The Bilirubin Overload Test with Special Reference to Liver Damage Produced by India Ink.* (26527)

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Data are presented on the effects of various agents on the bilirubin overload test in the rat. This test has been used previously in the rat to study hepatic regeneration(1). It differs from the bilirubin test in man in that the amount of bilirubin administered is sufficient to overload the maximum excreting ability of the liver. Special reference will be made to liver damage due to India Ink, since the test permits ready dissociation of the action of shellac and carbon particle fractions.

Material and methods. Sprague-Dawley male rats, weighing from 120 to 400 g, were anesthetized lightly with ether. Three mg per 100 g body weight of unconjugated bilirubin (Eastman Kodak) dissolved in 0.1 M sodium carbonate were given intravenously. Serum bilirubin levels were routinely read at 3 and 30 minutes, and 1,2,4 and usually 24 hours following injection, but only the 30 minute, 1 and 2 hour points were utilized in assessing the test. Serum bilirubin was estimated by a micro-method after Malloy and Evelyn(2) utilizing 25 lambda of serum, except that the diazo reagent was modified by addition of 0.6 ml instead of 0.3 ml of sodium nitrite. Direct-reacting bilirubin was read at 1 minute. The various agents studied were administered 30 minutes prior to injection of bilirubin unless stated to the contrary (Table I). The dose of sulfobromophthalein sodium (BSP) was 3 mg per 100 g body weight and the serum levels read at 30 minutes.

In the India Ink studies, the shellac fraction was prepared by centrifugation of Higgins American India Ink at 35,000 rpm for 1 hour. Microscopy showed that it was clear of carbon particles. A carbon suspension was prepared by suspension of India Ink precipitate in a 5% gelatine normal saline solution by means of an ultrasonic generator. Since, however, complete suspension was difficult, Germantown Lamp Black was later substituted, but the results were similar. Routine paraffin and frozen sections were made of the liver and stained by standard technics. Additional animals were used to study early histological lesions.

Results. Control animals. In the normal rat, there was a rise of total serum bilirubin level to a maximum around 3 minutes after which there was a rapid fall for 2 hours, followed by a slow fall to normal at 24 hours (Fig. 1). No direct-reacting bilirubin could be detected. Repeated injections of unconjugated bilirubin at half hour intervals in 10 animals did not alter the initial rapid fall of serum bilirubin levels indicating the tremendous hepatic excretory reserve in this animal. Cannulation of the common bile duct in 10 rats showed that approximately two-thirds of the exogenous bilirubin was excreted during the first 4 hours and the remainder during the next 24 hours. Anesthesia by intraperitoneal pentobarbital did not alter the normal curve. In normal animals, no BSP serum levels were present at 30 minutes.

Experimental group. Our results are summarized in Table I. Two abnormalities were observed: 1) A delay in the fall of total bilirubin serum levels, which was significant at 30 minutes after bilirubin injection although it could be demonstrated sooner. While a single value in animals with abnormal retention might be within the upper range of values seen in control groups, differences were best appreciated by evaluation of the entire curve, since overlap at all 3 times was not seen. Analysis by the Mann-Whitney "U" test showed the differences between control and abnormal groups to be highly significant. 2) A more striking finding in animals with bilirubin retention was the invariable appearance of a direct reacting bilirubin in the serum, which reached its maximum in approximately 30 minutes and fell to normal levels at 2 to 4 hours.

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TABLE I. Effect of Different Agents on Bilirubin Overload Test Showing Results Obtained at 30 Minutes and at 24 Hours after Administering Agent.

