4. Hoch, F. L., Vallee, B. L., *Analyt. Chem.*, 1953, v25, 317.

5. Slater, E. C., *Sarcosomes (Muscle Mitochondria), Symposia of Soc. for Exp. Biol.*, 1953, v10, p113.

6. Ziegler, D. M., Linnane, A. W., Green, D. E., Dass, C. U. S., Ris, H., *Biochim. Biophys. Acta*, 1958, v28, 524.

Received January 28, 1960. P.S.E.B.M., 1960, v103.

Development of Lipemia in Rats Following Intravenous Injection of India Ink.* (25667)

LAVINA LICKLEY AND FRANK C. MONKHOUSE
Department of Physiology, University of Toronta, Toronto, Canada

In the course of studies of the role of the liver in destruction of clearing factor, Higgins' India Ink was injected into rats. It was observed that blood samples withdrawn one to 2 hours after injection were lactescent. This lactescence was associated with an increase in level of total fatty acids. The lipemia occurred in fasted rats thus indicating that the India Ink was either stimulating an increase in rate of mobilization of fat from the depots or blocking the exit of the mobilized fat from the blood stream. Since the reticuloendothelial system is readily saturated with the India Ink particles, the lipemia could have resulted from inhibition of the normal function of these cells. It has been reported (1) that the reticuloendothelial system functions in uptake of cholesterol from the blood. The evidence(2,3) is against these cells playing any major role in uptake of chylomicrons. The following experiments were carried out in an attempt to determine the mechanism by which India Ink induced lipemia.

Methods and materials. Total fatty acids. The lipids were saponified by heating in presence of alkali and alcohol. The alcohol was removed and the fatty acids were freed by addition of acid in excess. The fatty acids were transferred to a solvent and, after evaporation of the solvent, were titrated by standard alkali. The details were as follows: 0.2 ml of plasma was added to 5 ml of alcohol-ethyl ether mixture (3:1 v/v) and heated to boiling in a water bath. The mixture was then cooled and filtered into a graduated test tube. The residue was reextracted, filtered into the same test tube, and total volume of the extracts made up to 12.0 ml. Five ml of the filtrate was placed in a 15 x 85 mm test tube with screwcap stopper and 0.1 ml of saturated NaOH was added. The mixture was heated in a boiling water bath for 30 min. It was then cooled and while cooling sufficient 33% sulphuric acid solution was added to give an acid reaction. Six ml of petroleum ether was added. The tube was stoppered tightly and shaken vigorously for 1 minute; then 3.5 ml of water was added and the tube shaken again. The mixture was allowed to settle, then two 2 ml aliquots of the clear supernatant were placed in 15 x 85 ml test tubes. The solvent was removed by evaporation and 3 ml of a 0.004% alcohol solution of thymol blue was added. Titration was carried out with 0.02 N KOH. Results are expressed in reference to a standard solution of oleic acid (100 meq/liter).

India Ink. Higgins' India Ink was obtained from the Higgins' Ink Co. Chin Chin India Ink and Pelikan Carbon (C11/1431a) were obtained from the Heinz Jordan and Co. Ltd. These were diluted 3:5 with saline and 1 ml was injected into the exposed jugular vein of each rat. Unless otherwise stated the term India Ink will refer to Higgins' India Ink only.

Rats. The animals used were female rats of the Wistar strain weighing 200-250 g, maintained on a standard chow diet and fasted over-night prior to injection.

Protamine and heparin were supplied by the Connaught Medical Laboratories, Toronto.

Results. In the first experiment India Ink

* This work was supported by a grant from the Ontario Heart Foundation.

TABLE I. Effect of India Ink on Total Fatty Acids in Plasma of Fasted Rats.

