by the 12th day. No change in wet liver weight was noted on the choline-deficient diet. There was no change in the DNA content per unit weight of liver on any of the diets, but as the total liver size decreased on the low-protein diet, there was a corresponding fall in liver DNA/kg body-weight. Fat-free dry liver weight, lipid and triglyceride were expressed per g DNA.

There was a highly-significant reduction in the fat-free dry liver weight per g DNA on the low-protein diet at 6 and 20 d. Surprisingly after 12 d on the low-protein diet the fat-free dry liver weight was similar to that of the control. There was no change in fat-free dry liver weight after 2 d on the choline-deficient diet, but there was a significant decrease by 6 d.

Total lipid per g DNA increased progressively with the period on the low-protein diet. By 12 and 20 d, virtually all the increase could be accounted for by a highly-significant increase in triglyceride. On the choline-deficient diet the increase in lipid just failed to reach significance by the second day ($P = 0.053$) but there was a highly-significant increase by 6 d. Only half this increase could be accounted for by an accumulation of triglycerides. Using the criterion of Seakins (1971) that a doubling of triglyceride content represents a significant increase in fatty infiltration two-thirds of the animals had fatty livers by 12 d on the low-protein diet, and all the animals by 6 d on the choline-deficient diet.

The calculated values for percentage disappearance rate of ICG are shown in Table 3,

Table 2. *The composition of the livers of the rats on low-protein (LP₆, LP₁₂, LP₂₀) and choline-deficient (CD₂, CD₄) diets*
(Mean values with their standard errors)

Dietary Group	Wet liver weight			DNA						FFDLW: DNA		Lipid: DNA		Triglyceride: DNA	
	g	g/kg body-wt		mg		mg/g wet-wt		Mean	SE	Mean	SE	Mean	SE	Mean	SE
		Mean	SE	Mean	SE	Mean	SE								
Control	11.3	0.4	43	1	22.2	0.9	2.0	0.03	139	4	13.2	0.7	1.77	0.1	
LP ₆	10.1	0.5	43	2	21.0	1.3	2.1	0.06	114	6**	16.0	1.0*	2.8	0.3**	
LP ₁₂	9.7	0.5	39	2	19.7	1.4	2.0	0.05	136	4	16.3	0.5**	5.1	0.5**	
LP ₂₀	11.2	0.5	38	1**	23.4	1.2	2.1	0.06	120	2**	17.1	0.9**	4.9	0.7**	
CD ₂	10.2	0.4	45	1	20.4	1.1	2.0	0.07	132	4	15.5	1.0	3.1	0.6**	
CD ₄	10.7	0.4	45	1	21.4	0.9	2.0	0.05	118	2**	23.6	1.0**	7.3	0.5**	

LP₆, low-protein diet for 6 d; LP₁₂, low-protein diet for 12 d; LP₂₀, low-protein diet for 20 d; CD₂, choline-deficient diet for 2 d; CD₄, choline-deficient diet for 4 d; FFDLW, fat free dry liver weight. Mean values were statistically significantly different from control value: * $P < 0.05$, ** $P < 0.01$.

Table 3. Percentage disappearance rate (PDR) of indocyanine green from the blood at three dose levels of indocyanine green in the rats given low-protein (LP₂, LP₁₂, LP₂₀) and choline-deficient (CD₂, CD₆) diets

(Values are the median and range for five rats/group)

Dose (mg/kg body-wt)...	PDR (%/min)					
	20		10		5	
	Median	Range	Median	Range	Median	Range
Control	9.4	8.0-10.7	18.7	16.9-20.3	24.8	20.4-29.5
LP ₆	6.0	4.9-6.9	9.6	8.5-10.7	13.7	11.2-16.1
LP ₁₂	7.0	6.5-7.2	11.5	11.4-11.7	14.0	13.1-14.9
LP ₂₀	5.6	5.3-5.8	9.3	8.3-10.5	13.5	13.3-15.2
CD ₂	8.4	6.8-9.9	16.8	14.2-18.0	22.4	18.2-25.2
CD ₆	9.6	8.4-10.3	17.9	13.9-20.4	22.7	19.8-26.7

LP₆, low-protein diet for 6 d; LP₁₂, low-protein diet for 12 d; LP₂₀, low-protein diet for 20 d; CD₂, choline-deficient diet for 2 d; CD₆, choline-deficient diet for 6 d.

