Isolated Rat Liver Preparation. Bile Production and Other Basic Properties.*† (19012)

R. W. BRAUER,‡ R. L. PESSOTTI,‡ AND P. PIZZOLATO.

From the Department of Pharmacology, School of Medicine, Louisiana State University, and the Pathology Laboratory, Veterans Administration Hospital, New Orleans, La.

The liver, more perhaps than any other organ, reflects in its structure and function changes impressed upon any part of the organism of which it is a part. Its close integration into the body economy, and its target position with respect to many drugs or diseases, render at once important, and difficult of achievement, an understanding of the functioning of this organ *per se*. The present paper is concerned with the description of an isolated liver preparation which retains a sufficient proportion of its normal physiological functions, and which survives long enough, to warrant study of its properties; it shows promise of permitting separation and analysis of many of the factors which affect the performance of this organ, an analysis of which is basic to the formulation of a usable concept of "liver function." In the past, perfusions of the isolated liver have been carried out in general as parts of biochemical investigations (1). Histological integrity of the tissue, bile production, and Kupffer cell activity were not studied, or were found to be poorly preserved after even short periods of perfusion. Furthermore, with one exception(2), survival of perfused liver tissue has been too short for many of the studies envisaged here.

The present preparation is based upon the following considerations: 1. The vascular tree of the liver is sensitive to perfusion pressures; unless sufficiently low inflow pressures are maintained at every moment some functions and structural integrity are soon lost(3). 2. The hepatic arterial supply is not essential for normal function of the rat nor even of the dog liver, provided adequate antibiotic levels are maintained in the blood supplied to the organ(4,5). 3. Survival of hepatic cells in organ culture is possible for periods of several days at least(2). 4. The heart-lung-liver preparation in the dog—contrast to saline perfused liver preparations—will produce bile, though none too well and not for very long(6). Thus, erythrocytes in the perfusate are, and innervation apparently is not, essential for bile production.

Methods. The perfusion medium is prepared in 3 separate solutions as follows:

A—2.5 g crystalline bovine plasma albumin,§ 37.5 mg $NaHCO_3$, 460 mg NaCl, 21 mg KCl, 6 mg $CaCl_2$, made up to 50 cc with distilled water.

B—4 cc 0.1 M Na_2HPO_4, 1 cc 0.1 M KH_2PO_4.

C—25 cc whole rat blood, containing 0.5 mg of heparin. Solutions A and B are mixed and equilibrated in the perfusion pump with 95% O_2 and 5% CO_2 mixture. C is then added. To secure constant glucose and antibiotic levels 0.012 cc/min. of the following solution are infused throughout each experi-

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‡ Present address: U. S. Naval Radiological Defense Laboratory (Pharmacology Section), San Francisco, Calif.

1. *e.g.* Kellaway, C. H., *Edinburgh Med. J.*, 1947, v54, 333. Fiessinger, N. and Bernard, H., *Arch. Int. de Pharmacodynamie*, 1934, v49, 398. cf. also 17.

2. Werthessen, W. T., personal communication.

3. Brauer, R. W., and Pessotti, R. L., *J. Pharm. and Exp. Ther.*, 1949, v97, 359.

4. Markowitz, R., Rappaport, A., and Scott, A. C., *Am. J. Dig. Dis.*, 1949, v16, 344.

5. Brauer, R. W., and Pizzolato, P., unpublished data, also Rappaport, A., personal communication, 1950.

6. Brauer, R. W., unpublished data. cf. Salzburg, P., and Watson, C., *J. Biol. Chem.*, 1941, v139, 593.

§ The authors wish to thank Dr. Edwin E. Hayes of Armour and Co. for making this material available in the amounts required for this work.

