Biochemical Pharmacology, 1961, Vol. 7, pp. 271–276. Pergamon Press Ltd., Printed in Great Britain.

BLOOD LEVELS AND CLEARING ACTIVITY OF A SYNTHETIC HEPARINOID

L. LEVY and G. E. CRONHEIM

Riker Laboratories, Inc., Northridge, Calif.

(*Received* 16 *February* 1961)

Abstract—A new synthetic heparinoid, sulfopolyglucin (SPG), is compared with heparin with regard to clearing activity and anticoagulant activity. The anticoagulant activity of SPG follows a curve similar to blood radioactivity and metachromasia measurements. SPG differs from heparin in time of appearance and duration of elevated clearing factor levels in the blood. Some theoretical aspects of the clearing factor differences are discussed.

IN RECENT years it has been found that heparin as well as many heparinoids are capable of clearing the plasma of visible lipemia in man and in animals.[1] This clearing action has been attributed to an enzyme called "clearing factor" or "lipoprotein lipase". It is believed that this enzyme is normally present in the blood in small amounts, but that upon injection of heparin or heparinoids, the amount in the blood increases many-fold. Considerable work by many investigators has gone into characterizing this enzyme, but at the present time little is known biochemically aside from some requirements for its activity and its localization in tissue.

Sulfopolyglucin (SPG) is a sulfated polysaccharide of 5–15 glucose units connected by α-1:4- and α-1:6-linkages with up to 3 sulfate groups per glucose unit. The SPG discussed in this paper has approximately 2·5 sulfate units per glucose residue. SPG resembles herapin in that both compounds have anticoagulant and lipemia-clearing activity.[2] However, quantitative differences exist in the activity and the metabolism of these two substances, as will be shown in this paper.

METHODS

Mongrel dogs were used throughout, and at least four dogs were used for each dose reported. All injections of heparin sodium USP (Lipo-Hepin Riker, 140 USP units/mg) and of SPG, as the potassium salt, were given intravenously, unless specifically noted. Radioactive SPG containing ^{35}S and non-radioactive SPG (21 USP anticoagulant units/mg) were synthesized in this laboratory and had a specific activity between 10^5 and 10^6 counts/min per mg. Plasma samples used for radioactivity measurements were either directly plated or digested with Pirie's reagent, precipitated as $BaSO_4$, and then plated. All counts were made on a Nuclear Chicago Gas Flow Counter at infinite thinness. The injection solutions were treated for radioactive measurements in the same way as the blood and urine samples, in order to make valid comparisons. Clearing activity was measured by a modified Grossman procedure using Ediol as the substrate.[2] Anticoagulant activity was measured by the Lee–White whole blood-clotting time. The spectrophotometric measurement of SPG used a modified Jaques

metachromasia procedure.[3] Ascending chromatography was used with water–ethanol–ammonium hydroxide (54:45:1) as the solvent. The chromatograms were developed with Azure A or sodium rhodizinate. These chromatograms were subsequently placed in cassettes with Kodak no screen X-ray film and developed according to conventional methods.

RESULTS

In order to determine how rapidly SPG left the blood, radioactive material was injected intravenously into dogs and blood samples were taken periodically for various measurements. In Fig. 1 is presented the results of a group of experiments using dogs (anesthetized with pentobarbital) which were given saline infusions, in order that there would be a urine flow of at least 1 ml/min. Ten milligrams of radioactive SPG per kg and all other doses (1, 3 and 5 mg/kg) given in this series of experiments had a