	No.	Dose*	Route	30 min. Mean	30 min. Range	30 min. Significance	30 min. Direct bilirubin	60 min. Mean	60 min. Range	60 min. Significance	60 min. Direct bilirubin	120 min. Mean	120 min. Range	120 min. Significance	120 min. Direct bilirubin
Controls	20	—	—	28.2	22–33	—	0	15.5	11–19	—	0	7.0	5–10	—	0
Simultaneous BSP test	5	3.0 mg	—	26.4	21–33	N.S.	0	15.2	11–19	N.S.	0	7.0	6–9	N.S.	0
Sodium salicylate	5	25.0 mg	I.P.	27.6	26–31	"	0	14.4	14–15	"	0	6.2	6–7	"	0
India Ink & constituents															
India Ink	5	.2 ml	I.V.	48.2	33–60	H.S.	8.0 (5–13)	39.4	30–48	H.S.	4.2 (2–7)	29.0	25–34	H.S.	1.8 (1–3)
After 24 hr	5	*idem*		43.0	38–52	"	6.8 (6–9)	33.2	28–41	"	2.8 (2–5)	21.6	16–29	"	0
Shellac	5	"	"	47.8	37–59	"	9.6 (5–12)	38.6	30–45	"	5.4 (2–9)	29.8	26–34	"	2.6 (1–5)
After 24 hr	5	"		40.8	34–45	"	6.4 (4–9)	33.6	29–38	"	3.6 (1–6)	20.8	17–23	"	0
Carbon suspension	5	"	"	28.8	20–33	N.S.	0	14.6	12–17	N.S.	0	6.6	5–9	N.S.	0
Gum acacia	5	"	I.S.	30.0	26–34	"	0	17.2	15–19	"	0	7.0	6–9	"	0
Carbon tetrachloride	5	.06 ml	I.P.	36.0	27–41	H.S.	5.4 (4–6)	26.2	19–30	H.S.	3.2 (2–4)	15.8	11–20	H.S.	0
After 24 hr	5	*idem*		28.0	23–32	N.S.	0	17.6	13–21	N.S.	0	8.0	7–9	N.S.	0
Preceded by dibenzyline	5	"		35.8	33–40	H.S.	3.4 (3–4)	22.6	18–28	H.S.	2.4 (2–3)	13.6	10–19	H.S.	0
50% ethanol	5	.6 ml	I.G.	34.0	33–37	"	4.4 (4–5)	22.4	21–25	"	2.4 (2–3)	14.2	12–16	"	0
After 24 hr	5	*idem*		32.8	18–59	N.S.	0	15.0	8–30	N.S.	0	7.8	5–10	N.S.	0
Chlorpromazine	5	1.0 mg	I.P.	32.0	28–38	"	0	13.6	11–16	"	0	7.0	5–9	"	0
Chloroform	5	.06 ml	"	24.2	21–28	"	0	13.8	10–19	"	0	6.4	6–7	"	0
Choline deficient diet 21 days	5	—	—	36.2	33–39	H.S.	4.5 (4–5)	19.0	17–21	H.S.	2.4 (2–3)	12.0	11–13	H.S.	0

* Dose/100 g body wt.

N.S. = $P > 0.10$
H.S. = $P < 0.01$

I.V. = via femoral vein
I.S. = via intrasplenic route
I.P. = via intraperitoneal route
I.G. = via intragastric route

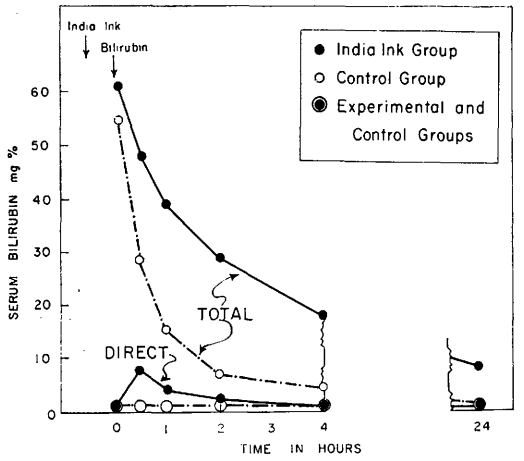

FIG. 1. Graph showing typical abnormal curve following whole India Ink administration 30 min. before inj. of bilirubin.

In animals given India Ink or shellac, total bilirubin serum levels were elevated at 24 hours and bilirubin overload indicated retention. With CCl_4 and ethanol, with one exception 24 hour serum bilirubin levels were normal and no retention or direct bilirubin could be demonstrated. Administration of India Ink, shellac, and carbon suspension to a further 22 animals by intra-splenic injection or in larger doses showed no significant differences, although histological lesions in the liver with whole India Ink tended to be more severe. Cannulation of the common bile duct in 10 rats given India Ink and 9 animals given CCl_4 showed a delay in bilirubin excretion over a 24 hour period, and a reduction in total quantity of bile excreted.

