months average age. 2. The incidence of leukemia was higher in mice inoculated intraperitoneally (89%) than in those injected subcutaneously (69%). 3. Of 122 young, adult C3H mice inoculated intraperitoneally, or intracranially with leukemic filtrates, 37% developed leukemia (none parotid tumors) at 8.8 months average age. 4. Of 310 newborn C3H mice inoculated when less than 16 hours old with the same leukemic filtrates, 64% developed either leukemia (56%), or parotid tumors (8%), at 3.8 months average age. Two mice in this group developed both leukemia and parotid tumors.

1. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 27.
2. ———, *ibid.*, 1951, v78, 342.
3. Woolley, G. W., Small, M. C., *Cancer*, 1956, v9, 1102.
4. Furth, J., Buffett, R. F., Banasiewicz-Rodriguez, M., Upton, A. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 165.
5. Dulaney, A. D., Maxey, M., Schillig, M. G., Goss, M. F., *Cancer Research*, 1957, v17, 809.
6. Hays, E. F., Simmons, N. S., Beck, Wm. S., *Nature*, 1957, v180, 1419.
7. Gross, L., *Ann. N. Y. Acad. Sci.*, 1957, v68, 501.
8. ———, PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 767.
9. ———, *ibid.*, 1955, v88, 64.
10. ———, *ibid.*, 1953, v83, 414.
11. ———, *Cancer*, 1953, v6, 153.
12. Friend, C., *J. Exp. Med.*, 1957, v105, 307.
13. Burmester, B. R., Cottral, G. E., *Cancer Research*, 1947, v7, 669.
14. Eckert, E. A., Beard, D., Beard, J. W., *J. Nat. Cancer Inst.*, 1955, v15, 1195.
15. Beaudreau, G. S., Bonar, R. A., Beard, D., Beard, J. W., *ibid.*, 1956, v17, 91.
16. Gottschalk, R. G., *Cancer Research*, 1946, v6, 270.
17. Bryan, W. R., *J. Nat. Cancer Inst.*, 1955, v16, 285.

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## Plasma Heparin Levels in Man Following Intravenous Fat Emulsions. (23723)

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Clearing of alimentary lipemia(1) and decrease in serum low density lipoproteins(2) following injection of heparin have raised the question of the possible physiologic role of heparin in the blood transport phase of fat metabolism. The concept has been strengthened by identification(3) of the tissue enzyme, lipoprotein lipase, of which heparin is probably a component(4), and demonstration (5) of a similar lipolytic enzyme in the plasma of some normal individuals. The inverse correlation found between human plasma heparin levels and serum low density lipoproteins(6) added further evidence. This report will present data showing an increase in the level of plasma anticoagulant substances (heparins) following administration of intravenous fat emulsions in man, indicating that fat intake is a stimulus for release of heparin into the bloodstream.

In preliminary experiments(7) increase in plasma metachromatic substances was found in 6 of 10 individuals following intravenous injection of cottonseed oil emulsion. The method used for extraction and separation of endogenous circulating heparin in the earlier study was a modification of the technic suggested by Nilsson and Wenckert(8) in which heparin is adsorbed on tricalcium phosphate gel, then eluted with citrate, and the octylamine-brucine method of Jaques(9) applied to the eluate. However, the use of this method of heparin extraction was not continued since other metachromatic substances, such as chondroitin sulphate, may be present in blood. Moreover, it was found that the octylamine

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precipitation of endogenous heparin, whether protein-bound or after preliminary tryptic digestion of the protein, varied in its completeness. Thus the investigation of the effect of intravenous fat on plasma heparin had to be repeated using a more reliable method. Furthermore, a disruption of mesenteric mast cells was observed in rats following intraperitoneal injection of fat emulsions containing phosphatide stabilizers(10). This raised the possibility, albeit improbable, that the increase observed in circulating heparin values in man after administration of intravenous fat emulsions containing soybean phosphatides was the result of mast cell damage with consequent heparin release rather than a normal physiologic response to fat intake. This necessitated the use of control emulsions containing phosphatides but no fat. The control studies were additionally valuable since they were also a check upon the contingency that variations in heparin levels resulted from the emotional stress associated with the experimental procedure.