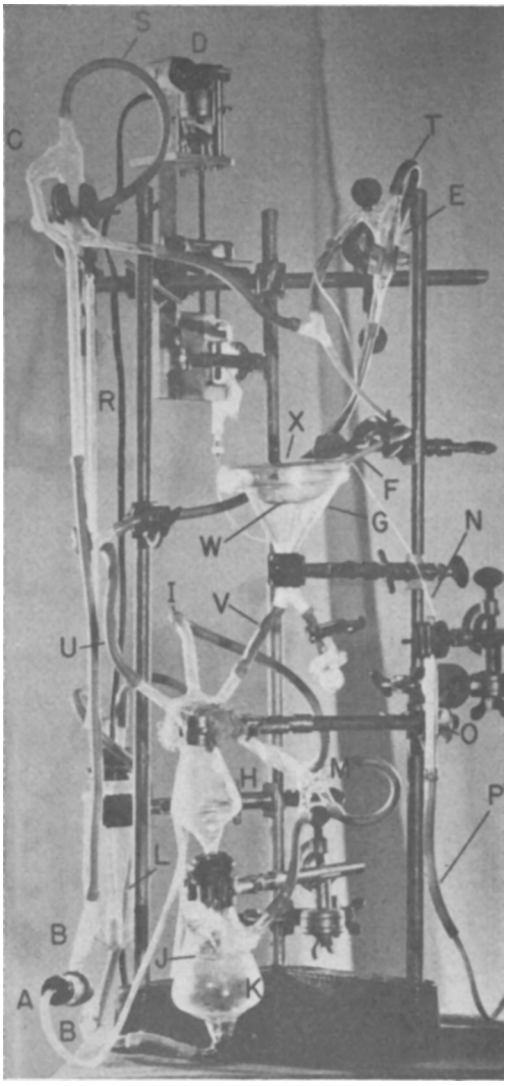

FIG. 1. Apparatus for isolated liver experiments. Perfusate path-K-B-A-B (valves and pulsating rubber finger pump)-C-E-(pressure control heads—open to atmosphere through gauze filters at ends of S and T)-U-H (falling film oxygenator)-J (nylon bag filter)-K. Supply to the liver tapped off between C and E through F. Oxygen-CO_2 passes through saline bubbler L, manifold, and trap M, to line I entering H (gas escapes through capped vent in back of H), also to reservoir K. Continuous infusions are delivered by pump D, catheter, and needle to E. For other letters and details of operation see text.

ment:

D—25 mg terramycin hydrochloride (Pfizer & Co.), 50 mg dextrose, 500 mg crystalline bovine plasma albumin, 92 mg NaCl, 4.2 mg KCl, 1.2 mg $CaCl_2$, made up to 10 cc with distilled water. Where desired the dye sodium tetrabromphenolphthalein disulfonate (BSP)¶ (3 mg/cc) or other drugs are added to this solution. The solutions are sterilized by filtration. Blood is obtained under aseptic conditions by heart puncture.

The perfusion pump used is a modification of those described in references(7) and takes into account the first point made above as well as the reduction in size allowed by the use of rat tissues. The general arrangement shown in Fig. 1 is operated inside a constant temperature cabinet. The circuit consists of a perfusate reservoir K, a modified Carrel-Lindbergh pump, BAB, a pressure and pulse pressure regulating system C-E (note the flaring connections found important for erythrocyte preservation), a falling film aerator H, and a return through a nylon bag filter J to the reservoir. The blood supply to the organ is tapped from the pressure regulated supply between C and E through F, and returns to the system via funnel G and aerator H. The gas mixture is bubbled through saline in the saturator L before being supplied to the oxygenator and the reservoir via M and I. All glass parts of this system except H are coated with silicone‖ for better blood preservation. Sterilization of all parts except A is carried out by autoclaving the assembled system (15 lb, 20 min.). A is cleaned, kept under Zephiran Cl (1:500) for 3 hours or more, flushed with saline and connected into the system with aseptic precautions. Antibiotics and other materials supplied to this circuit continuously are administered by the continuous infusion pump D. The pulsating air stream which operates pump A is supplied by a mechanical pump (*e.g.* the Palmer Ideal Respiration Pump) at about 80 strokes per minute and with such pulsation amplitude as to roughly equalize the perfusate streams

¶ The authors wish to thank Dr. H. B. Dunning of Hynson, Westcott and Dunning for supplying the BSP used in this work.

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‖ The authors are indebted to the General Electric Co., Schenectady, for supplying "Dry Film 9987", the organo silicone used in this work.

flowing through F-V and bypass U; the absolute flow through C may vary within wide limits without affecting the performance of the perfused liver. Blood flow through the organ can be determined by clamping V and noting the time elapsing before the fluid level in G reaches a set mark (corresponding to 10 cc). Bile is collected in a Wintrobe hematocrit tube O; bile flow rates are measured as rates of rise of the fluid column in this tube.