There was no correlation between degree of parenchymal damage and the abnormal bilirubin tests as indicated by studies with India Ink. Intravenous and intra-splenic injection of whole India Ink led to coagulative necrosis throughout the liver, beginning at 4 to 6 hours and reaching a maximum at 24 hours (Fig. 2). This necrosis appeared focal in the early stages, but later was predominantly periportal. It was usually associated with large intrasinusoidal masses of India Ink-laden Kupffer cells. The absence of significant necrosis in the rats given the shellac suggested that it was due to infarction following sinusoidal obstruction. Some portal edema was also present. While the shellac produced identical abnormalities in the bilirubin test, the degree of necrosis and portal edema was minimal or absent (Fig. 3). The degree of necrosis produced by the carbon suspension was much less than with whole India Ink. This was probably due to less carbon reaching the liver as much more was filtered by the lungs and spleen as compared to whole India Ink. In 2 livers after intra-splenic injection of carbon suspension, there were considerable carbon

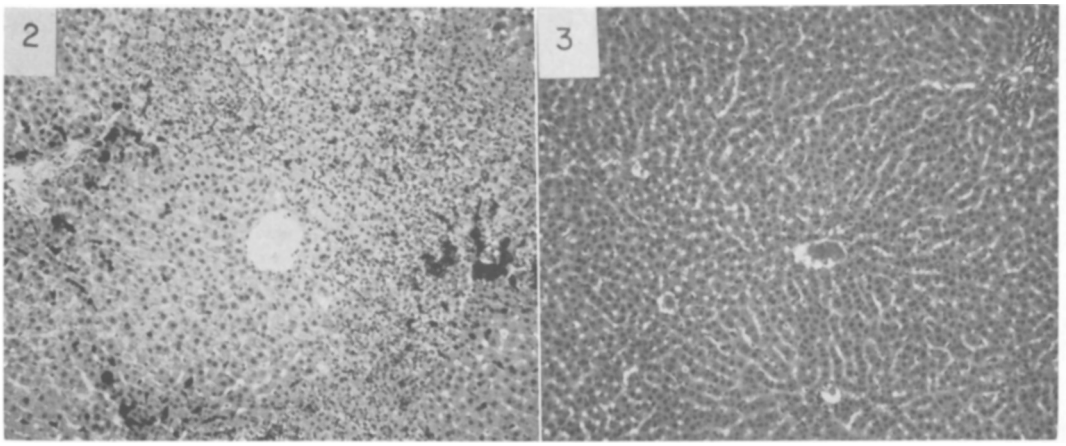

FIG. 2. Severe periportal coagulative necrosis in liver 27 hr after administration of whole India Ink. Note ink-laden phagocytes completely obstructing sinusoids at periphery of lobule. H. & E. × 120.

FIG. 3. Liver 27 hr after administration of shellac fraction of India Ink showing no significant histological abnormality. Bilirubin curve was identical to that given by whole India Ink. H. & E. × 120.

deposits in the sinusoids and Kupffer cells with some necrosis, but bilirubin tests were normal. Although typical moderate degenerative changes were present in the livers 24 hours after CCl_4, bilirubin curves were normal. Administration of dibenzyline (0.5 mg/100 g) 30 minutes prior to CCl_4 in 5 rats rad no significant effect on the test. No significant histological lesions were noted in the ethanol treated animals at 1 and 2 hours. The BSP test gave essentially similar results to the bilirubin overload test except in rats on a choline deficient diet when serum levels were normal at 30 minutes.

When BSP was administered simultaneously with bilirubin in the same syringe, no obvious bilirubin retention was observed (Table I).

Discussion. The experimental bilirubin overload test can indicate hepatic abnormalities early. It has the advantage of testing a physiological mechanism of the liver, and it may be also a more sensitive index than the BSP test as indicated by findings in choline deficient rats. It cannot be correlated with degree of liver necrosis, possibly due to the great reserve ability of the rat liver to excrete bilirubin, thus masking mild or moderate necrosis. The nature of the delayed excretion is uncertain. Due to its rapid onset and the variety of agents which produce it, it may be a non-specific vasomotor response causing anoxia as has been suggested to occur in CCl_4 poisoning. Administration of dibenzyline, however, 30 minutes prior to CCl_4 had no effect on the test. The source of the direct-reacting bilirubin is unknown. It may represent leakage into the serum from partially damaged cells, since with BSP and fluorescein there is considerable parenchymal storage prior to excretion(3). However, while there was destruction of liver cells in both India Ink and CCl_4 treated rats, direct-reacting bilirubin at 24 hours was observed only in animals treated with India Ink or shellac, and could not be correlated with histological damage. The second possibility is that it represents an extra-hepatic source of production. Such a source of conjugated bile pigment has been suggested by Hoffman *et al.*(4). However, it is difficult to appreciate why such a pigment should not appear after simple overloading. It would seem more probable to regard this pigment as hepatic in origin, but it is necessary to establish if it is a mono- or di-glucuronide.