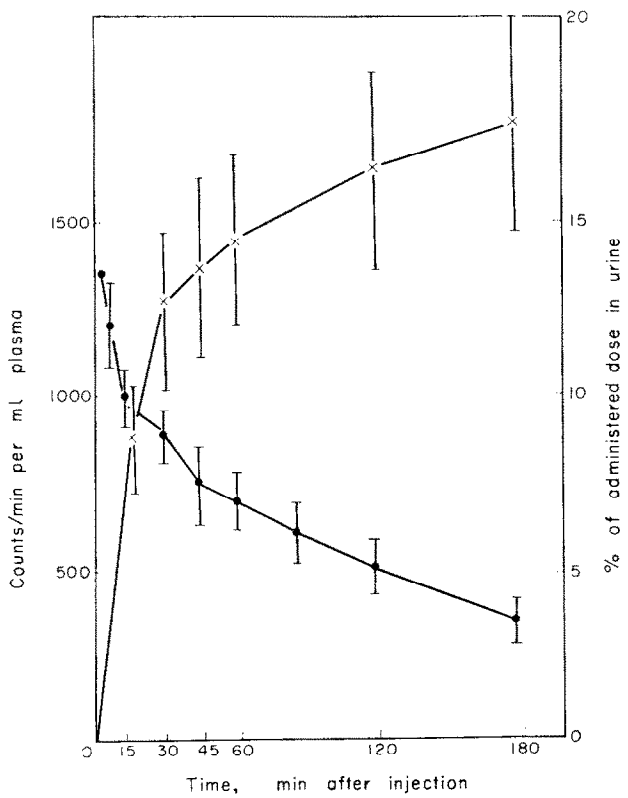

FIG. 1. These curves represent the mean (±s.e.) of at least four dogs after a single intravenous injection of 10 mg of radioactive SPG per kg. The percentage of the injected dose which appeared in the urine is represented by ×——×, while the counts/min per ml of plasma is represented by ●——●. The radioactivity in the plasma was 61 per cent of the total radioactivity of whole blood.

similar time-course in the blood, in that an initial rapid drop was followed by a slower fall. If the first and more rapid fall is a reflection of the dilution of the injected SPG by body fluids, then the slope of the second curve indicates either excretion or tissue distribution, or both. The urinary excretion curves in these same acute experiments

appear to be reciprocals of the blood levels (Fig. 1). On the basis that the second slope seen in Fig. 1 indicates a biological half-life of SPG in the blood of about 120 min, one would not expect to find measureable radioactivity in that tissue at the end of 12 hr or six biological half-lives. This has been verified repeatedly in that there has never been any radioactivity in the blood 12 hr after the injection, regardless of the size of the dose. According to the literature,[4] the decay curve for radioactive heparin is almost exactly the same as that presented in Fig. 1 for SPG. Loomis,[5] using radioactive heparin from the dog, found about 20 per cent of the radioactivity of the intravenously administered dose in the urine in 2 hr. The blood radioactivity curve of Loomis was also similar to that reported in this paper for SPG.

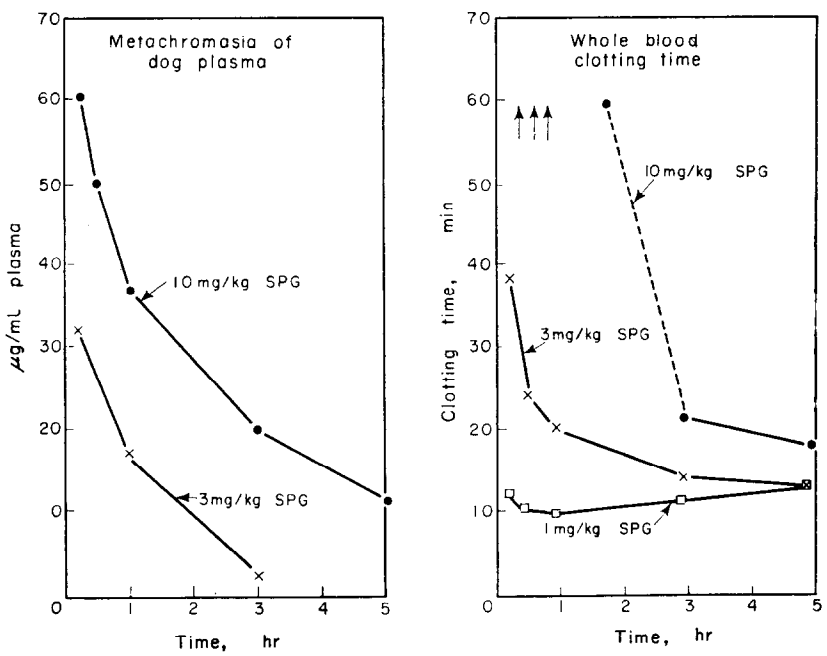

FIG. 2. These curves represent the mean value of at least four dogs. For additional information see text.