Non-fasted Sprague-Dawley rats were used as blood and liver donors. Livers were removed with sterile technic under sodium pentobarbital anesthesia (39 mg/kg intraperitoneally) after insertion of a catheter** into the bile duct, a cannula into the vena porta, and an outflow catheter (3", 3 mm i.d.—this at once assures free flow, and negative pressures at the hepatic veins) into the thoracic vena cava. The organ is placed diaphragmatic side down into a silicone lined porcelain dish (W) with a 5 mm hole in the bottom through which the return cannula in the vena cava is passed. Peculiar aspects to be noted about the operative technic are the following: (a) The esophagus is resected close to the stomach; this permits retraction of the esophagus into the thorax during the critical last stage of removal of the liver and speedier transfer to the pump. (b) Heparin (0.2 mg) is administered 2 minutes before occlusion of the vena cava; experience has shown that this results in more satisfactory clearing and better survival of the organ. (c) Circulation to the liver is seriously reduced and eventually totally stopped from the moment the portal vein is occluded to the moment the liver is inserted into the perfusion stream; it is important that this ischemic interval be kept as short as possible—3 to 5 minutes is considered satisfactory in this laboratory. Performance and survival are seriously reduced if ischemia exceeds 10 minutes. Samples taken for analysis include bile, drawn from the collecting tube every 15 minutes, or for special purposes collected in fresh tubes for 5 or 10 minute intervals; blood drawn at U for mixed, and at V for hepatic venous samples; and liver specimens obtained at "biopsy" or terminally. Determinations include total plasma proteins (8), blood glucose(9), blood and bile bromsulfalein contents(10), and hematocrits. Bile total solid content is determined by weighing drops of bile placed on polystyrene film,†† drying over silica gel, and reweighing. Volumes of 5 microliters suffice for this determination. Histological examinations here reported were made on formalin fixed sections, paraffin embedded and stained with hematoxylin and eosin.

Results. Isolated livers perfused under the conditions described, quickly recover from the operative ischemia. Within 3 minutes the organs appear uniformly pink. Simultaneously bile formation is resumed. Fig. 2 shows changes in rate of bile formation which are representative of the majority of experiments: an initial recovery period is followed by a relatively brief period of rapid bile flow which terminates after 2 to 3 hours and is succeeded by a prolonged period of less rapid but steady bile production. This phase may last from 5 to 12 hours, and, in turn, leads into the terminal phase of dwindling bile flow. Paralleling the bile flow are changes in appearance of the organ: the first phase is marked by resumption of blood flow and of a uniform, pink and healthy appearance. This is preserved through the succeeding two phases. The terminal phase is accompanied by the development of an increasing number of punctate hemorrhages which may eventually spread over most of the surface of the organ, and by increasing congestion and impaired circulation. Microscopically the first phases are marked by excellent preservation of the tissue, so much so that it is not readily distin-

** The authors are indebted to the Irvington Varnish and Insulating Co. for supplying the Polystyrene plastic tubing (0.025" I.D., 0.004" wall thickness) employed in this work.

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†† The authors are indebted to Dielect, Perth Amboy, N. J. for supplying this material 0.0008" thick.

