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[2] Millikan and Bowen, *Proc. Nat. Acad.*, **11**, 119 (1925).

[3] Bowen and Millikan, *Physic. Rev.*, **25**, 591 (1925).

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THE ANTI-STERILITY VITAMINE FAT SOLUBLE E[1]

BY HERBERT M. EVANS AND GEORGE O. BURR

UNIVERSITY OF CALIFORNIA

Read before the Academy April 27, 1925

When rats are reared on various "synthetic" food mixtures consisting of fat, carbohydrate and protein in separate and relatively pure form together with an appropriate salt mixture and the vitamines A and B,[2] they grow well and have every appearance of health. Depending somewhat on the exact character and the proportions of the constituents of the food, they sooner or later exhibit complete sterility. In many instances a transitory period of fertility, variable in its length, follows the attainment of sexual maturity. This is usually the case with the male. But in the case of young from such females, secured either from their early fertility period or by fertility induced by certain food extracts hereinafter described, we usually observe complete sterility from the very beginning of sexual life. The sterility is a dietary deficiency disease for it can be cured or prevented by a change in dietary regime, a change involving the addition of certain single natural foods high in a new food factor, vitamine E,[3] or the addition of very much smaller amounts of extracts of those foods. The sterility disease affects males and females differently.

In the male it eventually leads to destruction of the germ cells (eventually the entire seminiferous epithelium) but this is not the case with the female, where the ovary and ovulation are unimpaired throughout life but where a highly characteristic disturbance occurs in gestation, the death and resorption of the developing young. It is necessary to insist on the peculiar character of dietary sterility thus produced in the female through lack of vitamine E, for it is only by ascertaining the existence of typical "resorption gestations" that one may be assured that he is dealing with deficiency in the specific substance E. Many other dietary delinquencies cause sterility in the female, but they all do so by interference with other steps in the reproductive mechanism than those involved in lack of E, usually by preventing oestrus, ovulation, fertilization or implantation but not by resorption after implantation has occurred. In order to establish

female sterility as due to absence of fat soluble vitamine E it is necessary to establish with certainty the existence of oestrus and ovulation, coition and implantation. Such information is best secured by use of the newer methods of studying the vaginal smear, by mating animals at the appropriate time in the oestrous cycle, by subsequent detection of the "bouchon vaginale" and residual sperm, of the cessation of cycles and finally on the 14th to 16th day, of the occurrence of erythrocytes in the smear, a positive sign of implantation. In gestations where E is low or absent, the embryos seem at first normal but sooner or later, often by the eighth day, retardation in development can be substantiated. Evident abnormality, especially monstrosity, does not occur. At some time between the twelfth and twentieth day, foetal death occurs, usually on the twelfth or thirteenth day, but for some days thereafter, the maternal part of the placenta continues to live. There may also be continued gain in the mother's weight until the twentieth or twenty-first day. This would appear to speak decisively for peculiar need on the part of the developing young for the new vitamine as against placental injury as the cause of death. Furthermore, it would appear that the maternal placenta is not altered structurally sufficiently to account for belief in impairment of its function. Subnormality is not only seen in the embryo but in the foetal parts of the placenta, both in yolk sac and allantois, especially in the former; in the yolk sac, underdevelopment of the endodermal villi and blood islands is conspicuous, whereas, in the embryo one may note impairment in the mesenchyme and its chief derivatives, the blood vessels and blood cells. The exact time of foetal death appears to vary in the case of individual mothers and what is more remarkable, in the case of some embryos as contrasted with others in the same gestation. Thus dead and living young may occupy neighboring sites in the same uterine horn. Embryos may succumb shortly after implantation, or again only shortly before term.

Large numbers of females have been reared on various "pure" food regimes and bred shortly after the sixtieth day of life. Only those exhibiting a typical resorption were now employed to trace the distribution and abundance of the new food factor E in natural foods. Shortly after the failed or resorption gestation a small amount of a single natural food stuff was now added to the ration or fed separately from it and the fate of the next gestation followed with similar care. In many instances a normal sized litter of vigorous young resulted. In others, no alteration of the sterility was secured. We have thus charted the considerable and inconsiderable possession of E on the part of common foods. It is present but never highly concentrated in a great variety of animal tissues, musculature, fat and viscera, included in the latter being pancreas, spleen, liver, heart, hypophysis and placenta. One of the most remarkable things about the content of E in animal tissues is the fact that the vitamine is low in the

