tourniquet shock in rats is described. The method yields graded and reproducible results.

2. The percentage survival of rats to tourniquet shock was progressively decreased as the time of tourniquet application was increased. Following 3½ hours of tourniquets 100% of the rats survived, while after 4 hours of tourniquets only 3% survived.

3. Rats which had tourniquets applied for 4 hours with periods of release for 1 to 6 hours, followed by a second application of tourniquets, showed 100% in contrast to 3% survival for those without replacement, while 87.5% survived when the tourniquets were replaced after 7 hours.

4. The degree of hemoconcentration developing in rats in tourniquet shock was approximated by hemoglobin determinations. Increases in hemoglobin ranged between 27 and 52%.

15184

Inactivation of Estrone by Liver. Assay Method *In vivo* for Dietary Hepatic Injury in Rats.*

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Pellets of estrone implanted in the spleen will not induce estrus in ovariectomized rats fed a normal diet.[1] Estrone draining from the pellet through the splenic vein into the liver is inactivated by the normal functioning liver parenchyma, either through direct chemical destruction[1] or through a closed "hepato-intestinal cycle."[2] Failure of the liver to inactivate estrogen has been demonstrated by Biskind and Biskind[1] in rats maintained on a diet deficient in the vitamin B complex. Estrus in such animals appeared within 1 to 3 weeks and was interpreted as an indication of impaired liver function. Such functional change resulting from avitaminosis is in its etiology and pathogenesis different from the specific hepatic injury (necrosis and cirrhosis) produced in rats by the administration of a synthetic diet low in lipotropic factors (methionine and choline),[3] but containing all known vitamins including the members of the vitamin B complex. On the other hand, however, it has been shown[4] that with regard to inactivation of estrogen by the liver, rations deficient in lipotropic factors will have the same effect as rations free of vitamin B complex. In spite of this analogy only the disturbance elicited by rations deficient in lipotropic factors with its characteristic pathological manifestations is considered the true dietary hepatic injury.[3]

In previous experiments[4] the onset of functional disability of the liver, measured by the failure of inactivation of estrone, became evident in rats fed a diet low in lipotropic factors after an interval when severe histological changes are known to make their appearance. This interval was much longer than that observed in rats fed a vitamin B-free diet.[1,4] Whereas the impaired inactivation of estrone in rats kept on a vitamin B-free diet could be reversed and normalized by supplements of vitamin B complex,[1,4] large doses of yeast were needed to achieve the same result in rats

* Aided by a grant from Wyeth, Incorporated, Philadelphia.

1 Biskind, M. S., and Biskind, G. R., *Endocrinol.*, 1942, **31**, 109. Here also literature.

2 Cantarow, A., Paschkis, K. E., Rakoff, A. E., and Hansen, L. P., *Endocrinol.*, 1943, **33**, 309.

3 György, P., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 492; Webster, G., *J. Clin. Invest.*, 1941, **20**, 440; Blumberg, H., and McCollum, E. V., *Science*, 1941, **93**, 598; Lillie, R. D., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1941, **56**, 1255.