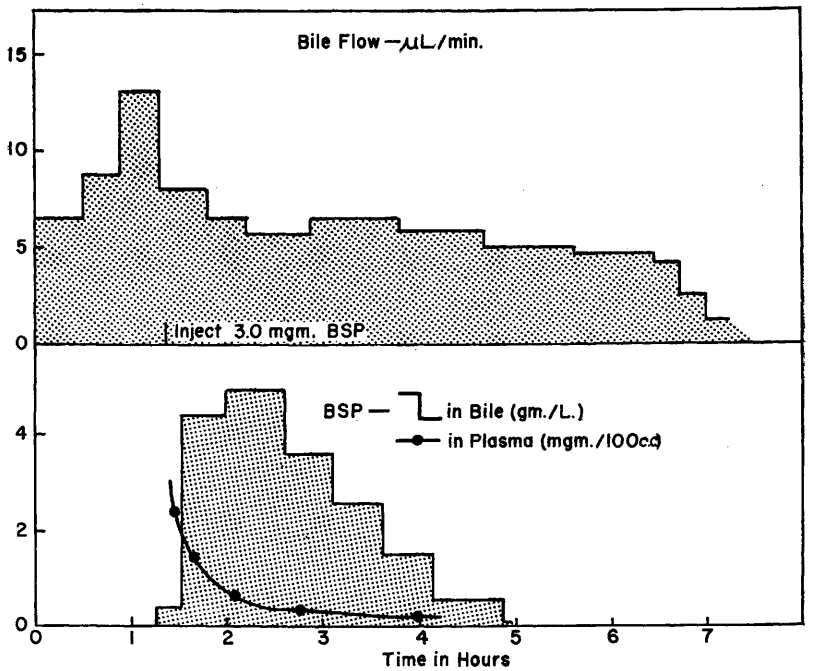

FIG. 2. Representative course of bile production in isolated liver showing four phases of activity, and course of plasma and bile BSP concentration after single BSP injection (Rat 50-50 ♂ 220 g).

guished from freshly cut liver of healthy animals (Fig. 3). Occasionally, cell free spaces are seen, especially around the central vein; these may possibly denote early edema. During the terminal phase, in addition to extravasation and congestion, regions of central necrosis may appear. With improved media there has been some tendency to find relatively intact histology even after cessation of bile flow.

Bile production by the isolated liver is affected by a variety of factors. Some of these, notably perfusion pressure, oxygen tension, pH of the perfusate, and temperature, will be discussed in greater detail in a subsequent communication dealing with the mechanism of bile production. For present purposes it may suffice to indicate that the following is a satisfactory set of operating conditions: Temperature, 37°C; gas mixture, 95% O_2—5% CO_2; perfusate pressure, 11.5 cm H_2O; pH, 7.4. None of these conditions are critical since they appear close to the optima for each variable.

Survival of the isolated liver as indicated by the period of bile production is positively correlated with the body weight of the donor. Thus, rats weighing between 160 and 200 g, yielded livers surviving for a mean period of 4 hours (3.5-4.5 hr), between 200 and 233 g, for 6.6 hours (5.0-7.5 hr), between 233 to 266 g for 8.3 hours (5.3-13 hr), and those between 266 and 300 g for 10.4 hours (8-17 hr). In 3 animals starved for 24 hours before removal of the livers, survival of the isolated organs was significantly shorter than for non-fasted animals of the same weight (290 g, 3.5 hr; 240 g, 3 hr; 240 g, 3.5 hr). On the other hand, enrichment of the medium described above with amino acids and vitamins (to approximate White's(11) medium) resulted in series of exceptionally long survivals ranging from 15 to 30 hours.

Bile secretion rates, concentrations, and pressures are summarized in items 7 and 8 of Table I, showing values for the steady bile flow phase. During the 2nd—the rapid flow phase—both flow rates and solid concentra-

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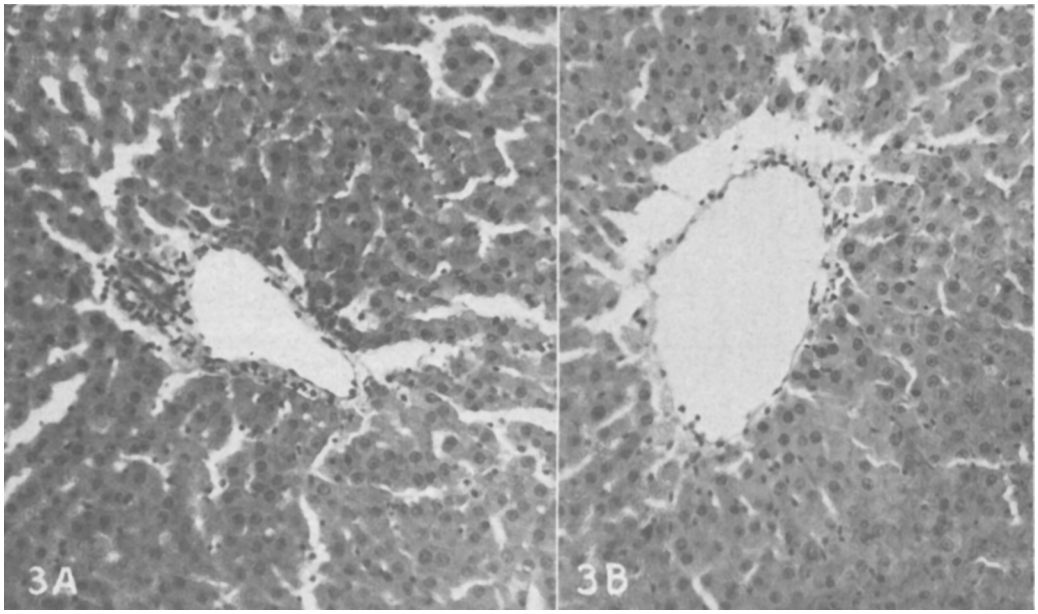