The experiments depicted in Fig. 1 do not give a good indication of whether the whole molecule or only the radioactive portion (sulfate residues) disappears from the blood. In order to determine whether part of the molecule was hydrolysed, metachromasia and anticoagulant activity were used as additional measurements. In Fig. 2, curves for both these parameters are shown; the data upon which these curves were based were derived from another set of dogs. It is apparent that the shape, as well as time-characteristics, of the two curves in Fig. 2 are similar to each other, as well as to the blood radioactivity curves seen in Fig. 1. In addition, anticoagulant time-curves for heparin USP had the same general shape and time characteristics as those for SPG, although the heparin had, on a weight basis, about seven times the anticoagulant potency of SPG.

Since, so far, all measurements of disposition showed a similarity between SPG and heparin, the slopes of the time-effect curves for lipemia-clearing activity (Fig. 3) were unexpected. For both doses illustrated here (2 and 5 mg/kg), heparin and SPG reached the same maximum activity, but the elevated levels for SPG were much more prolonged. The heparin peak occurred during the first hour, while the SPG peak was reached after about 3 hr. When higher dosages of these compounds were used, the peak activity for heparin was still early, but considerably elevated, while the peak activity of SPG was elevated to a lesser extent, but was reached with still greater delay.[2]

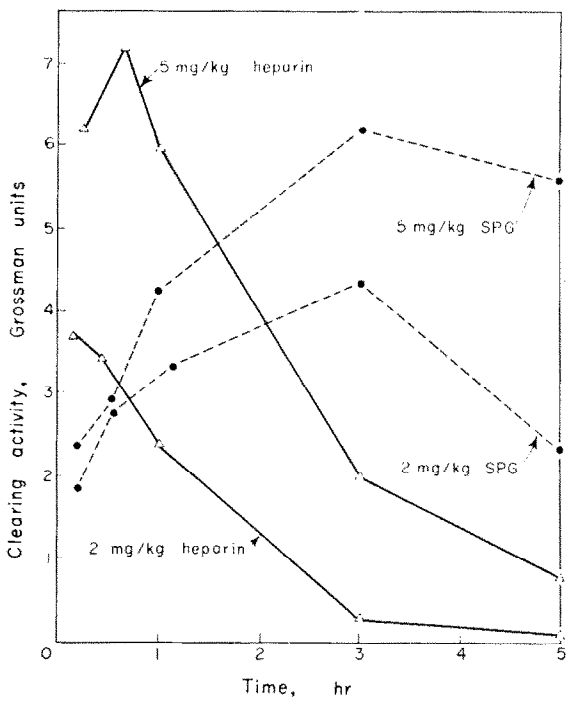

FIG. 3. These two curves show the average appearance time of lipoprotein lipase in the blood after a single intravenous injection of either SPG ●– – –● or heparin △——△.

Chromatograms of the urine of dogs receiving radioactive SPG, which were subsequently made into radioautographs, showed only the excretion of SPG. Development of duplicate chromatograms, using Azure A for identification of metachromatic material and rhodizinate for identification of inorganic sulfate, revealed the rapid appearance of only metachromatic material in the urine. Within 15 min after injection of an intravenous dose of SPG-^{35}S, a single metachromatic stain was found on the chromatogram which, when exposed to X-ray film, was also radioactive. After the injection of ^{35}S-heparin,[6] following which large amounts of ^{35}S-inorganic sulfate were found in the urine, no radioactive material differing from heparin was found on any of our films.

DISCUSSION

Korn[7] has proposed that heparin and heparin-like compounds are an essential part of the clearing factor or lipoprotein lipase molecule. The evidence presented in

this paper that the clearing factor curves for SPG and heparin are different in their temporal characteristics, is by itself not inconsistent with Korn's hypothesis, but the disappearance and excretion curves make this hypothesis unlikely.