*Procedure and methods.* The fat emulsion used was Lipomul† which contains 15 g cottonseed oil, 4 g glucose, 1.2 g soybean phosphatide and 9.3 g Pluronic F 68 per 100 ml. The control emulsion was identical in all respects except for the absence of the cottonseed oil. Intravenous injections were given at a rate of 20-25 drops per minute for one hour (approximately 100 ml) and then discontinued. During administration of the fat there was a slight rise in temperature in 2 individuals, but no other reactions occurred. There was no systemic evidence of hemolysis with this small total dose of intravenous fat although slight hemolysis was noted during separation of the plasma in a few instances when the lipemia was maximal. (Hemolysis was not a possible source of error in these studies since we have found on several occasions that heparin was not present after the hemolysis of washed erythrocytes.) Hospitalized patients were used in this study. No subjects were selected who had evidence of fat metabolic or hematologic disorders, and none had received heparin. Blood was drawn in the post-absorptive state just prior to the onset of the intravenous emulsion, 15 minutes and one hour after it was started, and approximately one hour after it was discontinued. It was placed in oxalated tubes, the plasma was immediately separated, and duplicate analysis of 5 ml aliquots was started the same day. The procedure used for extraction and assay of endogenous circulating heparin has been previously published(11). The method is based upon tryptic digestion of total serum or plasma proteins previously precipitated by methanol-acetone, dialysis after denaturation of the enzyme, and subsequent lyophilization or evaporation. The anticoagulant assay is performed as previously described(12) using recalcified sheep plasma according to the U.S.P. #14 technic. However, since the final extract after dialysis is at neutrality, no adjustment of pH is necessary. The evaporated (or lyophilized) material is dissolved in 1.0 ml of isotonic saline for the anticoagulant assay. Determination of the clotting endpoint is facilitated when evaporation rather than lyophilization has been used.

*Results.* The findings are presented in Table I. Fourteen individuals received both Lipomul and the control solution (Lipomul without fat), and in 4 additional subjects only Lipomul was given. The averages of duplicate analyses are shown. The maximum deviation from the mean encountered in this study was 1 unit per cent, or 11% from the mean. Accordingly changes in heparin differing less than 1 unit % from the fasting level were considered possibly due to variations in the method.

Of the 14 subjects who received both intravenous solutions, 9 showed increases in circulating heparin after Lipomul and in 4 of these heparin levels were also increased after the control injection. Twelve of the total number of 18 patients had increased plasma heparin levels after intravenous Lipomul, in one patient (L.A.) apparently a decrease occurred. The increase usually was found in the 15 minute sample, but occasionally did not appear until one hour after the intravenous was begun. In one patient the rise in heparin

† Generously supplied by Dr. E. A. Hawk of Upjohn Co., Kalamazoo, Mich.

TABLE I. Endogenous Plasma Heparin Values before and after Intravenous Lipomul, with and without Fat, in 18 Patients.

