multiplied extensively in tissue cultures of mouse testes and human testes. It is not clear whether the virus multiplied in the minute amounts of muscle from vessel walls, etc., or in some other tissue. Sections indicate that some cultures contained a small bit of striated muscle. In view of the relatively high titers of MM virus found in infected muscle-containing tissues in previously reported experiments(2) it seems quite possible that very small amounts of this type of tissue might give rise to easily detectable amounts of virus in tissue cultures.

The differences in rate of growth and period of survival of MM virus in cultures of human and mouse tissues may have clinical significance. If the slow rate of growth in human tissues *in vitro* is representative of the rate of growth *in vivo*, there should be more time for protective antibodies to be formed in infected persons than in mice or hamsters. The rapid decline in amount of virus in tissue cultures of mouse tissues may result from rapid killing of all susceptible cells, a factor that might contribute to the known severity of an infection in animals of that species.

Conclusions. MM virus multiplied extensively in 9 serial passages in mouse testicular tissue *in vitro* and in 4 serial passages in human testicular tissue *in vitro*. Fluid from the last tissue of each series represented a 10^{-13} dilution of original inoculum. These experiments do not exclude the possibility that the virus may have multiplied in the very minute amounts of muscle tissue present in the cultures. The virus multiplied more slowly and persisted longer in human tissue than in mouse tissue.

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Comparison of Excretion of Bromsulphthalein and Sodium Cholate after Intravenous Injection, Separately and Combined. (18440)

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(Introduced by W. J. Darby)

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Recently, interest has arisen in comparative studies of liver function tests, and the concept of association rather than dissociation of these tests has been brought to light(1,2). Although the bile salt tolerance test has been investigated intensively(3-9), it has not been subjected to a comparison with the bromsulphthalein test. The administration of bile salts has been observed to cause a retention of bromsulphthalein(10-12), but the blood

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concentrations of the injected bile salts have not been studied, and the effect of the bromsulphthalein on the excretion of bile salts has not been reported. Macdonald(13) has indicated that the bromsulphthalein excretion test afforded more accuracy in determining hepatic reserve if several samples of blood were drawn at intervals rather than the usual single sample.

In order to facilitate multiple determinations of bile salts in serum, a method was sought which was not only accurate but relatively short. A modification of the procedure reported by Minibeck(14), which has been virtually unnoticed in this country, was employed. We have applied this technic and are reporting comparative data on the bromsulphthalein and the bile salt tolerance tests when conducted separately and simultaneously on dogs in health and following hepatic damage.

Methods. Full grown mongrel dogs weighing from 8 to 12 kilos were used. The sodium cholate solution for intravenous injection contained 50 mg of cholic acid per ml and sufficient sodium hydroxide to adjust the pH to 7.4. This solution contained 25% glucose for the purpose of diminishing pain and thrombosis(5). It was passed through a Selas porcelain filter, porosity 02 and stored in rubber capped vials in the refrigerator. A dose of 50 mg of cholic acid per kilo of body weight was used. The bromsulphthalein solution contained 50 mg of the dye per ml of solution.‡ A dose of 5 mg bromsulphthalein per kilo of body weight was given. For the combined cholate and bromsulphthalein injections the 2 solutions were mixed before injecting. Injections were made into a superficial leg vein and samples of blood were drawn from a different vein, usually the femoral. Cholic acid was analyzed by the following modification of the method of Minibeck(14): 8.5 ml of 95% ethyl alcohol were pipetted into a test tube etched at the 10.0 ml volume mark, and 0.5 ml of barium oxide-acetate solution was added.§ 1.0 ml of the serum to be analyzed was added dropwise with shaking. The tube was heated over a small flame to boiling, and boiling was continued for 2 minutes. The mixture was cooled and alcohol was added to the 10.0 mark. It was then filtered, and 3.0 ml of the filtrate were transferred to a centrifuge tube. The solution was evaporated to dryness under reduced pressure at 40-45°C. Approximately 4 ml of the purified ethyl acetate‖ were added to the dry residue. 0.1 ml of a mixture of calcium oxide in ethyl acetate was added.¶ The tube was heated at 90°C for 2 minutes, centrifuged, and the ethyl acetate decanted. A second and third portion of 4 ml of ethyl acetate (but no additional calcium oxide) were added and, as before, heated, centrifuged, and decanted. 10.0 ml of acetic acid reagent** were added to the residue and the solution was stirred thoroughly with a glass rod and placed in a water bath at 37-40°C for 30 minutes along with 0.2 ml of 10 mg % standard cholic acid solution†† in 95% ethyl alcohol. The Coleman No. 12 photofluorometer with primary filter B-2 and secondary filter PC-2 was employed. The standard cholic acid solution was used to set the instrument. The reading was compared to a standard curve prepared by determining the fluorescence of graded amounts of cholic acid in 10.0 ml of the acetic-sulfuric reagent. The result was expressed in terms of cholic acid per 100 ml of serum.

