the action of the agent by releasing enzyme from cells in tissue culture (MBIII strain mouse lymphoblast) have also given negative results.

Summary. 1. An infectious agent similar to that described by Riley(1) has been demonstrated in 2 transplantable mouse tumors (CD/5 lymphoma and DBA/59 mammary carcinoma). The presence of the agent is detected only by a rapid rise in the plasma lactic dehydrogenase beginning about 29 hours following infection, reaching maximum levels in 9 days and persisting for up to 9 months. 2. Levels of the enzyme in brain, heart, kidney, liver, lung, pancreas, skeletal muscle, spleen, submaxillary gland, thymus and red and white blood cells were determined. No significant differences in any of these tissues were noted between normal and infected mice. 3. Hematocrit values were slightly but significantly lower than normal in mice bearing the LDH agent. These results are discussed in terms of possible sources for the increased level of plasma lactic dehydrogenase which results from infection with the agent.

1. Riley, Vernon, Lilly, F., Huerto, E., Bardell, D., Science, 1960, v132, 545.

2. Riley, Vernon, *ibid.*, 1961, v134, 666.

3. Notkins, A. L., Berry, R. T., Moloney, J. B., Greenfield, R. E., *Nature,* 1962, v193, 79.

4. Notkins, A. L., Greenfield, R. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 988.

5. Snell, George D., *J. Nat. Cancer Inst.,* 1953, v13, 1511.

6. Riley, Vernon, PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 751.

7. Wacker, W. E. C., *New Eng. J. Med.,* 1956, v255, 449.

8. Wroblewski, F., LaDu, F. S., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 210.

9. Maile, John B., *Laboratory Medicine, Hematology,* 2nd Ed., C. V. Mosby Co., St. Louis, Mo., 1962, p804.

Received May 7, 1963. P.S.E.B.M., 1963, v114.

Non-Enzymatic Dissolution of Fibrin "s" by Acidic Dyes, Surfactants, and Naphthalene Sulfonates.* (28611)

KURT N. VON KAULLA

Department of Medicine, University of Colorado School of Medicine, and the Belle Bonfils Memorial Blood Bank, Denver

We have found that many synthetic organic compounds induce marked fibrinolytic activity when dissolved in human plasma(1, 2,3). This activity is dependent upon the chemical structure of the compound(1,2). In addition, a factor is required which is present in human plasma or serum, but not in bovine plasma. The active compounds, however, do not induce dissolution of fibrin "s," the urea-soluble fibrin which is formed when, for example bovine fraction I is clotted by thrombin in the absence of calcium. Calcium is required for the function of the enzymatic fibrin stabilizing factor which converts fibrin "s" into urea-insoluble fibrin "i"(4). Preliminary observations indicated that minor molecular modifications, such as the introduction of a second hydrophylic group, deprived compounds of their ability to generate enzymatic fibrinolytic activity in human plasma, but endowed them with the new capacity to dissolve fibrin "s." This finding prompted an investigation of the relationship between the presence of strong hydrophylic groups in an organic molecule and its capacity to dissolve fibrin "s."

Material and methods. Preformed standard clots(5) were made from thrombin-clotted bovine fibrin, and the compound to be tested was dissolved either in human citrated plasma (variety III) or in buffered saline (variety IV)(6). The solutions were

* Supported by grants from Am. Heart Assn., Wyoming Heart Assn., and the Belle Bonfils Memorial Blood Bank, Denver.

carefully adjusted, if necessary, to pH 7.42 immediately before they were placed on top of the clots. Total volume of the standard clot was 0.42 ml and its height was 70 mm (Fig. 2). The digestion of 0.06 ml of clot corresponded to the dissolution of a cylindrical clot 10 mm high. No compound which caused the slightest retraction is included in this study. Unheated fibrin plates could have been used but the standard clot method is superior because it permits numerous readings and keeps the progressive dilution of the compound at a minimum.

The chemical compounds were used in the purest form commercially available or as experimental preparations donated by the manufacturers.† Where applicable, sodium salts were tested.