It has been shown(5,6) using the BSP test, that in the dog, the shellac fraction is responsible for the physiological activity of India Ink with the assumption that the carbon particles are harmless. It would appear, however, that the carbon particles in association with shellac may cause marked hepatic damage due to a mechanical effect. Unfortunately, we were unable to procure other fractions of the shellac from the manufacturers for testing.

Failure to demonstrate increased retention of bilirubin or BSP, when the tests were carried out simultaneously in contrast to the observations of Cantarow *et al.*(7) with prolonged infusion in dogs is probably due either to the fact that high levels of both substances were maintained only for a short period, or to the tremendous reserve of the rat liver.

Summary. The bilirubin overload test is described and the result obtained with a variety of hepatotoxic agents is presented. Hepatic injury can be demonstrated at a very early stage, but the test is of no value in indicating degree of histological liver damage. The biological effect of whole India Ink on the liver can be divided into: 1) Micro-infarction due to obstruction of the sinusoids by India Ink-laden Kupffer cells, an effect not detectable by the bilirubin overload test; and, 2) effect of the shellac which causes only minimal histological damage but which gives an abnormal bilirubin test.

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Reactiveness of Fetal Pituitary to Stressful Stimuli. Does the Maternal ACTH Cross the Placenta? (26528)

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Normally the concentration of ascorbic acid in the adrenal of healthy adult animals is reduced by adrenocorticotrophin (ACTH) released from the anterior pituitary gland. The same pituitary-adrenal relationship probably exists in the fetus, since ACTH release by the fetal pituitary and responsiveness of fetal adrenals has been shown(1-4). However, the question has not been fully resolved as to whether the source of the ACTH to which the fetal adrenals respond is the fetal or the maternal hypophysis. The question of placental permeability to ACTH arises. It is generally believed that the pituitary hormones, being proteins, do not cross the placental barrier under normal conditions. Yet some authors claim that under certain experimental conditions ACTH may cross the placenta(5,6). The authors have shown that subcutaneous injection of epinephrine into embryos after the 19th day caused a significant depletion of ascorbic acid from the adrenals of the fetus(7). In this paper the effect of epinephrine injection into the fetus was determined in normal and in decapitated fetuses.

Materials and methods. All experiments were carried out on rats on the 22nd or last day of pregnancy under ether anesthesia. The sequence of operation on the embryos was from ovarian to the cervical end of one horn of the utreus, then from the ovarian to the cervical end of the other horn. First, 2 control untreated embryos were removed through a small incision in the uterine wall, blotted on filter paper and weighed on a torsion balance. After decapitation, adrenals were removed and cleaned under 10-fold magnification, pooled and weighed in a closed vessel on an automatic analytical balance. Later the ascorbic acid content of the adrenals was determined by a modification(8) of the Roe and Kuether method. The next pair of embryos was similarly treated to also serve as control. The values from these 2 pairs of embryos established the 100% value for adrenal ascorbic acid concentration for the experimental embryos of the same female rat. In all experiments 4 adrenals were pooled for ascorbic acid determination. The next 4 fetuses were decapitated as follows: A circle of stitches of a thick surgical thread was placed on the uterine wall just above the head of the fetus. An incision in the middle of this circle allowed the head to peep through. The stitches were tightened around the throat of the fetus, then the head was cut off just above the ligature. This decapitation does not disturb the normal intrauterine position of the fetus. Removal of the 4 control fetuses takes only 1 to 2 minutes. It takes about 4 minutes to decapitate the first pair of fetuses (Decapitation I) and 4 additional minutes to decapitate the second pair of fetuses (Decapitation II). When the fetuses were to be further treated, epinephrine (20 μg/fetus), or ACTH* (50 milliunits/fetus) was injected subcutaneously in aqueous solution into the fetus through the uterine wall immediately after decapitation. The remaining fetuses were left untreated to determine to what extent the adrenals were stimulated by the laparotomy of the mother as well as by the operations on and injections into the other embryos. Exactly one hour after

* ACTH "Prolek" 1 mg contains 25 I. U.