viscera. It is lower in the liver than in the musculature. A daily feeding of half the total liver of rats reared on natural foods will not invoke fertility. There is failure also when the entire heart, spleen brain, kidney or testes are fed daily. The musculature and fat, on the other hand, while not a concentrated source of E, contain in their totality several times the minimum requirement for a successful gestation. E is present but extremely low in milkfat. Nine per cent of this, which is included in our basic ration, together with 15% lard, fails to prevent sterility, though with lard absent, 24% succeeds. Whole milk powder may constitute one third of the ration by weight and sterility result. Yet when whole milk powder is the sole food, its fat content, 28%, is sufficiently high to insure adequate E. There is definite evidence of a higher E content of milk when from cattle with access to fresh alfalfa pasturage. Cod liver oil, though high in vitamines A and D, is notably lacking in E. Throughout the life of animals, 9% by weight of the ration, may be constituted by cod liver oil, a single drop of which daily is adequate for A requirements and yet sterility result. In contrast with the paucity of E, even in its most abundant depots in animal tissues is its concentration in the organs of certain plants, especially in seeds and green leaves. It can be demonstrated to be unhurt after careful desiccation of such leaves (lettuce, alfalfa, pea, tea). Thus in a series of experiments, one and one-half, one and finally one-fourth gram daily of the lettuce leaf powder proved efficacious in cures. E is high in some cereals. We have found it in oats, corn and, especially, wheat, where it is low in the endosperm but concentrated in the embryo. The richness of wheat germ in E is extraordinary. We have found no other naturally desiccated substance comparable to it in value; 250 mg. daily evokes cures. In the case of both wheat germ and lettuce leaf, ether extraction of the carefully desiccated substance removes E quantitatively and secures for us oils which are efficacious in *daily*, single drop (25 mg.) administrations. E is probably present in most commercial oils so that when the latter constitute a high proportion of the diet, for instance when fed as 15% displacing lard, fertility results. Such results have been secured with Wesson oil, cocoanut oil, olive oil. Cotton seed oil when hydrogenated constitutes the substance called Crisco. As is well known, it is practically devoid of vitamine A and has hence frequently been employed instead of lard in researches where an A free diet was essential. Yet, when the fat content of our basic diet is represented by Crisco, fertility invariably results, this being in fact a curative regime. Crisco, cotton seed oil, corn oil, olive, cocoanut, walnut, peanut and flax seed oils can all be fed daily in quantities five times the required minimum of wheat germ oil without restoring fertility.

Proof of the Existence of Vitamine E in the Tissues of Animals Reared upon Natural Foods and of Its Depletion in Those Reared upon Synthetic

Diets.—We have completed a series of cannibal experiments. Sterile females reared upon "pure" food regimes were sacrificed daily and their tissues (liver, musculature and fat) fed to other females reared in an identical fashion and likewise of proven sterility. At the same time, normal females of proven fertility were similarly sacrificed and fed to other sterile "pure" food females. In all instances, the tissues of rats reared on a natural food regime were able to invoke fertility in their sterile sisters. Of even greater significance would seem the demonstration that in no instance could a cure be obtained by the administration of the same tissues from sterile females.

The Survival of Fertility in Animals Shifted from a Diet Possessing Vitamine E to One Deprived of It.—If animals are reared on a diet of natural foodstuffs and after their fertility is established, shifted to a pure food ration, they preserve their fertility for three or four months, when they lose it. Similarly, when sterile animals are cured with foods possessing the new vitamine, not only is the next gestation normal, but in some circumstances, the next two or three gestations. The survival of normal fertility is roughly dependent upon the amount of E in the curative regime. When by quantitative experiments we have determined the minimal dose of any "curative" food, i.e., one capable of immediately restoring fertility, we have been able to see the immediate loss of this fertility in the next gestation on the pure food regime.

Presence of Vitamine E in the Tissues of Normal Newborn Young.—Vitamine E is transferred from mother to offspring during intrauterine life for the tissue of new born rats acts as a cure of female dietary sterility.

Proof of the Normal Use or Wastage of Vitamine E in the Usual Metabolic Processes of the Body.—Groups of females have been reared on a natural food regime and their fertility established by trial gestations, after which they were all shifted to our standard pure diet. Half of them were bred immediately and in all instances were able to give birth to young in the next two succeeding pregnancies, the third uniformly failing. As soon as the advent of sterility was demonstrated, presumably by the exhaustion of E due to the drain of the repeated pregnancies, the other half of the animals were bred. These sisters were by this time likewise sterile. This half had been shifted to the pure food at the same time as had the first half of the group, but had been shielded from the drain of reproduction and especially placental function. It hence seems clear that the body stores of vitamine E are employed in normal metabolic processes at approximately the same rate, whether or not we have the drain of gestation.