Results. a) *Naphthalene sulfonates.* The introduction of a second SO_3-group into a naphthalene monosulfonate abolished its ability to induce fibrinolytic activity in plasma (1,2), but conferred upon it the ability to dissolve fibrin "s." The introduction of 3 isopropyl side chains caused an identical change. Of interest is also 1-amino-2-naphthol-4-sulfonic acid which was one of the very few compounds capable of inducing some fibrinolytic activity in plasma as well as being able to dissolve fibrin "s." The kinetics of the dissolution of fibrin "s" standard clots by naphthalene sulfonates is shown in Fig. 1A.

b) *Acidic dyes.* A number of dyes possess several SO_3groups and might therefore dissolve fibrin "s." The diagnostically important dyes bromosulphalein (disodium phenoltetrabromophthalein sulfonate) and Evans Blue (tetra-sodium salt of 4,4'bis[7-amino-8-hydroxy-2,4-disulfo)-naphthyl-azo]-3,3'bitolyl), the naturally occurring dye Amaranth (1-[4-sulfo-1-naphthyl-azo]-2-naphthol-3,6-sulfonic acid) and the old trypanocidal drug Naphuride®‡ (bis(m-aminobenzoyl-m-amino-p-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate)-carbamide) were studied. All 4 compounds, when dissolved in buffered saline, exerted very strong fibrin "s"-dissolving activity. The marked activity of Naphuride® was not particularly surprising since its ability to alter proteins was recognized long ago(7). The activity of bromosulphalein and Evans Blue was unexpected. Evans Blue, being effective at a 5 millimolar concentration, was the most active compound studied. Fig. 1B shows that the action on fibrin develops rapidly, *e.g.*, 24 mM bromosulphalein and 5 mM Evans Blue dissolved fibrin cylinders 32 mm (0.205 ml) and 64 mm (0.375 ml) high in 2 and 7 hours, respectively.

c) *Surfactants.* The introduction of small side chains into the sulfonated naphthalene ring transformed the compound into an agent which was moderately active against fibrin "s." To evaluate the role of the side chain further, numerous surfactants with sulfonic acid radicals as hydrophylic groups and aliphatic chains of various length as hydrophobic groups were tested and the studies eventually extended to other surfactants. Fig. 2 demonstrates the dissolution of cylinders of fibrin "s" 120 minutes after a 2.5% solution of the surfactants has been applied on top of the clots. Fig. 3 shows the rate of dissolution of clots by 6 different anionic surfactants of various activity. Cal Research 61R-6016 and Benax 2A1 dissolved a fibrin cylinder 1 cm high (0.06 ml of digested clot) in less than 2 hours whereas 5% Aerosol OS solution did not produce the same effect in 12 hours.

Generally speaking, most of the fibrin-dissolving surfactants were anionic, none nonionic, and only 2 cationic (Arquad and bis-carboxymethyl-laurylamine). None of these surfactants was able to induce fibrinolytic activity in human plasma. Some amphoretic surfactants, however, quite readily dissolved fibrin "s" and, in addition, induced a moderate, but clear-cut fibrinolytic activity in human plasma. Deriphat 160-C was the most active compound of this group.

The following surfactants, tested as 2.5% and 5% solutions in buffered saline, dissolved fibrin "s." They are listed in order of activity. The letters in parentheses are explained in the following section. Aerosol OS (2.5%)

† Cal Research compounds: California Research Corp., Product BCO, BDO: DuPont de Nemours Co., Deriphat 160-C pharmaceutical grade: General Mills.

‡ Suramin, Germanin.