If there is acquisition of the SPG molecule by the lipoprotein lipase, it would not be expected that the radioactivity of the blood would decrease while the level of enzyme activity in the blood increased. However, it is possible that the amount of radioactivity associated with the increase of lipoprotein lipase is too small to alter the measurements. The aforementioned possibility, in addition to evidence obtained by Robinson[8] and Korn[7] that samples of plasma from animals treated with heparin or dextran sulfate could be made to lose their lipoprotein lipase activity *in vitro* and *in vivo* with protamine or heparinase, make the combination of heparinoid with enzyme possible. However, the level of endogenous clearing factor in plasma is very low, and it seems more important to direct attention to the appearance of the enzyme in blood. Thus, it is more attractive to consider that heparin and other highly anionic substances cause either a release of clearing factor from the sites of storage or its increased synthesis, or both. Lipoprotein lipase has been found in various tissues, such as the heart and adipose tissue, and it is also believed to be in the vascular walls, as well as in other tissues.[1] Since SPG is chemically different from heparin, in that it is more acid, contains only glucose units, and has a lower molecular weight, it is possible that some of the primary sites for the release of clearing factor are affected by heparin and not by SPG. This would be consistent with other data which indicate that increasing intravenous doses of heparin only raise the peak-level of clearing activity, but do not extend the time-course, while higher intravenous doses of SPG not only raise the peak-level of clearing activity, but also extend the duration of elevated clearing activity in the blood. If, as an example, the sites in the vessel walls were the primary ones affected by heparin, while the sites in fat tissue were primarily affected by SPG, then the differences would be more readily understood.

Another possible factor to consider, in attempting to account for the differences in time-curves, is the rate of destruction of clearing factor. It has been suggested that the site of destruction of clearing factor *in vivo* is the liver. Since heparin and heparinoids have a great affinity for the liver,[4, 10–12] the action of SPG as well as heparin could cause alterations in the normal rate of destruction of clearing factor. Thus information consistent with this idea is derived from the observation that animals repeatedly given the same dose of SPG during long periods of time, tend to have higher and longer-lasting clearing activity in their blood than would be expected from the less frequent administration at similar amounts.[2] Since this cumulative drug action does not appear when heparin is administered repeatedly, the differences in the clearing activity curves might be explained by the action of SPG on the disposal mechanism for clearing factor in the liver and perhaps elsewhere.

It is possible that still other mechanisms not mentioned here may operate. However, it is felt that either of the above hypotheses, alone or together, could explain the difference between heparin and SPG. Experiments are in progress to clarify the similarities and differences between heparin and SPG.

Acknowledgement—The authors wish to thank M. Draper for synthesizing the radioactive compounds used in these experiments. We wish also to acknowledge the technical assistance of C. De Fusco and T. Hooper.

REFERENCES

1. D. S. ROBINSON and J. E. FRENCH, *Pharm. Rev.* **12**, 241 (1960).
2. E. WINDSOR and G. E. CRONHEIM, *Symposium on Thrombosis and Anticoagulant Therapy, Dundee, Scotland* (1960). In press.
3. L. B. JAQUES and H. S. BELL, *Meth. Biochem. Anal.* **7**, 292 (1959).
4. H. B. EIBER, I. DANISHEFSKY and F. S. BORELLI, *Proc. Soc. Exp. Biol., N.Y.* **98**, 672 (1958).
5. T. A. LOOMIS, *Proc. Soc. Exp. Biol., N.Y.* In press.
6. I. DANISHEFSKY and H. B. EIBER, *Arch. Biochem. Biophys.* **85**, 53 (1959).
7. E. D. KORN, *J. Biol. Chem*, **226**, 827 (1957).
8. D. S. ROBINSON, P. M. HARRIS and C. R. RICKETTS, *Biochem. J.* **71**, 286 (1959).
9. H. SCHOLL and G. SCHLETTER, *German Med. Mthly* **3**, 13 (1958).
10. P. E. MORROW, H. C. HODGE, W. F. NEUMAN, E. A. MAYNARD, H. S. BLANCHET, SR., D. W. FASSETT, R. E. BIRK and S. MANRODT, *J. Pharmacol.* **105**, 273 (1952).
11. E. HAUSEMANN, E. G. HOFFMAN, R. LITTERLE and M. WIEDERSHEIM, *Experientia* **8**, 153 (1952).
12. H. WEIGEL and K. WALTON, *Nature, London* **183**, 981 (1959).