Table 4. The derived values for maximal removal rate (R_{max}) and the affinity (K_m) of indocyanine green by the liver

(Values are means with 95% confidence limits)

Dietary group	R_{max} (mg/g per min)		K_m (mg/g)	
	Mean	95% confidence	Mean	95% confidence
Control	2.4	2.0-2.9	4.4	2.2-6.7
LP ₆	1.6	1.1-2.4	6.9	3.8-10.3
LP ₁₂	2.1	1.9-2.5	9.5	6.8-12.4
LP ₂₀	1.4	1.3-1.6	5.6	4.0-7.2
CD ₂	2.1	1.8-2.6	4.2	2.0-6.4
CD ₆	2.6	1.8-3.0	6.3	0.4-12.5

LP₆, low-protein diet for 6 d; LP₁₂, low-protein diet for 12 d; LP₂₀, low-protein diet for 20 d; CD₂, choline-deficient diet for 2 d; CD₆, choline-deficient diet for 6 d.

for each of the three dose levels used. The values are median and range for each group. It can be seen that the disappearance rate in the rats on the choline-deficient diet, was not different from the control at 2 and 6 d. In contrast, at 6, 12 and 20 d on the low-protein diet all animals had a depression in the percentage disappearance rate at all three dose levels. In Table 4, these results have been used to derive values for R_{max} and K_m . These are expressed as means and 95% confidence limits, as obtained by the method described by Barber *et al.* (1967). R_{max} was unchanged on the choline-deficient diet, but fell on the low-protein diet being 66, 88 and 60% of the control value at 6, 12 and 20 d respectively. Thus after 20 d on the low-protein diet R_{max} was significantly less than normal. The changes in K_m showed a different pattern. Once more there was no effect seen on the choline-deficient diet. On the low-protein diet K_m showed a tendency to increase at 6 d, was significantly increased at 12 d, but had returned to the normal range by 20 d.

DISCUSSION

Hepatic reserve can be applied to any of the many functions of the liver (Rikkers & Moody, 1974). In clinical situations it has been observed that in general wherever there is an identifiable derangement in hepatic function, there is a failure of the liver to remove ICG normally from the circulation (Leevy *et al.* 1967). This may be represented by either a change in the total receptor mass, R_{\max} , or the affinity of the dye for binding with the receptor, K_m . In both man and rat more subtle metabolic alterations, such as energy restriction, can modify the clearance of ICG from the plasma (Ohkubo *et al.* 1978). The mechanism whereby these changes are brought about is poorly understood.

On a choline-deficient diet the synthesis of the lipoprotein moiety of very-low-density lipoproteins is thought to be impaired. This leads to failure in the mobilization of fat from the liver with subsequent centrilobular accumulation (Mookerjea, 1965). Thus a dramatic increase in the fat content of the liver was seen by 2 d, being more marked by 6 d, but remarkably only 50% of the increase could be accounted for by triglyceride accumulation. Despite the considerable increase in fat there was very little change in the capacity of the liver to remove ICG from blood.

It is suggested that on low-protein diets fat accumulates in the liver as a result of decreased synthesis of the apolipoprotein (Flores, Pak *et al.* 1970; Flores, Sierralta *et al.* 1970; Seakins & Waterlow, 1972) although other mechanisms also appear to be operative (Seakins, 1971). On the low-protein diet total fat and triglyceride increased with time. Fat-free dry weight on the other hand showed a phasic response, with evidence of recovery at 12 d. It was considered that these results might be spurious, and the experiment was repeated with another series of animals and the same results were obtained. Marked changes were observed in both R_{\max} and K_m on this diet. The increase in liver fat showed a negative linear relationship to R_{\max} ($r = 0.98$, $P < 0.02$). When total fat was expressed as a proportion of fat-free dry weight (total fat: FFDLW) the negative relationship with R_{\max} was closer ($r = 0.99$, $P < 0.01$).

The mechanism whereby ICG is bound and transported across the hepatocyte for excretion into the bile is dependent upon receptor and carrier proteins which are thought to belong to the family of glutathione-S-transferases (Kaplowitz, 1980). They comprise up to 10% of the total protein content of the liver. The net content is dependent on the relative rates of synthesis and degradation, as is true for the proteins involved in lipid handling, and must relate to the over-all state of protein metabolism within the hepatocyte, and be responsive to the dietary state (Stein *et al.* 1976; Ohkubo *et al.* 1978). The phasic response on the low-protein diet, with apparent recovery at 12 d was a real phenomenon. This may reflect a changing pattern of protein synthesis and degradation in the liver with time, during the process of adaptation to a low protein intake (Garlick *et al.* 1975).

A number of pieces of evidence point to the fact that the liver lobule has three functional zones, periportal, mid-zonal and centrilobular. Toxic agents affect the different zones in a characteristic and specific fashion (Stoner & Magee, 1957); the enzymic activity of cells taken from the different zones varies (Shank *et al.* 1959); the varied distribution of these enzymes can be demonstrated histologically (Novikoff, 1959); in vivo there is differentiation in the uptake of fluorescent compounds by cells in the different zones (Gumucio *et al.* 1981). It has been suggested that the cells in the centrilobular zone are peculiarly susceptible to toxic metabolites because they contain much less glutathione than those in the other regions of the rat liver lobule (Smith *et al.* 1979). It is not clear how, or if, the glutathione content of itself affects the activity of the glutathione-S-transferase activity of a cell. However, the evidence presented here would suggest that as choline deficiency in the diet affects the centrilobular cells predominantly, and has little effect on ICG handling, then the removal

of ICG is not a major function of the centrilobular zone. In contrast, a protein-deficient diet affects ICG handling and also causes fat accumulation in the periportal areas. We would conclude that ICG removal is carried out by the cells in the periportal zone predominantly. Hence the capacity of the liver to take up ICG may be a sensitive indicator of the functional capacity of the periportal zone. If the insult extends to involve other areas of the liver more extensively there may not necessarily be a corresponding deterioration in the ability to take up ICG.

