12007

Presence of a Factor in Blood Which Enhances Bacterial-Growth Activity of Riboflavin.*

ROBERT E. ECKARDT,† PAUL GYÖRGY AND LORAND V. JOHNSON.

From the Department of Surgery (Ophthalmological Service) and the Department of Pediatrics of the Western Reserve University School of Medicine and of the University Hospitals of Cleveland.

Previous to 1939, the two principal methods of determining the content of riboflavin in biological materials were the rat growth method and some method which utilized the fluorescent properties of riboflavin. The first method had the disadvantages of being inapplicable for substances that contained very small amounts of riboflavin and of being cumbersome, time-consuming, and expensive. The second method was complicated by the presence of other pigments and by the uncertainty as to whether the fluorescent substance determined was in reality physiologically active riboflavin or some photoderivative of riboflavin. As a consequence, determination of riboflavin in the blood was impractical by either of these methods. It was, therefore, with considerable interest that the microbiological assay of Snell and Strong[1] was noted. Not only was this method applicable to substances containing minute amounts of riboflavin, but also it was quick and apparently specific for physiologically active riboflavin, since photoderivatives of riboflavin have no effect on the growth of bacteria. It was not long until the method had been applied to animal and human blood with fair success[2] and later with apparently complete success.[3]

It was our belief, however, that the specificity of the method had not been sufficiently proved to permit its application to such a complex substance as blood. Accordingly, we devised a method whereby

* We wish to express our gratitude to the Department of Bacteriology, Western Reserve University School of Medicine, for kindly placing the facilities of their department at our disposal for this work.

† S.M.A. Corporation Fellow and George Angell Fellow for research in Ophthalmological Chemistry.

1 Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem.* (Analyt. Ed.), 1939, **11**, 346.

2 Fraser, H. F., Topping, N. H., and Isbell, H., *Pub. Health Rep.*, 1940, **55**, 280; Feeney, R. E., and Strong, F. M., *J. Biol. Chem.*, 1940, **133**, xxxi; Strong, F. M., Feeney, R. E., Moore, B., and Parsons, H. T., *J. Biol. Chem.*, 1941, **137**, 363.

3 Spies, T. D., Stanbery, S. R., Williams, R. J., Jukes, T. H., and Babcock, S. H., *J. A. M. A.*, 1940, **115**, 523; Spies, T. D., Bean, W. B., Vilter, R. W., and Huff, N. E., *Am. J. M. Sc.*, 1940, **200**, 697.

the riboflavin in the blood was destroyed by irradiation under alkaline conditions, the blood was then neutralized, and known amounts of riboflavin were added to it. This note reports the results of that work.

Methods. Human venous blood was collected and citrated. It was laked by the addition of 9 volumes of water, the pH was adjusted to 9 or 10 with NaOH, and the blood was then irradiated for from 10 to 12 hours under a 300 Watt incandescent bulb at a distance of about 6 inches. For the irradiation, the laked blood was placed in the inner tube of a condenser, through the jacket of which water was circulated to prevent heating. Following irradiation, the blood was neutralized with 10% HCl to a pH of 6.8 to 7.0. This irradiated blood was then used in the following manner:

Fifty tubes were prepared, each of which contained 5 cc of the basal medium of Snell and Strong.[1] To one group of tubes, pure riboflavin in amounts of 0.0, 0.05, 0.075, 0.10, 0.15, 0.20, 0.25, and 0.40 gamma was added and the contents of the tubes were diluted to 10 cc. To the other tubes, 1 cc or 2 cc of the irradiated blood (1:10) was added, then riboflavin in amounts of 0.0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25, and 0.40 gamma was added, and the mixtures were diluted to 10 cc. All of the tubes were then sterilized in an autoclave for 15 minutes at 15 pounds pressure. When cool, each of the tubes was inoculated with 0.15 cc of the inoculum. The inoculum was prepared by centrifuging a 24-hour growth of *Lactobacillus casei* ‡ in the basal medium to which one gamma of pure riboflavin had been added. The cells were washed with 10 cc of 0.9% sterile saline solution, centrifuged, and resuspended in 4 cc of 0.9% sterile saline solution. One cc of this suspension was added to 100 cc of sterile saline solution, and this suspension was used for inoculating. This inoculum was adopted at the suggestion of Dr. M. Landy.[4]

All tubes were incubated for 72 hours at 37.5° C. They had been thoroughly shaken immediately following inoculation and were shaken again at 24 and 48 hours. The tubes were removed from the incubator, and those that contained the irradiated blood were centrifuged, the precipitate was washed once with 7 cc of distilled water, and the supernatant fluid titrated with 0.1 N NaOH to a definite blue end point with 6 drops of brom thymol blue (0.04%) as an indicator.