| Age | Sex | Time of blood sample | Heparin levels in units, % Lipomul | Control | Age | Sex | Time of blood sample | Heparin levels in units, % Lipomul | Control |
|---|---|---|---|---|---|---|---|---|---|
| 23 | ♀ | Before I-V | 12.3 | 12.5 | 44 | ♀ | Before I-V | 10 | 10.5 |
| | | I-V 15′ | 14.3 | 12.7 | | | I-V 15′ | 9.5 | 10.1 |
| | | ″ 60′ | 12.7 | 11 | | | ″ 60′ | 9.3 | 9.0 |
| | | 60′ after I-V | 12.2 | 11.7 | | | 60′ after I-V | 10 | 11.2 |
| 61 | ♂ | Before I-V | 10.6 | 9.6 | 26 | ♀ | Before I-V | 9.5 | 9.5 |
| | | I-V 15′ | 9 | 9.6 | | | I-V 15′ | 12 | 9.2 |
| | | ″ 60′ | 9 | 9.2 | | | ″ 60′ | 12.5 | 9 |
| | | 60′ after I-V | 9 | 9.7 | | | 60′ after I-V | 11.8 | 8.3 |
| 64 | ♀ | Before I-V | 10 | 8.8 | 28 | ♀ | Before I-V | 8.2 | 9.2 |
| | | I-V 15′ | 9.5 | 8.2 | | | I-V 15′ | 8 | 8.2 |
| | | ″ 60′ | 10.2 | 8.2 | | | ″ 60′ | 9.5 | 7.8 |
| | | 60′ after I-V | 11 | 8.2 | | | 60′ after I-V | 9 | 9.5 |
| 52 | ♂ | Before I-V | 10.2 | 10.2 | 66 | ♀ | Before I-V | 10.2 | 11.7 |
| | | I-V 15′ | 12.5 | 12.2 | | | I-V 15′ | 11.8 | 11.2 |
| | | ″ 60′ | 11 | 10 | | | ″ 60′ | 11.5 | 12 |
| | | 60′ after I-V | 12.5 | 10.2 | | | 60′ after I-V | 11.2 | 11.7 |
| 52 | ♀ | Before I-V | 9 | 10.5 | 61 | ♂ | Before I-V | 11.5 | 10.9 |
| | | I-V 15′ | 11.5 | 12.5 | | | I-V 15′ | 11.7 | 10.5 |
| | | ″ 60′ | 9.5 | 10 | | | ″ 60′ | 11.5 | 10.7 |
| | | 60′ after I-V | 10.2 | 10.8 | | | 60′ after I-V | 11.5 | 11.5 |
| 57 | ♂ | Before I-V | 11.2 | 12.2 | 43 | ♀ | Before I-V | 10.5 | 11 |
| | | I-V 15′ | 11 | 10.5 | | | I-V 15′ | 14 | 13 |
| | | ″ 60′ | 10.5 | 10.2 | | | ″ 60′ | 13.5 | 11.7 |
| | | 60′ after I-V | 10.5 | 9.5 | | | 60′ after I-V | 10 | 11.5 |
| 71 | ♂ | Before I-V | 11.5 | 10 | 46 | ♂ | Before I-V | 10.5 | 11.2 |
| | | I-V 15′ | 13.7 | 11.7 | | | I-V 15′ | 9.6 | 11.2 |
| | | ″ 60′ | 13 | 13 | | | ″ 60′ | 10.5 | 11.3 |
| | | 60′ after I-V | 9.5 | 11 | | | 60′ after I-V | 10.5 | 11.5 |
| 40 | ♀ | Before I-V | 10.8 | | 67 | ♂ | Before I-V | 9.5 | |
| | | I-V 15′ | 11.5 | | | | I-V 15′ | 11.5 | |
| | | ″ 60′ | 10.3 | | | | ″ 60′ | 13.7 | |
| | | 60′ after I-V | lost | | | | 60′ after I-V | 14 | |
| 78 | ♂ | Before I-V | 13.5 | | 54 | ♂ | Before I-V | 12.2 | |
| | | I-V 15′ | lost | | | | I-V 15′ | 15 | |
| | | ″ 60′ | 14.5 | | | | ″ 60′ | 12 | |
| | | 60′ after I-V | 13.2 | | | | 60′ after I-V | 9.5 | |

was found after the intravenous infusion was discontinued. In some individuals the increase in heparin was maintained after the fat drip was discontinued; in others it was not. In 2 cases, when a rise in heparin was found during the infusion of Lipomul, the level one hour after cessation of the intravenous was markedly below the fasting level. It is also of interest that in 3 of 4 patients in whom heparin levels rose after the control injection of Lipomul without fat, the increase was not sustained as it was in the individuals after Lipomul.