Recoveries of cholic acid and glycocholic acid added to human serum are given in Table

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‡ Hynson, Westcott and Dunning preparation.

§ 20 g of barium oxide, anhydrous, Merck, were added to 400 cc of water, warmed and filtered. Sufficient barium acetate was added so that the solution contained 0.4 g of barium acetate per 100 cc.

‖ Ethyl acetate was purified according to Fieser, Exp. in Organic Chem., 1941, p. 364.

¶ 3.3 g of finely pulverized anhydrous calcium oxide were mixed with 100 ml of purified ethyl acetate. This mixture was shaken immediately before using.

** Nine parts of concentrated sulfuric acid to one part glacial acetic acid.

†† Cholic acid was generously supplied by the Ames Co.

TABLE I. Recoveries of Bile Acids Added to Serum.

Bile acid	No. of determinations	Theoretical, mg %	Determinations, mg %	Avg analytical recovery, %	Avg deviation of single determination $\alpha+$, ± %
Cholic acid added to human serum	5	0.8	0.72	90.0	5.4
	12	4.0	3.82	95.4	4.97
	9	8.0	7.64	95.5	4.71
Glycocholic acid added to human serum	6	0.8	0.63	79.1	11.10
	6	4.0	3.75	93.8	3.4
	6	8.0	7.63	95.3	7.3
Cholic acid added to dog serum	7	0.8	0.59	73.5	13.6
	4	2.0	1.90	95.0	7.5
	8	4.0	3.59	89.6	3.7
	5	8.0	7.43	92.8	2.7
	3	37.5	40.3	107.4	2.5

$\alpha+$ Average deviation of single determination is calculated from the formula $\alpha = v/n$ in which v is the deviation of each determination from the mean and n is the number of determinations.

I. The desired volume of the standard cholic acid solution was evaporated to dryness in a test tube, and a known amount of serum was added, mixed thoroughly, and allowed to stand for 20 minutes. 1.0 ml of serum was then withdrawn and analyzed as described above. Recoveries of cholic acid added to dog serum are also given in Table I.

In Minibeck's procedure, the alcoholic filtrate was evaporated to dryness in atmospheric pressure. Recoveries were improved by taking the filtrate to dryness under reduced pressure at 40-45°C. In order to study the effect of the method of drying of the alcoholic filtrate on the determination, glycocholic acid was added to serum at various concentrations and the filtrate evaporated (1) under reduced pressure and (2) at atmospheric pressure on a boiling water bath. At the levels of 0.8, 4.0 and 8.0 mg %, recoveries under reduced pressure averaged 79.2, 94.0, and 95.8% of the theoretical amount respectively and, under atmospheric pressure, 50.2, 78.8, and 87.0% respectively. In another series, cholic acid was added at the level of 2.0 mg %; recoveries under reduced pressure averaged 101.2% and under atmospheric pressure 75.0% of the theoretical amount. Pure alcoholic solutions of cholic acid and glycocholic acid were not so easily affected by heat, and could be evaporated to dryness in boiling water without loss of fluorescence. The BSP analyses were made according to Gaebler(15) except that a Klett-Summerson photoelectric colorimeter was used. For the production of liver damage, carbon tetrachloride, Merck, in a dosage of 1.0 ml per kg of body weight was given by stomach tube. Approximately 13 hours after the poisoning, the BSP excretion test was done; at 16 hours the cholate excretion test, and at 20-24 hours the combined BSP and cholate tests were made. Immediately after the combined test, a needle biopsy specimen of the liver was taken. Later the livers were examined grossly and microscopically.