Saline inj.		India Ink inj.	
No. of animals	Fatty acids, mg %	No. of animals	Fatty acids, mg %
32	154 ± 43	37	380 ± 99

was injected into a group of rats and blood samples withdrawn by cardiac puncture at various times after injection. Total fatty acid content of each sample was measured. Lipid levels were increased at 30 minutes and reached a maximum at 60 to 90 minutes after injection. Levels were usually normal by 3 hours after injection. In subsequent experiments, unless otherwise stated, blood samples were taken between 60 and 90 minutes after injection of India Ink.

Table I shows a summary of the results from 70 rats, 32 of which received an injection of saline and 37 an injection of India Ink. There is obviously a highly significant increase in total fatty acids in the animals receiving the India Ink over the saline-injected controls.

Different groups(4,5) have reported that protamine injections cause lipemia in fasted rats. Experiments were carried out to compare development of lipemia following injection of India Ink with that following injection of protamine. For each experiment 16 rats were used, divided into 4 groups of 4 animals each. One group received saline, one group India Ink, one group protamine and the fourth group India Ink and protamine. All solutions were given intravenously *via* the exposed jugular vein, and blood samples taken by cardiac puncture. This experiment was repeated 3 times and the combined results of the 3 experiments are summarized in Fig. 1. Of the 48 rats, 6 died during cardiac puncture and insufficient blood was obtained from them for duplicate measurements. It is obvious from the results that India Ink caused a greater degree of lipemia than protamine and that the 2 together caused a more intense lipemia than either substance alone. This suggests that they caused lipemia by different mechanisms.

It was the opinion of Bragdon and Havel (4) that the protamine-induced lipemia was a result of inhibition of clearing factor. It was of interest, therefore, to determine whether or not heparin injected at the peak of the India Ink-induced lipemia would clear the

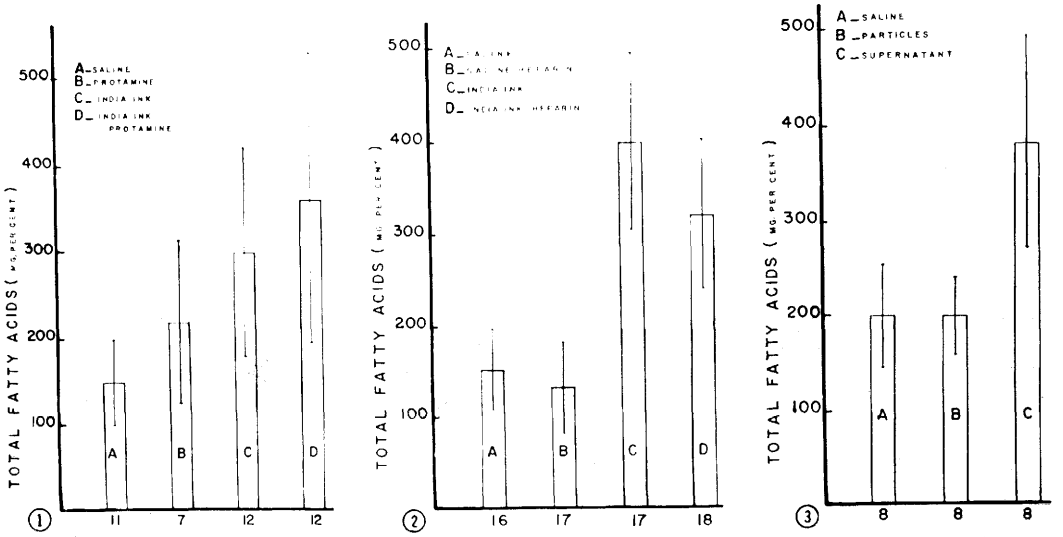

FIG. 1. Total fatty acid level in rat blood following intrav. inj. of various solutions. All samples taken at 60 min. after inj. Mean values and stand. dev. are shown. The number under each column indicates total No. of animals in each group.

FIG. 2. Effect of intrav. heparin on the lipemia caused by inj. of India Ink. Mean values and stand. dev. are shown. Number under each column indicates total No. of animals in each group.