FIG. 3. Liver Rat 50-55 ♀ 300 g, after 24 hr in the perfusion pump. (a) Periportal area showing well preserved hepatic cords with slightly dilated sinusoids. 160 ×. (b) Central vein showing intact hepatic cords and sinusoids. Occasional necrotic cells are found adjacent to the vein. The clear perivascular area is typical and may represent edema. 160 ×.

tions average slightly higher, namely in males 9.4 ± 0.61 μL/min. and 3.4 ± 0.4%, and for females 9.7 ± 0.71 μL/min. and 5.5 ± 0.38%.

Bromsulfalein is extracted from the perfusate by the isolated liver not only after a single injection, but also continuously during infusions. Fig. 2 shows the exponential decay of plasma BSP levels seen in about half of the experiments (a flattened curve such as is seen in infants and in certain types of liver injury is found in the other half); the course of bile BSP concentrations shown is typical of all preparations. Steady plasma and bile BSP levels can be reached in continuous infusion studies (see Table I, last two items). Bromsulfalein is excreted by the isolated liver in at least 3 separable components only one of which, accounting for about 20% of the dye recovered from bile, is indistinguishable from BSP(12).

The reticulo-endothelial system of the isolated liver remains functional; thus, India ink is quickly removed from the perfusate, and numerous engorged Kupffer cells can be found in sections of livers so treated (Fig. 4). Bromsulfalein extraction from the perfusate is reduced by India ink injection as shown in Fig. 5; perfusate BSP concentrations during continuous BSP infusion increase to higher plateaus after India ink administration. Bile flow decreases somewhat but biliary BSP concentrations are not readily affected.

Table I summarizes the performance of the isolated liver in the steady bile flow phase together with some of the relevant operating conditions. For purposes of discussion, comparison values for rat livers *in situ* in intact animals are included.

Discussion. The basic prerequisite for an isolated liver preparation that is to aid the understanding of the intact organism, is that all elements of the tissue must be maintained in a functional state. The most important result of the work here reported has been the demonstration that the isolated liver can form bile, and that bile flow can be maintained for extended periods of time. Conditions which were found compatible with bile formation also could be shown to result in preservation

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TABLE I. Conditions and Performance of Isolated Liver During the Third (Steady Bile Flow) Phase, with Reference Data for the Liver *in situ*.

Description		Isolated liver	Liver, *in situ*
Body wt—g		275 ± 8.9 (30)*	250–310
Plasma vol—cc		72.1 ± 1.27 (30)	9.6–11.8 †
Hematocrit—%		11.3 ± .34 (27)	47 ± .41‡
Plasma protein—g/100 cc		4.8 ± .19 (25)	5.6 ± .4 ‡
Perfusate flow through liver—cc/min		93 ± 5.8 (20)	8–12 §
Glucose in perfusate—mg/100 cc		210 ± 13.7 (18)	115–140 ‡
Bile flow—μL/min	M	6.4 ± .60 (15)	17.3 ± 1.5 ‡
	F	5.1 ± .54 (14)	12 ± .6 ‡
Bile solids—g/100 cc	M	2.7 ± .2 (9)	3.2 ± .33‡
	F	3.6 ± .3 (10)	5.2 ± .29‡
Secretion pressure—cm bile		14.6 ± .82 (8)	20.3 ± 1.1 ‡
Time to remove 50% of a single injection of BSP from circulation—min		17 ± 2.5 (6)	5.6 ± .5 ‖
BSP in plasma (cont. infusion .2 mg/kg/min) —mg/L		24.7 ± 4.7 (6)	17.4 ± 3.1 ‡
BSP in bile (cont. infusion .2 mg/kg/min)—g/L		5.7 ± .59 (14)	4.1 ± .42‡