An Excess of E Cannot Increase Fertility beyond Normal Limits.—The administration to sterile animals of foods or extracts of foods known to be twice to twenty times as rich in vitamine E as is required for the birth of living young does not increase litter size or weight, or in other ways im-

prove the performance of the reproductive mechanism beyond normal limits. This is in consonance with what we know of the action of other vitamines, there being little or no reliable evidence of advantage from an abnormally high quota of them, yet absolute need of the minimal quota and, for complete normality, for what we can call the effective quota.

Efficacy of a Single Curative Dose of Vitamine E Administered at the Beginning of Gestation.—Since the work which has previously been detailed showed a definite if transitory storage of E by the body, it seemed reasonable to suppose that a sufficiently high feeding of E on a single occasion—early in gestation—might suffice for that particular gestation. It was, in fact, found that success resulted from a single administration of the same total amount represented in twenty-two days of separate daily dosage with the minimal effective amount of wheat germ oil. The minimal effective daily dose was found to be about 25 milligrams and a single administration of 550 milligrams of the oil led in all tests to the birth of living young. Furthermore, curative foods or extracts of those foods can be fed as late as the fifth or sixth day of pregnancy and rescue the situation. Finally, the vitamine in the form of oil can be just as effectively administered parenterally—by subcutaneous or intraperitoneal injection—as by mouth.

Physical and Chemical Characteristics of the New Substance.—We come now to a consideration of the physical and chemical characteristics of the new substance. The vitamine may be called fat soluble, though its range of solubility is far greater than that of ordinary fats. While this solubility range may really be due to the solubilities of impurities as yet associated with the vitamine, it is a fact that the most concentrated fractions yet obtained have been almost completely miscible with solvents representing such a range as methyl alcohol, ethyl alcohol, ether, pentane, benzene, acetone, ethylacetate, carbon disulfide, etc. The vitamine is almost insoluble in water, yet we have repeatedly encountered its presence in watery solutions. There is enough left in the water after precipitation of calcium soaps, for instance, to be extracted with ether and effect cures. The distribution ratio between water and ether is very large, for a few extractions with an equal volume of ether effects quantitative removal. This has been established by a large number of feedings of the nonsaponifiable fraction, the residual soap always failing to produce fertility. The solubility of E in such substances as alcohol and pentane shows a large temperature coefficient and is so much greater than some of the contaminating substances—the sterols, for example—as to permit separation of the vitamine from them.

Vitamine E is remarkably stable to heat, light, air and many of the ordinary chemical reactions. As regards temperature, while the ashing of wheat germ completely destroyed the vitamine, yet heating of the germ

to 170°C so that it was greatly charred left the E unimpaired. Distillation of wheat germ oil, or a fraction out of it, in superheated steam at 180° C for several hours has not destroyed it. Distillation in vacuo up to 233°C has not in fact caused any lowering of the potency of the fractions so treated nor have any physical changes, like changes in solubility, been detected. We have not encountered evidence that daylight affects E in wheat germ oil but there would appear to be partial destruction by one hour exposure in thin layers to a powerful quartz mercury lamp. As regards oxidation, exposure of wheat germ oil for as many as twelve hours to a stream of air washed with acid and alkali and at 97°C, has not destroyed E. At normal temperatures the vitamine is remarkably stable to both acid and alkali and many chemical treatments. It dissolves unchanged for instance in saturated alcoholic hydrogen chloride. We have hydrogenated wheat germ oil in the presence of palladium at 75°C and no injury to the vitamine resulted. Further, alcoholic extracts of Crisco, a hydrogenation product of cotton seed oil, are always fairly rich in the vitamine. We have treated the germ oil with both 20% HCl and $N/10$ HCl for 20 hours at room temperatures without destruction of the vitamine. It is not destroyed by concentrated sulfuric acid. It resists the action of boiling 20% alcoholic KOH, though partial destruction would appear to occur on very prolonged hot saponification. The saponification with 20% alcoholic KOH can be carried out at 30°C without great loss of the vitamine which goes into the nonsaponifiable quota, five per cent of the oil, so that by this step alone a notable concentration of E is always attained. The nonsaponifiable quota is in turn chiefly (73%) sitosterol which is largely insoluble in pentane in the cold, an excellent solvent for vitamine E, which together with pigments and other materials can thus be washed out of the sterols, leaving them white. The sterols are inactive. The orange-red viscous oil obtained from the pentane can be treated with methyl alcohol, removing more extraneous material, the vitamine going in the alcohol portions which now can be mixed with petroleum ether and diluted to 90% MeOH, allowing an immediate separation into two layers, the petroleum ether invariably securing more of the vitamine, in fact all of it, if the distribution be done with successive fresh portions of the petroleum ether. Further purifications can now be carried out both with digitonin, boiling methyl alcohol and finally, distillations in vacuo—yet the concentration effected, of course, does not relatively compare with that effected with the first three steps of the procedure just outlined. The final yellow viscous oil does not develop crystals on long standing. It contains only a trace of ash and no nitrogen, sulphur, phosphorous or halogen. It is remarkably potent. When 5 milligrams are fed or injected under the skin of a female of proven sterility at the inception of a new gestation, normal litters of vigorous young are born and have been reared