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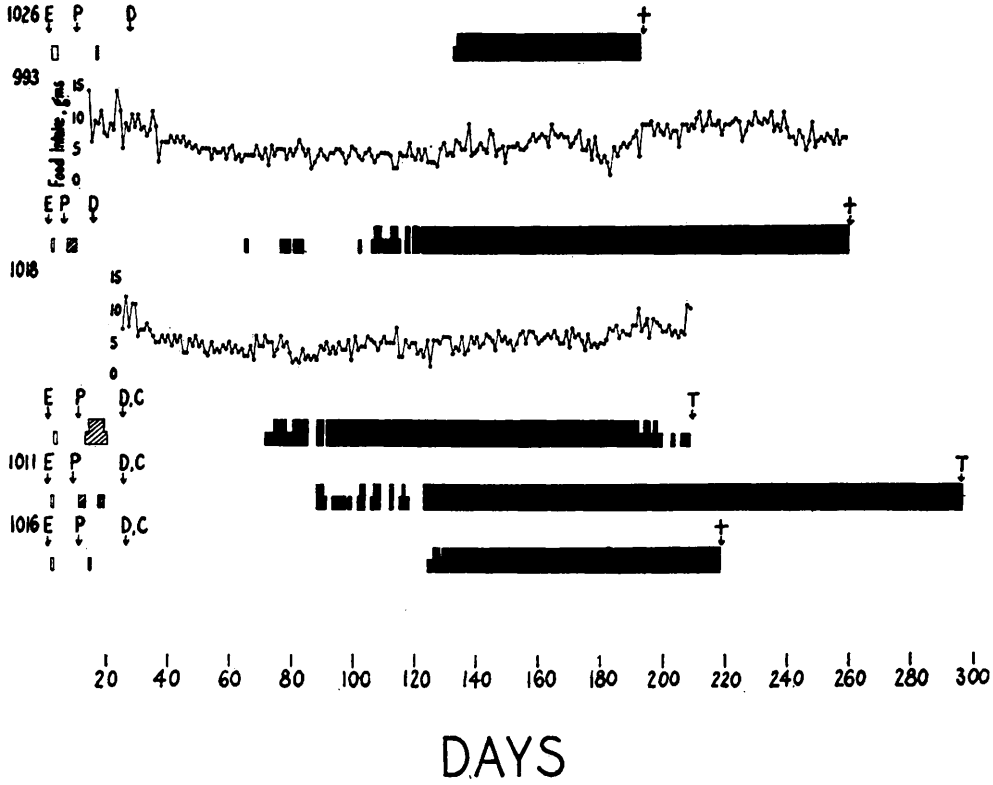

FIG. 1.

Series of rats receiving a diet low in proteins and high in fat. (5 of 9 rats in Group A). The blocked areas represent estrus. Partial estrus is depicted by a reduction in height of the block. Symbols are as follows: E—Test dose of estrone, P—Insertion of pellet, D—Diet started, C—Supplement of cystine, T—Experiment terminated. +, death of animal. The rats were castrated at zero time.

fed a diet low in lipotropic factors.

Reversal of the estrus reaction by the administration of large doses of yeast indicates that curative measures for dietary hepatic injury may be adequately assessed in the living animal.

Yeast as a curative factor in dietary hepatic injury is in good accord with previous findings obtained in prophylactic experiments of long duration (150 days). Addition of yeast to the basal experimental diet prevented the production of hepatic injury as controlled by macroscopic and microscopic examinations of the liver.[5] However, yeast is not the best and most characteristic representative of the lipotropic substances. Given as sole source of protein and not—as in our previous studies—as supplement to a diet with a low casein content as source of protein, yeast may even support dietary hepatic injury, especially necrosis.[6] Furthermore, yeast is rich in vitamins of the B group and in many other cell constituents. Thus, curative effect exerted by yeast has to be considered too complex to permit detailed analysis. Judging from prophylactic studies based on pathological findings in the dead animal a curative effect similar to that of yeast might be logically anticipated from simple substances such as methionine or methionine-containing proteins, for example casein or lactalbumin. In contrast, even manifold increased vitamin B supple-

[5] György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355. Also György, P., *Am. J. Clin. Path.*, 1944, **14**, 67, with bibliography.