FIG. 3. Comparison of effects of particulate matter and supernatant from India Ink on fatty acid level of blood. Ink was centrifuged at 90,000 g for one hr. Mean values and stand. dev. are given. Number under each column indicates total No. of animals in each group.

plasma and reduce lipemia. Rats were injected with India Ink and 60 minutes later were given 50 units of heparin intravenously. Thirty minutes after heparin injection blood samples were taken. The rats were divided into 4 groups. One group received saline at zero time and 60 minutes later, one group received saline at zero time and heparin 60 minutes later, one group received India Ink at zero time and saline 60 minutes later, and the fourth group received India Ink at zero time and heparin 60 minutes later. Blood samples were taken by cardiac puncture 90 minutes after start of experiment. The combined results of 3 separate experiments are summarized in Fig. 2. The rats which received saline and heparin showed a mean total fatty acid level slightly lower than rats which received only saline. This difference was not statistically significant. Heparin did significantly ($p>0.01$) reduce the lipemia caused by India Ink. This reduction, even after 30 minutes, is far from sufficient to restore the blood level of fatty acids to normal. In many instances the lactescence had disappeared completely. As stated earlier, the lipemia lasted a relatively short time. Microscope examination of liver sections showed that the Kupfer cells were loaded with carbon particles for many hours, even days, after injection. This raised the question whether the lipemia was caused by the carbon particles or by some ingredient or ingredients in the solvent part of the Ink. Pelikan Carbon made up in saline and gelatin(6) did not cause lipemia though it was taken up by the liver as well, if not better, than carbon from India Ink. India Ink was centrifuged in a Spinco high speed centrifuge at 90,000 rpm for 1 hour. The supernatant was pipetted off and the particles of carbon were resuspended in saline. The 2 solutions were injected into rats and the blood level of fatty acids measured one hour later. Results are shown in Fig. 3. It is obvious that the agent responsible for the lipemia was in the supernatant. The president of the Higgins' Ink Co. was kind enough to give us information on materials used in manufacture of their ink. Various combinations of these ingredients were tried. It was found that crude shellac (orange) dissolved in 2% sodium borate caused lipemia in rats of approximately the same degree as the supernatant from the India Ink. Sodium borate alone had no effect on lipid levels.

Discussion. Commercial India Ink has been used(7,8) and continues to be used(9), to saturate and thereby inhibit the phagocytic system within the animal body. Often the conclusions reached from such experiments have disregarded any effects caused by materials in the Ink other than carbon. Halpern *et al.*(6) showed that the shellac in commercial India Ink reacted with certain plasma proteins which, in turn, significantly altered uptake of carbon. Our results show that intravenously injected commercial India Ink can cause a significant increase in fat content of the blood. This lipemia-producing effect also appears to be due to the shellac content of the India Ink. The mechanism by which the shellac induces lipemia has not been determined, but differs from that of protamine. Protamine being an antiheparin inhibits both release of clearing factor(10) and its activity (4). Commercial India Ink does not inhibit release of clearing factor. If anything it prolongs the *in vivo* action of clearing factor by interfering with its destruction. When heparin was injected at the peak of India Ink-induced lipemia, lactescence was much more markedly decreased than total fatty acid level. This difference is greater than when alimentary lipemia is cleared by injection of heparin. It suggests that the India Ink (due to its shellac content) interferes with transport of fat out of the blood. Much more work has yet to be done to be certain of this. Most important to be considered here, we believe, is the demonstration that a lipemia can be produced rapidly by a method hitherto unused. In view of the current emphasis on fat metabolism and its relation to atherosclerosis any addition to the armamentarium for study in this field should be carefully considered.

