that rabbit serum contains active substances not present in the extract. It is relevant to consider the nature of these substances. The histamine content of rabbit serum (1 to 3 γ/cc[5,6]) is so great that it may be responsible for a considerable part of the activity of serum on the rabbit ear vessels. Evidence for this was furnished by Tsai *et al.*[7] who found that incubation of rabbit serum with crude histaminase reduced its vasoconstrictor activity by about one-third. As these workers pointed out, the residual activity indicates the presence of a non-histamine vasoconstrictor substance in rabbit serum. Such a substance must be responsible for the major part of the activity of cat and human sera upon rabbit ear vessels, since these sera contain insufficient histamine (0 to 0.06 γ/cc[5,6,8]) to account for an appreciable part of their vasoconstrictor activity.

Summary. A substance which stimulates smooth muscle was separated from dried rabbit serum and from the buffy coat of human blood by ether extraction. This substance is soluble in ether only in the presence of certain blood lipids. Aqueous solutions of the material stimulate the rat and rabbit intestine, cat nictitating membrane and tail vessels, but are devoid of effect on the vessels of the rabbit ear. The present findings, together with those of Tsai *et al.*,[7] suggest that several vasoconstrictor substances are present in serum.

5 Code, C. F., *J. Physiol.*, 1937, **90**, 349.

6 Anrep, G. V., Barsoum, G. S., Talaat, M., and Wieninger, E., *J. Physiol.*, 1939, **96**, 130.

7 Tsai, C., Wu, C. H., Fang, H. S., and Chen, T. I., *Proc. Chinese Physiol. Soc., Chengtu Branch*, 1943, **2**, 1.

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Further Studies on the Menstrual Discharge of Women.*

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Earlier studies on the menstrual discharge of normal women have been reported[1] with emphasis on its great toxicity, as little as 0.01 cc when fresh killing an immature rat within 48 hours. This paper summarizes certain corrections and additions that have evolved from continued investigation of this material.

It was stated that the toxin was most concentrated in the cells and debris of menstrual fluid. We have since found that the endometrial debris forms upon centrifugation, a middle layer between the serum and blood corpuscles. Even after being washed 3 to 6 times with saline, this layer is nearly 10 times as toxic as the serum, whereas the washed red blood cells are practically harmless. The amount of endometrial debris varies, being least when the rate of catamenial flow is greatest; the toxicity of different collections during menstruation, whether serum or whole discharge is tested, varies in direct proportion to the quantity of endometrial debris It would appear, therefore, that the toxicity is derived from the endometrial debris.

As stated, after fractional precipitation of whole discharge with $(NH_4)_2SO_4$ toxicity is most concentrated in the water-insoluble portion (after dialysis) of the englobulin precipitate. This precipitate, however, is apparently not a true englobulin, since it does not go into solution in the presence of electrolytes. When the whole discharge is not filtered before precipitation, the endometrial debris itself might be carried down and account for

* The Mrs. William Lowell Putnam Investigation of the Toxemias of Pregnancy, aided by grants from the Committee for Research in Problems of Sex, National Research Council.

1 Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 100.

the toxicity of this fraction. However, even when filtered serum from the discharge is ⅓ saturated with $(NH_4)_2SO_4$ and the precipitate dialysed, the resultant water-insoluble material is also insoluble in normal saline and lethal to immature rats in amounts equivalent to 0.1 to 2.0 cc of the original serum. Denaturation in the process of fractionation cannot be the explanation, since exactly the same technic applied to normal venous serum yields an englobulin fraction soluble in normal saline and non-toxic even in amounts equivalent to 10.0 to 15.0 cc of serum. After dialysis some of the englobulin precipitate from laked whole normal blood or from the serum of badly hemolysed or lipemic normal blood is insoluble in saline but does not harm immature rats in amounts equivalent to 5.0 to 10.0 cc of whole blood or serum. The fact that the serum of menstrual fluid almost invariably contains products of hemolysis, therefore, cannot account for its toxicity. Furthermore, whole discharge that has stood and become badly hemolysed is less toxic than when fresh and only slightly hemolysed. These observations strengthen our original intimation[1] that if the toxin is a decomposed protein it is formed *in vivo*. It may be a specific denaturation product of the endometrium consequent upon withdrawal of hormonal support.