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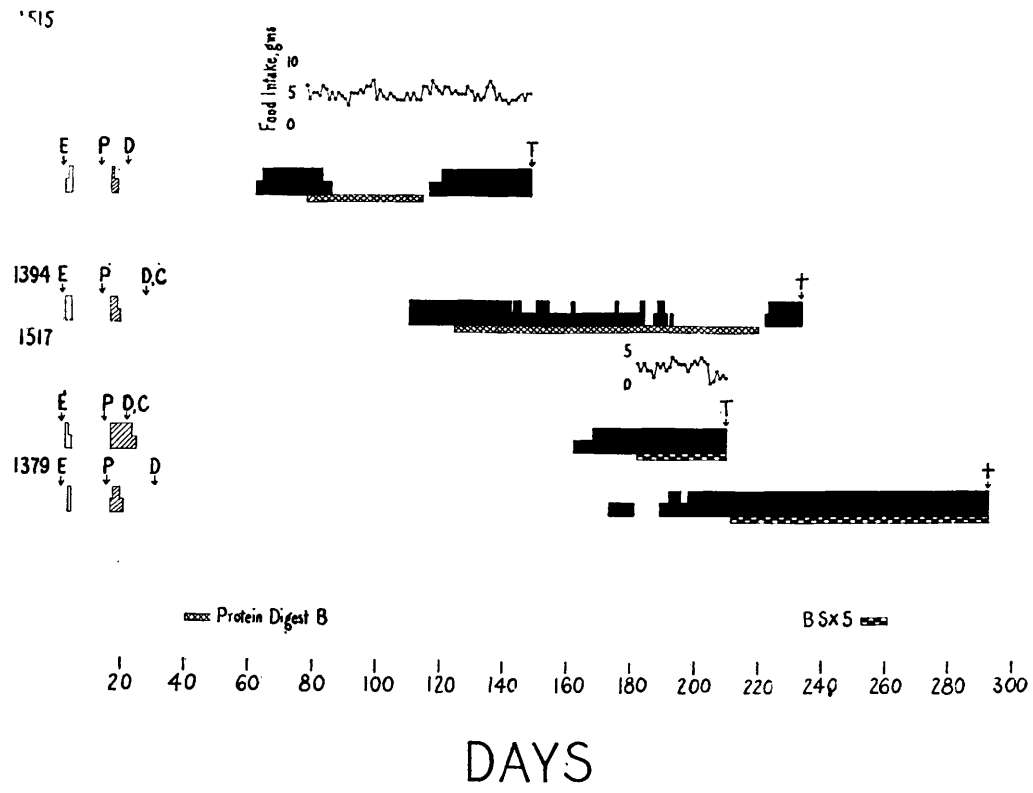

FIG. 2.

The effect of protein digest—lactalbumin digest (in 2 of 5 rats in Group D) and of fivefold vitamin B supplements (in 2 of 13 rats in Group E). The symbols have the same meaning as in Fig. 1.

ments should prove to be devoid of any curative effect.

The following experiments deal mainly with these special questions. They were undertaken to produce supporting evidence for the usefulness of the method of estrus reaction in the study of experimental dietary hepatic injury in the living animal.

Methods. Rats used were of 3 separate strains: 1. Sprague-Dawley (65 rats), 2. Wistar (9 rats), and 3. a strain obtained from a local dealer (17 rats). Animals were selected with a weight of 150-230 g, the majority weighing approximately 200 g. The preparation of the animals for the experiments, including surgical technic, was the same in the previous study.[4] The experimental diet was slightly modified, as follows: Casein (SMACO) 8, Crisco 38 (or, in some experiments, Crisco 30 and peanut oil 8), sucrose 50, salt mixture 4. The vitamin supplements were given separately: (a) Solution of B vitamins (1 cc daily) containing thiamine 20 γ, pyridoxine 20 γ, riboflavin 25 γ, Ca-pantothenate 100 γ. (b) Vitamins A and D in form of Ol. percomorph., 3 drops weekly. (c) Vitamin E as special supplement was only used in the experiments with the local and Wistar strains (3 mg in the form of natural concentrate† once a week). In the remaining experiments the vitamin E requirement was covered solely by the basal diet with its high content of Crisco, a fat rich in vitamin E.‡ More than half of the experimental animals received as a further separate supplement 50 mg of *l*-cystine daily. The purpose of this supplement was the acceleration and aggrava-

† Kindly furnished by Distillation Products, Inc., Rochester, N.Y.