Summary. Intravenous injection of commercial India Ink in rats caused a significant increase in total fatty acid content of the blood. The effect of a single injection lasted from 2 to 4 hours. The lipemia appeared to be caused by the shellac in the commercial ink. Pelikan Carbon made up in saline and

gelatin did not cause lipemia, though it readily saturated the reticular endothelial system. The mechanism by which the shellac induces lipemia is obviously different from that of protamine. India Ink does not prevent *release* of clearing factor (lipoprotein lipase). Heparin injected at peak of the India Ink-induced lipemia reduces the lactescence much more than it reduces total fatty acid level.

We wish to thank Dr. J. Campbell for his advice concerning determination of blood lipids.

1. Friedman, M., Byers, S. O., Roseman, R. H., *Am. J. Physiol.*, 1954, v177, 77.

2. Waddell, W. R., Geyer, R. P., Clark, E., Stare, F. J., *ibid.*, 1954, v177, 90.

3. Di Luzio, V. R., *Fed. Proc.*, 1959, v18, 37.

4. Bragdon, J. H., Havel, R. J., *Am. J. Physiol.*, 1954, v177, 128.

5. Scholtz, M. C., Page, I. H., *Circulation*, 1959, v16, 479.

6. Halpern, B. N., Benacerraf, B., Biozzi, G., *Brit. J. Exp. Path.*, 1953, v34, 426.

7. Brichner, R. M., *Bull. Johns Hopkins Hosp.*, 1921, v40, 90.

8. Markham, N. P., Florey, H. W., *Brit. J. Exp. Path.*, 1951, v31, 25.

9. Selye, H., Lemire, Y., Cantin, Marc, *Med. Experimentalis*, 1959, v1, 11.

10. Monkhouse, F. C., McClain, Florence, Baker, D. G., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 50.

Received January 29, 1960. P.S.E.B.M., 1960, v103.

Effects of L-Norepinephrine on Cardiac Metabolism of Dogs in Hemorrhagic Shock.* (25668)

DONALD B. HACKEL

Dept. of Pathology, Western Reserve University School of Medicine, Cleveland Metropolitan General Hospital, O.

Earlier studies in this laboratory have demonstrated a biochemical abnormality of the hearts of dogs in hemorrhagic shock, manifested mainly by a negative extraction of pyruvate by the myocardium. It was also shown that a high per cent of these dogs developed subendocardial hemorrhage and necrosis(1). Since it was hypothesized that these deleterious changes might be partly related to increased secretion of epinephrine during shock, it was felt that detailed studies of the effects of sympathomimetic substances in shock were indicated. We have previously shown that l-norepinephrine does not increase survival rate when given to dogs 10 minutes or longer after production of hemorrhagic hypotension (2), and we have further shown that incidence of myocardial lesions is greater in animals so treated(3). The present report is an extension of the earlier reports, and will be concerned with the effects of l-norepinephrine treatment on some aspects of the myocardial metabolism of dogs in hemorrhagic shock.

Methods. The methods briefly described here have been previously described in detail (1,2). Eighteen mongrel dogs were premedicated with subcutaneous morphine sulfate (3 mg/kg body weight) and anesthetized with equal parts of Dial-urethane† and sodium pentobarbital (0.25 ml of this mixture/kg body weight) given intravenously. Each dog was given 200,000 units of penicillin and 0.2 g of streptomycin intramuscularly, and 5 mg/kg of heparin intravenously before the experiment. The aorta, pulmonary artery and coronary sinus were catheterized *via* the femoral artery and jugular veins, respectively, under fluoroscopic control. Systemic arterial and coronary sinus blood samples were analyzed for O_2 and CO_2 (Van Slyke manometric technic), pyruvate(4), lactate(5) and glucose(6). Samples were drawn during the initial control period following which the dogs were bled from the femoral artery over a 10 minute pe-

* This work was supported by grants from U. S. Public Health Service and Cleveland Area Heart Soc.

† The Dial-urethane was generously supplied by CIBA Pharmaceutical Products, Inc., Summit, N. J.