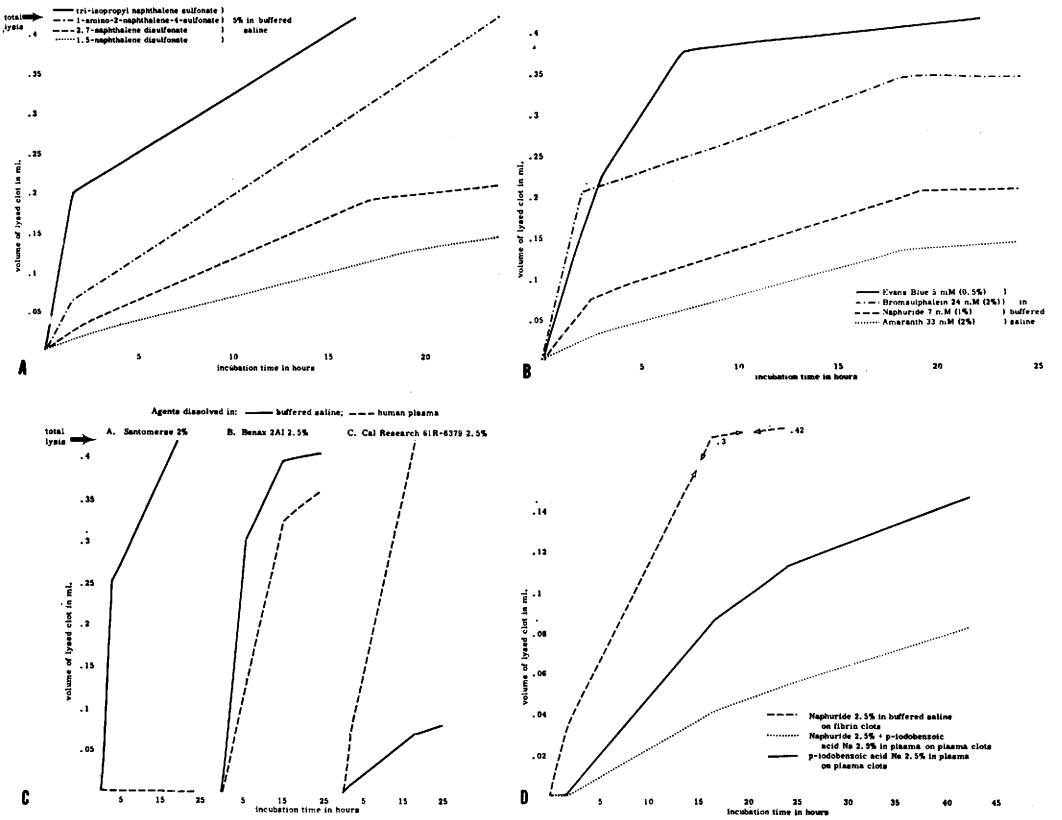

FIG. 1. Dissolution of standard fibrin clots by synthetic compounds. A: Dissolution rate of fibrin "s" by naphthalene sulfonates, 5% in buffered saline. "Total lysis": complete dissolution of clot. B: Dissolution rate of fibrin "s" by Evans Blue, Naphuride®, bromosulphalein, and Amaranth. C: Comparison of dissolution rate of fibrin "s" by 3 anionic surfactants dissolved in buffered saline and in human plasma. Human plasma inhibits Santomerse I, does not affect Benax 2A1, and enhances Cal Research 61R-6379. D: Comparison of dissolution rate of fibrin "i" (recalcified human plasma) induced by p-iodobenzoic acid Na in absence and presence of Naphuride®. Dashed line: dissolution rate of fibrin "s" by 2.5% Naphuride in buffered saline. Solid line: dissolution rate of fibrin "i" by 2.5% p-iodobenzoic acid Na in human plasma. Dotted line: dissolution rate of fibrin "i" by 2.5% p-iodobenzoic acid Na together with 2.5% Naphuride in human plasma.

was the most active compound, dissolving the entire standard clot (fibrin cylinder 70 mm high) within 10 hours, whereas Miranol J2M (5%) was the least active and required 24 hours to dissolve a column only 12 mm high. The 2.5% solution was ineffective.

Aerosol OS (I); Santomerse I (I); Cal Research 61R-6915 (N); Bis carboxymethyl Laurylamine; Benax 2A1 (N); Cal Research 61R-6376 (E); Aerosol 18 (E); Teepol (E); Cal Research 61R-6016 (N); Igepon T33 (I); Tide (I); Sodium-N-dodecyl Asparate (RC 5875) (I); Alamine 4D Disulfonate; Nekal BA-75 (I); Duponal WA (N); Sodium dodecyl sulfonate (E); Deriphat 160-C; Nacconal LAL (I); Duponal D (N); Deriphat 154 (I); Arquad 16 (I) Cal Research 61R-6377 (E); Product BCO (I); Sodium Hexadecyl sulfonate; Cal Research 61R-6378; Petrosul; Igepon AP-78 (N); Cal Research 61R-6370 (E); Deriphat 160; Miranol CM; Cal Research 61R-6380 (E); Lamepon (N); Product BDO (N); Arquad 12-50 (I); Triton X-200; Miranol J2M (N).