Thus in clinical situations one might expect ICG removal to be a useful test for liver function when the damage primarily involves periportal cells. In those situations where the damage is primarily centrilobular, ICG removal would tend to underestimate the severity of the damage. The pattern of liver damage in protein-energy malnutrition includes periportal infiltration of fat, and adaptive changes in many enzymes which are normally more active in the periportal zone (Trowell *et al.* 1954; Waterlow *et al.* 1960). Therefore the use of ICG may provide a useful indicator of early hepatic impairment in this condition.

The authors thank the Research Corporation of America and the Wellcome Trust for support.

REFERENCES

- Barber, H. E., Welch, B. L. & Mackay, D. (1967). *Biochem. J.* **103**, 251.
 Best, C. H. & Huntsman, M. E. (1932). *J. Physiol., Lond.* **75**, 45.
 Fleck, A. & Munroe, H. N. (1966). *Analyst, Lond.* **91**, 78.
 Fletcher, M. J. (1968). *Clinica. Chem. Acta* **22**, 393.
 Flores, H., Pak, N., Maccioni, A. & Monckeberg, F. (1970). *Br. J. Nutr.* **24**, 1005.
 Flores, H., Sierralta, W. & Monckeberg, F. (1970). *J. Nutr.* **100**, 375.
 Garlick, P. J., Millward, D. J., James, W. P. T. & Waterlow, J. C. (1975). *Biochim. biophys. Acta* **414**, 71.
 Garrow, J. P. & Pike, M. C. (1967). *Br. J. Nutr.* **21**, 155.
 Gumucio, J. J., Miller, D. L., Krauss, M. D. & Zanolli, C. C. (1981). *Gastroenterology* **80**, 639.
 Hunton, D. B., Bollman, J. L. & Hoffman, H. N. (1960). *Gastroenterology* **39**, 713.
 Hunton, D. B., Bollman, J. L. & Hoffman, H. N. (1961). *J. clin. Invest.* **40**, 1648.
 Kaplowitz, N. (1980). *Am. J. Physiol.* **239**, G439.
 Kessler, G. & Lederer, H. (1965). In *Automation in Analytical Chemistry*, p. 341 [L. T. Skeggs Jr, editor]. New York: Mediad Inc.
 Leevy, C. M., Smith, F., Longueville, J., Paumgartner, G. & Howard, M. M. (1967). *J. Am. med. Ass.* **200**, 236.
 Lineweaver, H. & Burke, D. (1934). *J. Am. Chem. Soc.* **56**, 658.
 McLean, A. E. M. (1962). *Lancet* **ii**, 1292.
 Mookerjee, S. (1965). *Can. J. Biochem.* **43**, 1733.
 Novikoff, A. B. (1959). *J. Histochem. Cytochem.* **7**, 240.
 Ohkubo, H., Musha, H. & Okuda, K. (1978). *Am. J. dig. Dis.* **23**, 1017.
 Paumgartner, G., Probst, P., Kraines, R. & Leevy, C. M. (1970). *Anns. N.Y. Acad. Sci.* **17**, 134.
 Rikkers, L. F. & Moody, F. G. (1974). *Gastroenterology* **67**, 691.
 Riggs, D. S. (1970). *The Mathematical Approach to Physiological Problems*. Cambridge: MIT Press.
 Seakins, H. A. S. (1971). Fat metabolism in protein malnutrition. PhD Thesis, University of the West Indies.
 Seakins, H. A. S. & Waterlow, J. C. (1972). *Biochem. J.* **129**, 793.
 Shank, R. E., Morrison, G., Cheng, C. H., Karl, I. & Schwartz, R. (1959). *J. Histochem. Cytochem.* **7**, 237.
 Smith, M. T., Loveridge, N., Wills, E. D. & Chayen, J. (1979). *Biochem. J.* **182**, 103.
 Stein, L. B., Mishkin, S., Fleischner, G., Gatmaitan, Z. & Irwin, M. A. (1976). *Am. J. Physiol.* **231**, 1371.
 Stern, I. & Shapiro, B. (1953). *J. clin. Path.* **6**, 158.
 Stoner, H. B. & Magee, P. N. (1957). *Br. med. Bull.* **13**, 102.
 Tagle, M. A. & Donoso, G. (1965). *J. Nutr.* **87**, 175.
 Trowell, H. C., Davies, J. N. P. & Dean, R. F. A. (1954). *Kwashiorkor*, p. 128. London: Edward Arnold.
 Waterlow, J. C. (1948). *Med. Res. Coun. Spec. Rep. Ser.* no. 263.
 Waterlow, J. C. (1975). *Am. J. clin. Nutr.* **28**, 1330.
 Waterlow, J. C. & Bras, G. (1957). *Br. med. Bull.* **13**, 107.
 Waterlow, J. C., Cravioto, J. & Stephen, J. M. L. (1960). *Adv. Protein Chem.* **15**, 131.