Attempts to purify the toxin have been hampered by its lability. The minimal lethal dose before and after various procedures aimed towards stabilization and purification has been established by tests on 19- to 21-day-old male rats weighing 20 to 25 g, our criterion being death within 48 hours after the subcutaneous injection of a given amount. Although the whole discharge, rapidly dried, sealed and stored in the refrigerator, may retain its original potency for years, the toxicity of wet material, even when quickly frozen and stored at −18°C, may almost completely disappear overnight. Partially purified toxin is particularly labile and loses potency while being dried or soon thereafter. Our purest preparation to date is a grayish-white powder made from filtered serum which had been ⅓ saturated with $(NH_4)_2SO_4$. The precipitate was washed twice with ⅓ saturated $(NH_4)_2SO_4$ and dialysed until sulphate-free (24 hours in running tap water). The dialysed material was centrifuged, the precipitate washed once with normal saline and dry-frozen in a Flosdorf-Mudd apparatus. When first prepared, 5.0 mg (equivalent to 0.75 cc of serum) suspended in saline was the M.L.D.; at the last testing, 6 weeks later, 20.0 mg were required.

We reported that pretreatment with progesterone, adrenal cortical extract or the serum of rabbits immunized against menstrual discharge protects rats against lethal amounts of toxin. These therapies cannot be considered specific because of the surprising discovery that even water or normal saline renders an immature rat resistant to an otherwise lethal dose of menstrual fluid if as much as 1.0 cc is injected subcutaneously twice daily for 2 days prior to giving the toxin. This is, of course, a large volume of fluid for such a small animal (nearly ⅕ of the body weight). Does increased adrenal activity therefrom provide protection? Desoxycorticosterone or adrenal cortical extract given together with a M.L.D. of toxin does not prevent death but prolongs significantly the length of survival. Whatever the explanation, the finding indicates that susceptibility to the toxin is markedly influenced by the animal's condition as regards water balance. Our earlier observations on the influence of hormonal status upon susceptibility may perhaps all be interpreted on this basis, since an effect of steroid hormones on water balance has become well recognized.

It was also reported that serum from rabbits which had received repeated injections of sublethal doses would not protect against the toxin if simultaneously administered. We have found that adequate immunity was never established in these early experiments. Until a precipitin titer of 1 to 1,000,000 or over is established, no protective action is demonstrable. Unless small doses are given at first, that is, no more than the M.L.D. for an immature rat, the rabbit is likely to die. Even with this amount loss of appetite and listlessness are noted and, after 2 or 3 injections at 5-day intervals, severe subcutaneous reactions develop with diffuse and extensive edema and brawny induration.

With continued injections the local and systemic reactions become much less intense, even though the dose is doubled. The inflamed areas from the early injections, some of which have ulcerated, become abscesses which drain and heal and no new ones appear. By this time the precipitin titer is very high and the serum prolongs the period of survival of an immature rat when given simultaneously with a M.L.D. of menstrual toxin. Into one of 2 littermate rats 1.0 cc of rabbit serum is injected subcutaneously and into the other 1.0 cc of saline. One-half hour later 1.0 cc of serum and saline, respectively, is injected and, in a different site, a M.L.D. of menstrual toxin. In 7 such experiments, using serum from 5 immune rabbits, the rats treated with serum lived 6 to 48 hours longer than the saline-treated rats. The longest interval between deaths of littermate rats simultaneously receiving a M.L.D. of toxin alone has been 3 hours. Moreover, in 17 experiments similar to the above except that serum was taken from rabbits before injections of menstrual toxin were started or before adequate immunity was established, no rat injected with rabbit serum lived more than 3 hours longer than its saline-injected littermate. The fact that transferable immunity may be produced with menstrual toxin seems to be clear evidence of its specificity.

With the idea that a substance similar to or identical with the toxin of menstrual discharge might result from other forms of cellular injury, we have investigated the englobulin fraction of the blood of severely burned rats, degenerating livers of guinea pigs and rats and bleeding decidua from spayed pseudopregnant guinea pigs. Our object was to immunize rabbits against toxin from some source other than human, first, with the thought that by means of cross precipitin reactions we might have a test for circulating menstrual-like toxin in humans when it may be present in small amounts, viz., at the time of menstruation and in late pregnancy toxemia,[2] and conceivably in shock. Secondly, if immune serum were to be administered to humans it would, of course, be necessary to eliminate human proteins from the immunizing material. To date we have not succeeded either in finding anything similar to the menstrual toxin or in obtaining from rabbits immunized against these materials serum capable of precipitating menstrual toxin or of delaying the death of rats when given together with a M.L.D.† These negative results, in contrast to those with menstrual fluid, supply additional controls and thereby strengthen the evidence in favor of the specificity of the menstrual toxin.

Conclusion. The toxicity of the menstrual discharge of normal women appears to be due to a specific product of the endometrium formed *in vivo*.

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2 Smith, G. V., and Smith, O. W., *J. Clin. Endocrinol.*, 1941, **1**, 470.

† While this work was in progress, Menkin reported the finding of an injurious factor in the englobulin fraction of inflammatory exudates of dogs and man which he named "necrosin."[3] From his description we surmised that his factor and our menstrual toxin might be identical. Investigation of this possibility is now under way.

3 Menkin, V., *Science*, 1943, **97**, 165.