Results. When the values obtained on each of the first three dogs were plotted, the curves followed a similar pattern. The averaged values of each experiment on the three dogs were used, therefore, in constructing Fig. 1 and 2, in which the BSP retention in one graph and cholic acid concentration in the other were plotted against time after injection.

In the healthy animals, the cholate and the BSP excretion rate compared closely with the findings of others(3,10,13,15). When the cholate and BSP were administered in combination, the excretion of BSP was greatly retarded during the first 15 minutes. At 30 minutes, however, there was only a slight difference from the BSP retention when BSP was injected alone. The cholic acid excretion, on the other hand, appeared not to be altered by the presence of BSP in the blood.

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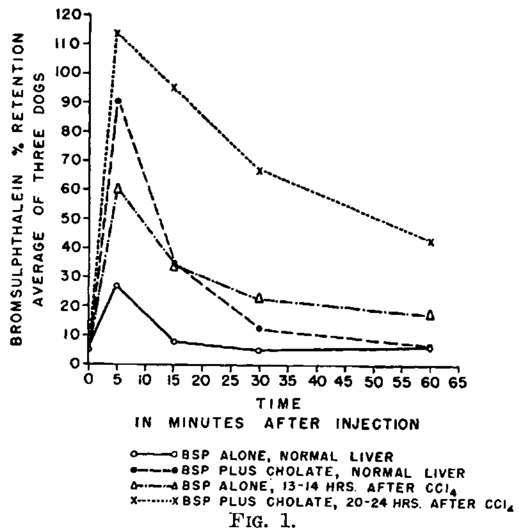

FIG. 1.

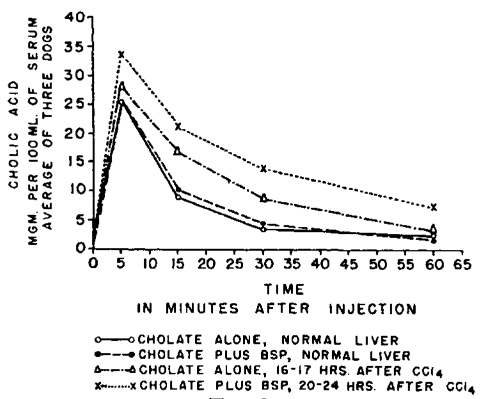

FIG. 2.

The reason for the delay in BSP excretion when BSP and cholate were given together was investigated. Since glucose was used in making the cholate solution, BSP was combined with a 25% solution of glucose adjusted to pH 7 with sodium hydroxide, and injected. The rate of the excretion of BSP was unchanged. Cholic acid, equivalent to 20 mg %, added to an aliquot of serum containing BSP, did not alter the intensity of absorption. In order to determine if mixing the BSP and sodium cholate before injection had an effect on the BSP retention, an experiment was performed on a fourth dog in which BSP was injected into a superficial vein of the right hind leg at the same time that sodium cholate was injected into a vein of the left hind leg. The retention of BSP and the concentration of cholic acid were comparable to those observed in studies in which the two solutions were mixed before injection. In this dog, as in the other 3, the BSP at 5 and 15 minutes after injection of both BSP and cholate was greatly elevated, while the cholic acid concentration was not affected by the BSP in the blood. The order of performing experiments in the first 3 dogs was: sodium cholate, BSP, and combined BSP and cholate. To determine if the sequence of injection might influence the BSP retention, the order was reversed in a fifth dog so that the first experiment performed on this dog was the combined injection of BSP and sodium cholate. The same results were observed as in the other dogs.