If the grading is based on a 1% solution, the less active compounds did not exhibit any clot-dissolving property, and the order of activity is different as seen from Fig. 3.

d) *Effect of human plasma on dissolution rate of fibrin "s" by surfactants and other*

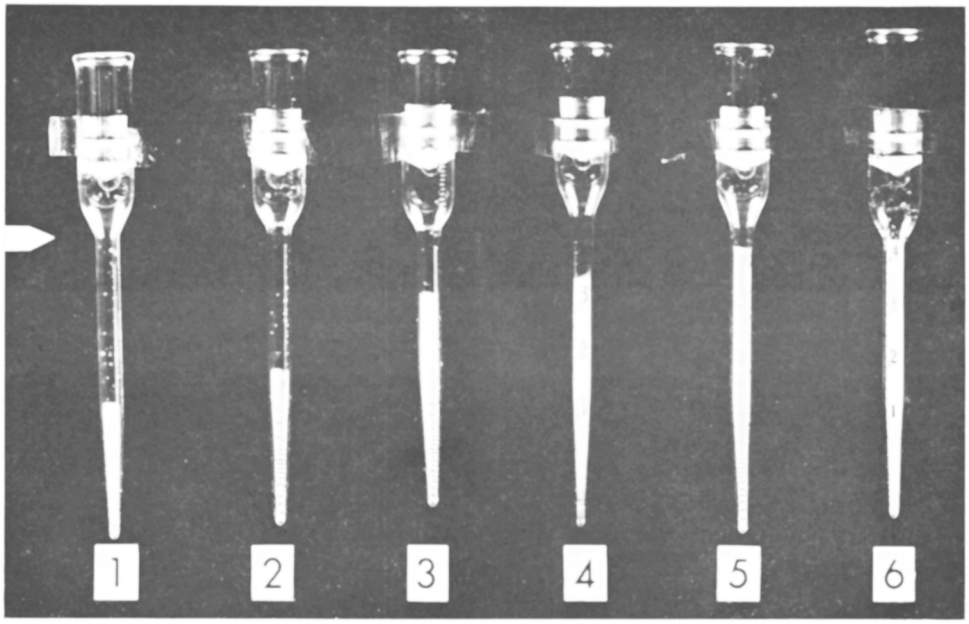

FIG. 2. Dissolution of preformed standard fibrin "s" clots by a 2.5% solution of Naphuride® and various surfactants dissolved in buffered saline. Incubation time 120 min. #1: Naphuride®. #2: Benax 2A1. #3: Cal Research 61R-6916. #4: Duponal WA. #5: Aerosol 18. #6: Control with buffered saline applied alone on top of the clot. Arrow: Original height of standard fibrin clot.

active compounds. Judging by their activity on fibrin "s" when dissolved in human plasma, the compounds fall into 3 distinct groups: 1) compounds which are inhibited by human plasma: (I) in above listing. 2) compounds which are not particularly affected by human plasma: (N). 3) compounds, the activity of which is markedly enhanced by human plasma (E). Compounds without a letter after the name were not tested in presence of human plasma. Fig. 1C depicts 3 representative examples for each group.

e) *Absence of synergism between synthetic compounds dissolving fibrin "s" and synthetic compounds inducing fibrinolytic activity in human plasma*. Compounds exerting strong dissolving action against fibrin "s" in presence of human plasma do not enhance, but actually inhibit the activity of compounds which induce enzymatic fibrinolytic activity. Fig. 1D reproduces a representative experiment. It is evident that addition of fibrin "s" dissolving Naphuride® to p-iodobenzoate (dissolved in plasma) reduces its fibrinolytic activity on plasma clots.