‡ The original culture of this organism was obtained from Dr. F. M. Strong of the University of Wisconsin.

[4] Landy, M., personal communication.

Results. A typical example of the results is given in Fig. 1. These results have been duplicated in 6 experiments with different samples of blood. It can be seen that the amount of acid produced by *L. casei* is much greater when irradiated blood is added to the basal medium than would be expected from the amount of riboflavin which had been added. That this growth is not due to the presence in the blood of riboflavin that has not been destroyed by irradiation is evidenced by the negligible growth obtained when irradiated blood alone was added to the basal medium. On the other hand, 1 cc of non-irradiated blood produces about 1.8 cc of acid, 2 cc about 2.5 cc of acid, and 4 cc about 4.5 cc of acid.

Comment. From these results, it is evident that blood contains some substance which, by itself, in the absence of riboflavin, is without effect upon the acid production by *L. casei* but which stimulates

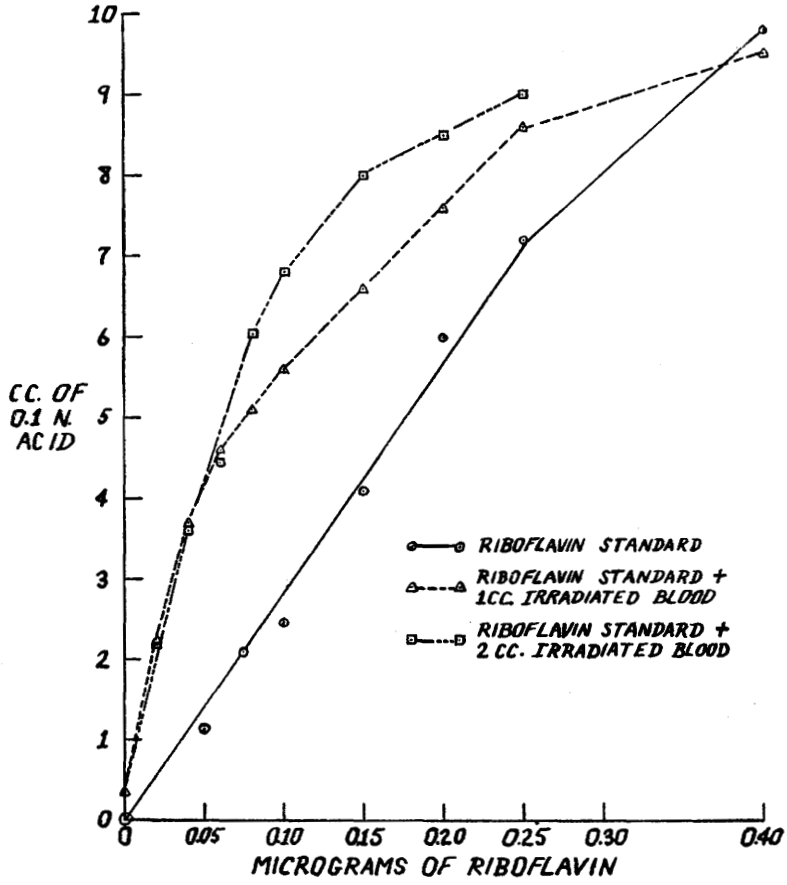

FIG. 1.

the growth of this organism in the presence of riboflavin. Preliminary studies indicate that this substance is not biotin, for a relatively pure biotin solution added to the basal medium in amounts of 2.5 rat units per tube produced no such effect. Furthermore, it seems unlikely that it is any known component of the vitamin B_2 complex, since doubling the amount of yeast extract produced no similar effect. This yeast extract should contain all the known components of the B complex except biotin and riboflavin, which were adsorbed to the lead sulphide in the course of preparation.[1]