A statistical analysis of the results is shown in Table II. In the 14 subjects who received both Lipomul and the control injection the different incidence (9:4) of increased circulating heparin is almost statistically significant. When the entire group of 18 individuals is analyzed, the results are statistically significant, the difference in proportions (12 of 18 : 4 of 14) being more than twice its standard error. Chi square analysis of the number of increased and decreased heparin levels in the 18 patients after Lipomul (12:1) shows that these results are definitely significant. It is also noteworthy that in 8 of the 12 patients in whom a rise in heparin levels after Lipomul was found, the increase was from 2-4.5 units %, or at least

TABLE II. Summary of Data and Statistical Analysis.

| | No. | >Heparin after Lipomul | >Heparin after control | Statistical method | Results | Confidence level, % |
|---|---|---|---|---|---|---|
| Patients who had I-V Lipomul and control (Lipomul without fat) | 14 | 9 of 14 | 4 of 14 | $Chi^2$ test | 3.6 | Almost 5 |
| | | | | S.E. of diff. in proportions | .188 | |
| | | | | | | „ |
| | | Avg 21% | Avg 18% | Diff. in proportions | .357 | |
| Total No. of patients who received I-V Lipomul | 18 | 12 of 18 | 4 of 14 | $Chi^2$ test | 4.6 | 5 |
| | | | | S.E. of diff. in proportions | .179 | 5 |
| | | Avg 22% | | Diff. in proportions | .381 | |
| | | | <Heparin after Lipomul | | | |
| | | | 1 of 18 | $Chi^2$ test | 8 | 1 |

twice the maximum deviation from the mean.

*Discussion.* The isolation of metachromatic substances from the blood, or evidence obtained by the use of protamine titration technics, does not prove the presence of circulating heparin. However, the demonstration of biologic (anticoagulant) activity of plasma extracts in normal humans is more convincing. This was independently accomplished several years ago by two groups(8,12) of investigators. Furthermore, electrophoresis on toluidine blue paper of the final extract obtained with the extraction method described in this study showed that it contained a metachromatic substance having the same mobility as commercial heparin(11). There are other acid mucopolysaccharides, chondroitin sulphate and beta-heparin, present in normal serum(13) and probably in the end-product we obtain. However, chondroitin sulphate has no anticoagulant activity, and beta-heparin possesses anticoagulant activity only with the thrombin method of titration. It has merely trace anticoagulant activity when tested on ox blood *in vitro,* in dogs and cats *in vivo,* or by the U.S.P. 14 method for heparin assay(14). Since the latter is the assay technic employed in this study, and the extract is dialyzed to remove false activity, it is reasonable to believe that the final titration is specific for biologically active anticoagulant heparin.

Lipoprotein changes have been demonstrated in humans following intravenous fat emulsions(15) that are identical with those induced by the injection of heparin in normal persons during alimentary hyperlipemia. It was subsequently found that plasma lipemia clearing and lipolysis was increased in some individuals after intravenous(16,17) and oral fat intake(18). The enhanced activity, when present, was identified by inhibitor studies as lipoprotein lipase(17,18). The results reported in this paper of an average increase of 22% in circulating heparin after intravenous fat in 12 of 18 individuals conform with and extend these previous observations.

It was observed in this study that changes in the level of lipemia clearing activity do not necessarily parallel the fluctuations in circulating heparin. Since heparin is apparently a component of lipoprotein lipase(4), this observation appears paradoxical. Evidence has been presented, however(17,18), which suggests that lipemia clearing activity is inactivated during the clearing process. The quantity of circulating clearing factor demonstrable at any given moment is then a resultant of its stimulation and production on the one hand, and its inactivation by the liver (19) and the clearing process itself on the other. This may account for the fact that endogenous plasma lipoprotein lipase is not found in many individuals.