‡ According to a personal communication of Dr. K. E. Mason (Rochester), 5% of Crisco in the diet represents the minimal protective level.

tion of cirrhotic changes in the liver.[5]

Results. The appearance of estrus showed great variations. It is remarkable that 35 out of a total of 91 experimental animals remained in anestrus for the whole duration of observation, up to 150 days or more. Two rats showed only temporary or irregular estrus. In the remaining group of 54 rats regular estrus was observed 39 to 195 days, with a calculated average of 86 days after the animals were put on the basal experimental diet. In several instances estrus when first noticed showed some fluctuation before going into solidly sustained complete estrus (Fig. 1, rats 993 and 1011).

On the assumption that normal liver "inactivates" estrone, lack or retarded appearance of estrus should indicate absence or delayed production of hepatic injury with its characteristic pathological attributes. Such behavior would be at variance with the reactivity of normal rats[5] and could only be explained by the presence of estrone pellets in the spleen preceded by ovariectomy in the present experimental group of animals.§

Rats showing the appearance of continuous estrus were kept under observation for 2-4 weeks without any change in the experimental conditions. At the end of this preliminary period the animals were divided in 5 subgroups (A to E). Subgroup A was kept as control on the unchanged original regime. Rats in B received *dl*-methionine‖ in daily doses of 50 mg, in C a digest of casein (Amigen) 1 g daily, in D a digest of lactalbumin (Lactamin)‖ 1 g daily. In subgroup E the daily basal vitamin supplements were increased fivefold. In instances when cystine was given before the appearance of regular estrus, it was discontinued as soon as treatment with lipotropic factors (methionine or protein digests) started.

Once established, full estrus persisted throughout the total period of observation in Group A (9 animals), in which the original experimental conditions remained unchanged, and in Group E (13 rats) in which the animals received a fivefold increase in the amount of basal vitamin B supplements. The solid curve of full estrus in such animals (See Fig. 1 and 2 with representative examples) served also as control for experiments in which estrus has disappeared under the influence of special curative measures. Of course, even without special treatment estrus, when artificially supported by a pellet of estrone implanted in the spleen, cannot last permanently and must cease as soon as the pellet is completely absorbed or becomes tightly encapsulated (Rat No. 1018 in Fig. 1). This last possibility has to be borne in mind in cases where estrus was seemingly suppressed after application of special curative measures. In order to avoid false conclusions a positive result should not be considered as such unless with discontinued therapy estrus reappears, thus proving the presence of properly functioning estrone pellets.

This special experimental procedure was fully observed in subgroups B, C, and D in which the therapeutic effect of methionine (16 animals), casein-digest (11 animals), and lactalbumin-digest (5 animals) was investigated. The results have shown unequivocally that methionine (Fig. 3) as well as the 2 protein-digests (representative examples for lactalbumin in Fig. 2) exerted a definite therapeutic effect which manifested itself in complete or at least in partial suppression of estrus. Interruption of treatment was followed in due course by reappearance of full estrus. The time required for the suppression of estrus by means of supplements of lipotropic factors showed considerable variation, ranging from a few days to 10 weeks, with an average of 31 days.

In a number of rats (in all subgroups) daily food intake was measured. No correlation could be found between appearance or disappearance of estrus and the curve of daily food intake.

Discussion. The assumption submitted previously[4] that impairment of estrone inactivation by the liver observed in rats on a cirrhosis-producing diet resulted from the specific dietary hepatic injury, is well borne out by the present studies. Fivefold increase in the amount of daily vitamin B supplements

§ This special problem will be discussed in a separate communication. Preliminary report appeared in *Federation Proceed.*, 1945, **4**, 155.

‖ Kindly furnished by Wyeth, Incorporated.