Citrated plasma apparently contains an equal quantity of the factor, for plasma irradiated in the same way as the whole blood exhibited the same effect. Blood treated with Tonsil,§ which is believed selectively to adsorb the riboflavin, behaves like irradiated blood. However, 2 cc of Tonsil-treated blood give the same results as 1 cc of irradiated blood, suggesting that the factor may be partially adsorbed by the Tonsil. These experiments with Tonsil further suggest that the factor is not a substance produced by the irradiation process. On the other hand, breast milk treated with Tonsil‖ and urine irradiated for 4 hours under alkaline conditions do not exhibit the same effect.

It is also apparent that substances to be assayed for riboflavin should first be tested to see whether or not this factor is present, by destroying the riboflavin, either by irradiation or by Tonsil treatment, and by then adding known amounts of riboflavin to them. Unless similar tests are performed, the determinations made by this method are of questionable value because of the possibility of the presence of this factor. Similarly, the values for the content of riboflavin in the blood which have thus far been reported in the literature represent not the true value of riboflavin in the blood but only an apparent value. Blood values must be re-determined by using as a standard curve a basal medium to which riboflavin-free blood, in amounts equivalent to those being used for assay, has been added before the riboflavin is added. The significance attached to variations in the riboflavin content of blood before and after treatment must now be reconsidered.

§ Tonsil is a special fuller's earth which may be obtained from L. A. Salomon and Bros., 216 Pearl Street, New York, N. Y.

‖ At our suggestion, Dr. M. Landy of the Research Laboratories of the S.M.A. Corporation, Chagrin Falls, Ohio, repeated our original observation on irradiated blood. Furthermore, he tested the effect of Tonsil treatment of blood and breast milk, and kindly supplied us with a copy of his data for comparative purposes. Our experiments with Tonsil were undertaken at his suggestion.

Conclusions. Human blood from which the riboflavin has been removed by irradiation contains some factor which enhances the growth-promoting (acid production) activity of riboflavin on *Lactobacillus casei* ϵ. In the future, investigators who make determinations of the riboflavin content of blood or other biological materials must take into consideration the presence or absence of this factor.

12008 P

Effects of Sex and Gonadotropic Hormones on Red Cell Counts of Rats.

ERWIN P. VOLLMER, ALBERT S. GORDON, IRVING LEVENSTEIN AND H. A. CHARIPPER.

From the Department of Biology, Washington Square College of Arts and Science, New York University.

In a previous communication,[1] we have reported that normal female rats, injected for 2-3 months with pregnant mare serum hormone showed an erythrocyte count well below normal. Since hypophysectomy is also followed by an anemia,[2] it seemed to us that a broad investigation of sex and gonadotropic effects on the blood picture might serve to clarify the so-called hemotropic action of the anterior pituitary.

In the first series of experiments, determinations were made upon groups of normal and castrated animals injected daily with different hormone preparations.* The red cell counts of 47 normal mature rats of our colony were found to vary from approximately 8.0-9.0 million per mm^3, and averaged about 8.5 million per mm^3. Eight normal females injected daily for 2 months with 15 r.u. Gonadin revealed an average red cell count of 7.5 million per mm^3. The average red cell count of 11 normal males treated for 2 months with 10-15 r.u. Gonadin was 10.1 million per mm^3. Eleven untreated castrate females, 2 months after the operation possessed an

1 Gordon, A. S., Levenstein, I., and Charipper, H. A., *Proc. Am. Physiol. Soc.*, 1940, 68.

2 Vollmer, E., Gordon, A. S., Levenstein, I., and Charipper, H. A., *Endoc.*, 1939, **25**, 970.

* We wish to thank Dr. Erwin Schwenk, Schering Corporation, for generous supplies of testosterone propionate, estradiol benzoate, and Pranturon (a human pregnancy urine preparation). We are also indebted to Mr. Donald Wonder, Cutter Laboratories, for Gonadin (a pregnant mare serum preparation).