The site(s) at which lipolysis of neutral-fat containing lipoproteins occurs *in vivo* is

not clear. Lipoprotein lipase activity has been demonstrated in tissue(3) and in the bloodstream(5.16). The serum enzyme activity as measured *in vitro* accounted for the clearing of only 5% of the fat actually removed from the blood(16), and the authors therefore postulated that tissue clearing factor was primarily responsible. However, circulating lipoprotein lipase activity is usually more abundant in plasma rather than serum(20), may be inactivated by the process of lipolysis as discussed above, and the maximum demonstrable in a test tube *in vitro* where the end products of the reaction are not removed is almost certainly the minimum which occurs in the bloodstream. Moreover, in several individuals as much as 5-6 milliequivalents per liter per hour of unesterified fatty acids was released upon incubation of lipemic plasma at 37°C *in vitro*(21). The fairly rapid release of additional heparin into the blood after intravenous fat suggests but does not prove that the lipolysis of chylomicra occurs predominantly in the plasma.

Since an increase in heparin levels was found in 12 of 18 subjects after Lipomul, and in only 4 of 14 after the control injection which also contained the soybean phosphatide, the latter appears to have been eliminated as the major cause of the rise in circulating heparin through its toxic effect on mast cells (10). Furthermore, emotional factors cannot be implicated. It seems reasonable that the results obtained were primarily a consequence of the rapid entrance of fat into the blood.

*Summary.* 1. Plasma heparin levels were determined in duplicate in 18 individuals before, during and after intravenous infusion of cottonseed oil emulsion, and in 14 of the group after control injection without fat. 2. There was an average increase of 22% in circulating heparin in 12 of the 18 subjects after the fat emulsion; an average increase of 18% in 4 of 14 patients after non-fat emulsion. 3. The results indicate that fat intake is a stimulus for the release of heparin into the bloodstream.

1. Hahn, P. F., *Science,* 1943, v98, 19.
2. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yakley, A., Simonton, J., White, S., *Circulation,* 1951, v4, 666.
3. Korn, E. D., *Science,* 1954, v120, 399.
4. ———, *J. Biol. Chem.,* 1957, v226, 827.
5. Engelberg, H., *ibid.,* 1956, v222, 601.
6. ———, *Acta Medica Scand.,* 1955, v151, 161.
7. Unpublished data.
8. Nilsson, I. M., Wenckert, A., *Acta Med. Scand.,* 1954, v150, Supp. 297.
9. Jaques, L. B., Monkhouse, F. C., Stewart, Mary, *J. Physiol.,* 1949, v109, 41.
10. Shoulders, H. H., Meng, H. C., *Fed. Proc.,* 1957, v16, 371.
11. Engelberg, H., Dudley, A., Freeman, L., *J. Lab. Clin. Med.,* 1955, v46, 653.
12. Freeman, L., Engelberg, H., Dudley, A., *Am. J. Clin. Path.,* 1954, v24, 599.
13. Bollet, A. J., Seraydarian, M. W., Simpson, W. F., *J. Clin. Invest.,* 1957, v36, 1328.
14. Yamashiva, I., *Acta Chem. Scand.,* 1954, v8, 1316.
15. Herbst, F. S. M., Lever, W. F., Waddell, W. R., *Science,* 1956, v123, 843.
16. Cleland, W. W., Iacono, J. M., *Fed. Proc.,* 1957, v16, 383.
17. Engelberg, H., in press.
18. ———, *J. Applied Physiol.,* 1957, v11, 155.
19. Jeffries, G. H., *Quart. J. Exp. Physiol.,* 1954, v39, 261.
20. Engelberg, H., *Am. J. Physiol.,* 1955, v181, 309.
21. ———, *Metabolism,* Nov. 1957, in press.

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