Discussion. Clots are dissolved at pH 7.4 by synthetic compounds in 2 ways: A) Urea-insoluble fibrin "i" is formed in recalcified human plasma and, in contrast to fibrin "s," possesses cross-linking covalent disulfide bonds which are produced by the enzymatic fibrin stabilizing factor(4) and calcium. This clot cannot be dissolved directly by synthetic compounds at this pH. Synthetic compounds, however, which induce fibrinolytic activity in human plasma, cause indirect enzymatic lysis of such clots. B) Soluble fibrin "s" is dissolved by 5 M urea, and by 0.5-2 M concentrations of LiCl, LiBr, NaBr, NaI, and guanidine HCl. None of these compounds induces fibrinolytic activity in human plasma (8). As shown in this paper, various surfactants and some acidic dyes also dissolve fibrin "s." Dissolution of fibrin "s" by the surfactant, Alconox(9), and by sodium dodecyl sulfate and cetyltrimethylammoniumbromide(4) have recently been reported. The dissolution phenomenon had been related to an increase of negative charges in the fibrin monomer (4). Surfactants are very effective hydrolytic agents for some proteins under certain

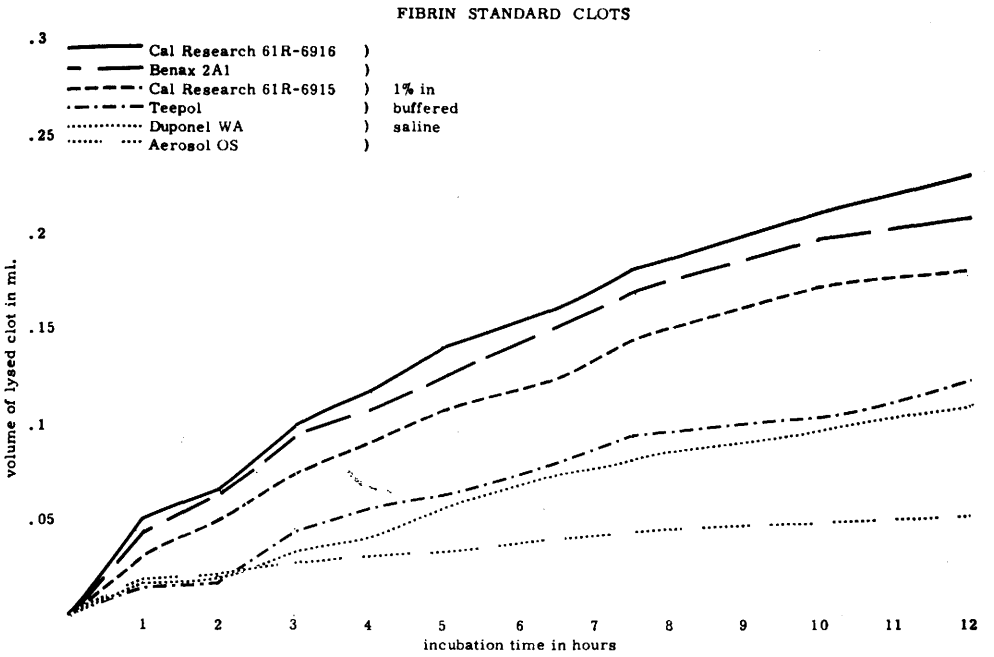

FIG. 3. Rate of dissolution of fibrin "s" by 1% solutions of anionic surfactants in buffered saline.

circumstances, increasing N-terminal groups up to 10-fold(10).

The action of surfactants on fibrin "s" in the presence of human plasma is more complex. Inhibition encountered with some compounds might be explained by either adsorption of the surfactants by the plasma proteins or the formation of more resistant fibrin clots, even in the absence of calcium, by diffusion into the fibrin clot of the applied plasma which is rich in fibrin stabilizing factor. The enhancement by human plasma of the fibrin "s" dissolving properties of other surfactants presupposes that these do more than rupture hydrogen bonds in the fibrin molecule. They must trigger something in the plasma which, in turn, enhances their action on fibrin "s." Induction of moderate fibrinolytic activity seems to be the most likely explanation. However, the partial suppression by the same surfactants of the fibrinolytic activity induced in plasma by synthetic compounds (which themselves do not dissolve fibrin "s") makes such an explanation doubtful. There is no evidence at present that a mutual binding of both compounds in the reaction mixture results in inhibition.

The rapid dissolution of fibrin "s" by certain dyes is not explained except to say that they, too, possibly introduce strong negative charges into the fibrin monomer. This phenomenon requires additional studies, since the bromosulphalein and Evans Blue are both injected intravenously in man for diagnostic purposes.