to adulthood. Sister control rats invariably continue sterile. Furthermore the daily administration of only three tenths of a milligram of this substance throughout the life of the male results in the retention of complete normality when animals are reared and held on pure foods—a normality proven from the weight and histological condition of the testis and by weekly functional tests throughout a year, and controlled by the invariable development of sterility at the end of three months in littermate brothers held on the identical ration save for omission of the vitamine trace.

OUTLINE OF FRACTIONATION OF 6 KILOS OF WHEAT GERM

6 kilos Wheat Germ

Extracted with U.S.P. Ether in Soxhlet

Ether extract. Active. Yield: 600 grams—10%	Ether insoluble residue. Inactive.

Saponified in the cold with 20% alcoholic KOH

Nonsaponifiable matter (N.S.M.) Yield: 5%. Contains all the active material.	Soaps and Glycerol. Inactive.

Crystallized from cold pentane

Pentane-soluble red oil. Yield: 33% of the N.S.M. Contains all the active material.	Pentane insoluble solids. Sitosterol. Yield 66% of N.S.M. Inactive.

Extracted with hot MeOH

Hot methyl alcohol solution. Active.	Methyl alcohol insoluble residue. Inactive. Yield: 3% of N.S.M.

Crystallized from cold MeOH

Cold MeOH solution. Active	Precipitate from cold MeOH. Almost entirely inactive. Yield: 10% of N.S.M.

Distributed between dilute MeOH and petroleum ether.

Petroleum ether soluble. Active. Yield: 13% of N.S.M.	Dilute MeOH soluble. Inactive. Yield: 4% of N.S.M.

Sterols precipitated by digitonin.

Sterol-free orange oil. Yield: 2–3 grams. Active.

Sterols from digitonide. Inactive.

Refluxed in hot 20% alcoholic KOH

N.S.M. Contains all of the active material

Fatty acids. Inactive.

Sterols again precipitated by digitonin.

Sterol-free oil. Active.

Sterols. Inactive.

Treated with boiling MeOH

Orange solution in MeOH. Active. Yield: 700–1000 grams. (Proven active in single doses of 5–10 mgs.)

Residue insoluble in hot MeOH. Inactive. Yield: 50–100 mgs.

Distilled in vacuo

Fraction I	*Fraction II*	*Fraction III*
Up to 200° at 0.8 mm. Low activity. 38% of the total.	200–233° at 0.5 mm. Highly active. 27% of the total.	Residue above 233°. Highly active. 35% of the total.

[1] Aided by grants from the Committee for Research on Sex Problems of the National Research Council and from the Bureau of Dairying, U. S. Department of Agriculture.

[2] We have employed various proportions of casein, lard, butter and cornstarch, the vitamine B being secured from daily administration of 0.4 to 0.6 gram of whole dried yeast and A from the butter employed. In many instances, however, milkfat was omitted and the A requirements met with various levels of cod liver oil which varied from a single drop daily to two per cent by weight of the ration. The salt mixture employed was after E. V. McCollum and consisted of NaCl 0.173, $MgSO_4$ (anhyd.) 0.266, $NaH_2PO_4 + H_2O$ 0.347, K_2PO_4 0.954, $CaH_4(PO_4)_2 + H_2O$ 0.540, Fe citrate 0.118, Ca lactate 1.300.

[3] Provisionally designated in previous publications from this laboratory as vitamine X. We now so designate it on account of its serial position, following the alphabetic terminology proposed by McCollum, who names the antirachitic factor D.