* No. in parentheses indicate No. of experiments.
† Calculated from data of reference 15.
‡ Data from reference 16.
§ In the absence of data for the rat the data of 17 for blood flow per g liver for man and dog have been used to calculate a reasonable range of values. Actual values are probably somewhat larger in the rat.
‖ Data from reference 3.

of the macroscopic and microscopic anatomy of the tissue, in the maintenance of functional Kupffer cells, and of continued detoxication activity, at least as indicated by the excretion of modified forms of BSP. Thus, evidence is on hand that the above basic condition is met by the preparation here described. The second key question to be answered for such a preparation concerns the "normalcy" of operating conditions and behavior. Table I shows some quantitative comparisons between the present preparation and rat livers *in situ* in intact animals. Items 3 and 5 probably represent the most important differences between the conditions to which a liver is exposed *in situ* and in perfusion. The low hematocrit values are useful in prolonged perfusions, to prevent sludging and liver edema, and to protect erythrocytes against mechanical destruction. Under these conditions high flow rates are required to supply adequate oxygen to the tissue; below 60 cc/min. bile flow and survival alike are impaired. The value of 90 cc/min. is below destructive pressure and flow levels and has been found satisfactory for most studies. Item 9—a key characteristic of BSP extraction curves after single dye injections—probably illustrates one of the few situations where lower perfusate flow rates would result in a closer approach to normal; the flat extraction curves probably result from imperfect utilization of the multi-plate extractor mechanism of the liver at the high throughput rates (3). Items 10 and 11 show that BSP handling *per se* is not seriously impaired in the isolated liver. Items 7 and 8 show that bile

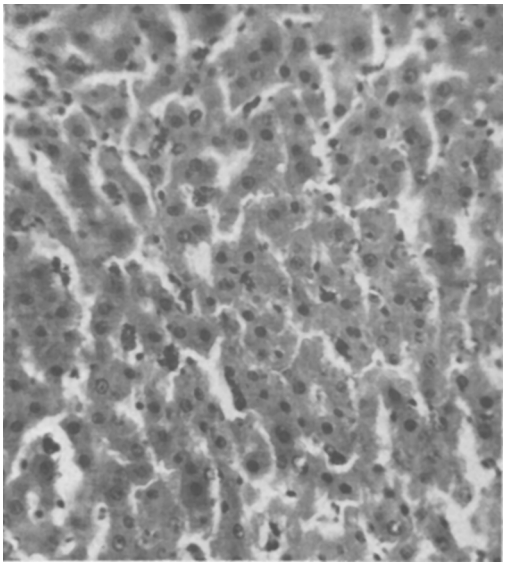

FIG. 4. Rat 50-59, ♀, 338 g. Section of liver six hr after India ink administration showing reticuloendothelial cells containing phagocytized India ink particles. 200 ×.

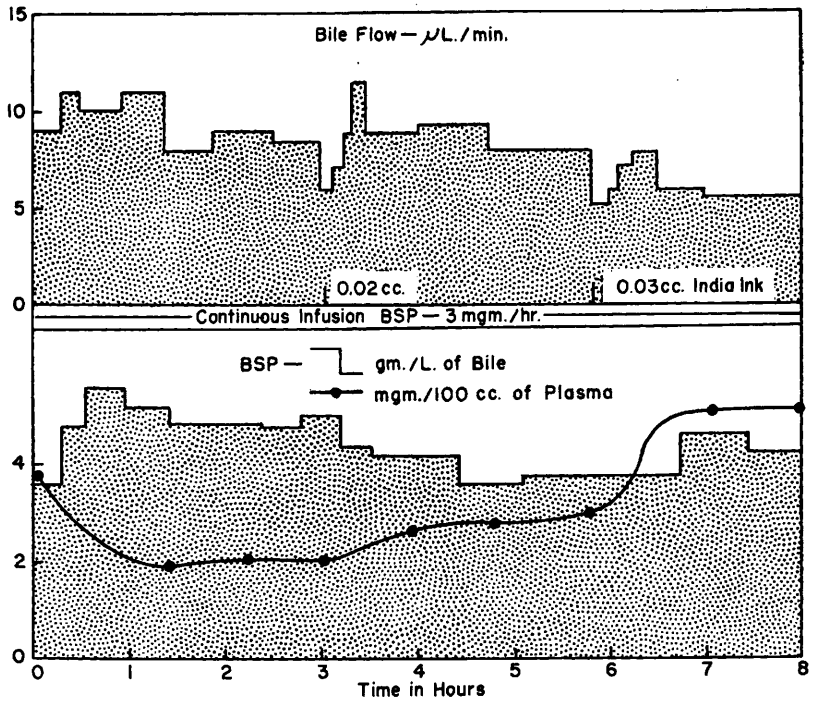

FIG. 5. The effect of India ink on bile flow, BSP uptake and excretion. Rat 50-59, ♀, 338 g.