Introduction of a second SO_3-group changes the capacity of mono-naphthalene sulfonic acid to induce fibrinolytic activity into an ability to dissolve fibrin "s." This is best explained by assuming that naphthalene-disulfonates exhibit some characteristics of a surfactant; introduction of the second SO_3-group into the naphthalene ring coincides with the loss of the hydrotropic properties of the molecule(6). Further studies of such structural peculiarities may pave the way for synthesis of fibrinolytic agents. In this regard, additional investigations with amphoteric surfactants (*e.g.*, Deriphat 160-C), which induce both rapid dissolution of fibrin "s" and moderate fibrinolytic activity in human plasma, are indicated. For instance, introduction of a hydrophylic group with unsaturated bonds would be of interest, because

fibrinolytically active compounds carrying a hydrophilic substituent with double or triple bonds are more active than their counterparts with a saturated hydrophilic substituent(2).

Summary. Naphthalene-disulfonic acids, acidic dyes, and numerous surfactants dissolve fibrin "s" at low concentrations. Human plasma inhibits some surfactants, does not affect others, and enhances markedly the activity of a third group. Of the amphoteric surfactants, Deriphat 160-C dissolved fibrin "s" and, in contrast to other surfactants, induced enzymatic fibrinolytic activity in human plasma.

1. von Kaulla, K. N., *Thromb. Diath. Haem.,* 1962, v7, 404.

2. ———, *Chemistry of Thrombolysis: Human Fibrinolytic Enzymes,* C. C Thomas, Springfield, 1963, p261.

3. von Kaulla, K. N., Smith, R. L., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 530.

4. Loewy, A. G., Gallant, J. A., Dunathan, K., *J. Biol. Chem.,* 1961, v236, 2648.

5. von Kaulla, K. N., *Thromb. Diath. Haem.,* 1961, v5, 489.

6. ———, *Arch. Biochem. Biophys.,* 1962, v96, 4.

7. Kocian, V., *Arch. f. exp. Path. u. Pharm.,* 1936, v182, 313.

8. Shulman, S., Katz, S., Ferry, J. D., *J. Gen. Physiol.,* 1953, v36, 759.

9. Fleming W. H., Norman, P. S., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 636.

10. Colacicco, G., Investigations concerning the nature and significance of the submolar quantities of N-terminals in proteins. Thesis. Columbia Univ., New York, 1960.

Received May 9, 1953. P.S.E.B.M., 1963, v114.

Maintenance of Pregnancy in Protein-Deficient Rats with Short-Term Injections of Ovarian Hormones.* (28612)

WARREN G. KINZEY§ AND HERBERT H. SREBNIK
(Introduced by C. W. Asling)
Department of Anatomy, University of California, Berkeley

In absence of dietary protein most pregnant rats resorb their litters by mid-gestation (1). Pregnancy can be maintained, however, by daily injections of estrone and progesterone from day 3 to 20 of gestation(2), an observation suggesting that reproductive failure may be due to reduced levels of pituitary and/or placental gonadotropins. This study examines the functional capacity of placenta to maintain pregnancy in protein-deficient rats when ovarian hormone administration is terminated by mid-gestation. Since hypophysectomy also interrupts pregnancy when performed before establishment of placenta (3), pregnant hypophysectomized rats were injected concurrently with protein-deficient rats.

Materials and methods. Virgin Long-Evans rats, 75-85 days old and weighing 175-220 g, were bred with normal males and fed protein-free diet† *ad lib.* beginning the day of finding sperm in vaginal smear (day zero). Animals were kept in individual cages, on screens, until autopsy on day 21 of pregnancy. Injections of 1 μg esterone and 4 mg progesterone‡ in 0.3 ml sesame oil were given once daily for varying periods. Control rats received sesame oil only or were uninjected. Three additional groups of rats of comparable age and weight were fed purified complete diet containing 24% casein. Two groups were hypophysectomized by parapharyngeal route on day 5 of pregnancy and injected as above; a third group was intact and uninjected. Vaginal smears were examined daily during gestation for the presence of erythrocytes (the placental sign). At autopsy uteri were removed, examined for implantation

* Supported by Univ. of California Research Grant.

§ Present address: Dept. of Zoology, Univ. of Calif., Davis.

† Composition of purified diets and of fat-soluble vitamin supplement was reported previously(4).

‡ Estrone was obtained from Parke, Davis and Co., progesterone from Nutritional Biochemicals Co.