After the livers were damaged with carbon tetrachloride, the rate of excretion of BSP became much more abnormal than the cholate excretion rate. The BSP test in each dog definitely indicated liver disease. In the presence of liver damage, the cholic acid concentration in the serum 15 minutes after the injection was greater than one-half the 5 minute concentration. When the liver was normal, however, the 15 minute concentration was less than one-half that at 5 minutes. The cholate excretion test was performed approximately three hours after the BSP test, at which time the liver damage would be predicted to be more severe. In the combined injection tests the cholic acid concentrations in 2 dogs were somewhat higher than when cholate alone was injected. In a third dog, however, there were no significant differences. In the presence of hepatic damage, the combined test resulted in a BSP retention during the first 30 minutes which paralleled roughly that in the BSP alone but which was 35-60% higher. In 2 dogs the BSP retention in the combined test was definitely higher after carbon tetrachloride than when the liver was normal, whereas in a third animal the five minute levels were the same, but the dye was excreted slower after carbon tetrachloride had been given.

Microscopic examination of the liver biopsies taken 20-25 hours after administration of the carbon tetrachloride revealed in each dog a coagulation necrosis with nuclear fragments and infiltration of a few leucocytes involving principally the central zone of the lobules.

Discussion. A modification of the method described by Minibeck was found to be satisfactory for the determination of cholic acid and glycocholic acid. One disadvantage of this procedure is that bile acids containing less than three hydroxyl groups did not give significant fluorescence. Desoxycholic acid had only 14% of the fluorescence of an equivalent weight of cholic acid. Chendesoxycholic acid had 30%, alpha hyodesoxycholic acid 5%, and dehydrocholic acid 2.5% of the fluorescence of cholic acid. For the purposes of determining cholic acid, however, the method was adequate. When sodium cholate was given simultaneously the excretion of BSP was greatly delayed, but there was little effect on the cholate excretion. Apparently the liver preferentially excreted the cholate before excreting the BSP. Sodium cholate and BSP may, therefore, be handled in a similar manner by the liver. It appears possible that the BSP test might be rendered even more sensitive by simultaneously giving cholic acid. Plans have been made for investigating this problem.

Lichtman(16) states that it is the consensus that the BSP is a valuable test of liver damage except when there is biliary obstruction. Mateer and co-workers(17) pointed out that in obstructive jaundice, due to back pressure and interference with the flow of bile from the ducts, there must be a simultaneous backing up of the BSP in the serum, which is due to extra-hepatic obstruction rather than to impairment of cellular function. It has been proven that cholate concentrations reached levels as high as 13 mg % in jaundice(18). In addition to a purely mechanical interference with the excretion of BSP, it appears from the present study that the high serum concentrations of cholate in obstructive jaundice may also impede the excretion of BSP. The effect of bilirubinemia on retention of BSP is unsettled. Dragstedt and Mills(19) reported that intravenous injection of bilirubin caused a retention of BSP. Cantarow and co-workers(20), however, did not confirm this observation; on the contrary, they suggested that the BSP was excreted preferentially over the bilirubin.

Summary. 1. A modification of Minibeck's method was found to be satisfactory for the determination of cholic acid and glycocholic acid added to serum. 2. In healthy dogs when bromsulphthalein and sodium cholate were injected together, the concentration of bromsulphthalein in the serum was greatly elevated five and fifteen minutes after administration. The cholate concentration, however, was normal. 3. In early central necrosis of the liver produced by carbon tetrachloride, the bromsulphthalein excretion was definitely delayed while the cholate excretion was only slightly changed. The bromsulphthalein test appeared to be more sensitive in detecting early hepatic damage. During the first thirty minutes the bromsulphthalein retention in the combined test paralleled roughly the retention when bromsulphthalein was given alone, but was 35-60% higher.

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