production by the perfused liver is quantitatively comparable to that in the intact animal. This is especially evident if one recalls that optimal bile production occurs during phase 2 and not during phase 3 upon which Table I is based. The transition between these phases may be due to depletion of circulating bile acids caused by the lack of entero-hepatic circulation in the perfusion system, or to biochemical changes similar to these described by Lundsgaard(13). The glucose and total protein levels maintained (Items 4 and 6) are convenient but non-critical under present operating conditions.

It is felt that the mechanism and procedure here described are such that mechanical factors no longer control the duration of an experiment. All evidence at this time points to depletion of essential tissue components as the factor resulting in transition from phase 3 to phase 4: the relation between body weight of donor and liver survival, the effect of preoperative starvation, and the effect of the addition of nutrients alike point in this direction.

The present preparation is primarily designed for the study of bile formation, of the vascular reactions, and of the excretory mechanisms of the isolated liver. Extended survivals resulting from improvements in procedure should make the perfused liver a useful tool in the study of hepatotoxic agents, of reticulo-endothelial cell turnover and activity, and of nutrition and depletion of the liver.

Summary. (1) It has been shown that the isolated, perfused rat liver can be maintained under conditions which permit the continuous production of bile for periods up to 25 hours. (2) Livers thus maintained preserve an apparently normal histology up to the terminal

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phase. (3) The reticulo-endothelial cells of such perfused livers retain their ability to remove particulate matter from the perfusate stream. (4) Bromsulfalein is extracted from the perfusate by the isolated liver, and is re-excreted into the bile in high concentration and in several separable components, analogous to those found in the intact animal. (5) Data have been presented to permit a comparison of the conditions of operation and the performance of the isolated rat liver, and the liver *in situ* in the intact animal. (6) Details of the method of preparation, the perfusion pump, and the perfusion media have been presented, together with specific data concerning the course of typical experiments. (7) The range of applicability of the preparation in its present state of development and directions of further exploration have been discussed.

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Nucleic Acid Changes in Rat Nerve Tissue After Parenteral Administration of Vitamin B_{12}. (19013)

W. F. ALEXANDER AND B. BACKLAR.

From the Department of Anatomy, St. Louis University School of Medicine, St. Louis, Mo.

The beneficial effects of vit. B_{12} in treating nutritional anemias(1,2) and neurological disturbances(3,4) have been demonstrated. The biochemical mechanisms and the cytological localization of the activity of this vitamin as yet are not fully known. Facilitation of the formation of ribonucleic acid in liver cells protected by large doses of vit. B_{12} against depletion by poisoning with carbon tetrachloride has been reported by Koch-Weser and Popper(5). On the assumption that the intensity of the basophilic reaction indicates the quantity of nucleoprotein present, Stern, Taylor, and Russell(6) studied weanling rat livers stained with methylene blue. These investigators found that there was almost no basophilia in rats on a diet containing soybean oil meal as the only protein source. The addition of vit. B_{12} to this diet produced a decided liver basophilia. Liver extract gave a slightly greater response. In 1950, after our work had begun, Seigel and Worley(7), using basophilia as an index of the presence of nucleic acids, confirmed the results of Stern *et al.* A nucleic acid abnormality in bone marrow cells due to pernicious anemia also has been corrected by local injection of vit. B_{12}(8). The present investigation has been undertaken to determine whether parenteral administration of B_{12} produces definite cytochemical changes in the nucleic acids in nerve tissue in rats. Several stains were used to visualize any possible changes in basophilia, total nucleic acids, pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA).

Materials and methods. Two experiments were carried out. Table I contains a summary of the dietary groups and the quantities of vit. B_{12}* administered. Food consumption records were kept daily and the animals weighed every other day. The basal diet was

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* The vit. B_{12} used in this study was furnished through the courtesy of Mr. A. W. Veazey of Merck & Co.