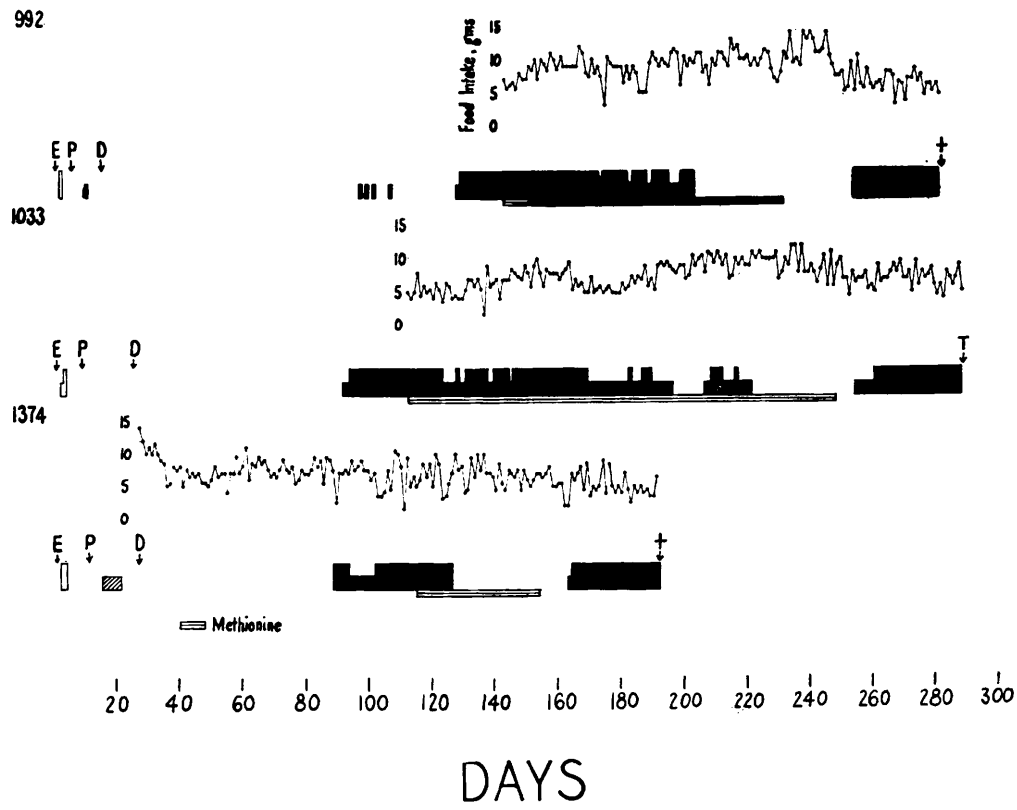

FIG. 3.
The effect of methionine (in 3 of 16 rats in Group B). The symbols have the same meaning as in Fig. 1 and Fig. 2.

had no effect on estrus appearing in this group of rats. Thus it can be denied that the liver injury produced by cirrhosis-producing diet has led to impairment of estrone inactivation solely by increasing the need for the B vitamins. Such relative vitamin B deficiency would have been the counterpart of the similar effect of absolute lack of B vitamins.[1]

With the demonstration of the therapeutic effect exerted by lipotropic factors such as methionine or methionine-containing protein digests (casein and lactalbumin) the specific character of the impairment of estrone inactivation in this group of rats is well substantiated. The same lipotropic factors play a leading part in the prevention of dietary hepatic injury, proven by macroscopic and microscopic evidence.[5] The present observations should arouse special interest for two reasons: 1. They were made on living animals, and 2. They indicate strongly the possibility of therapeutic reversal of the underlying hepatic changes, in contrast to past routine studies of prophylactic nature. Inasmuch as treatment was initiated soon after the functional hepatic disturbance became apparent, it is improbable that major fibrotic changes in the liver could have been developed before therapy was started. In consequence, the restored suppression of estrus should not be construed as an outward sign of completely restored architecture. Morphological cure may be deduced only from direct histological examination. On the other hand the methods of estrone inactivation could become useful in the evaluation of possible therapeutic factors on living animals suffering from dietary liver injury. It is conceivable to regard estrus appearing in rats fed a cirrhosis-producing diet as the equivalent of pseudo-estrus of vitamin A deficiency, based on the inability of cirrhotic livers to utilize vitamin A of the

diet. This explanation, however, disregards the important fact that estrus disappeared even in cirrhotic rats as soon as the estrone pellet was completely absorbed. Lack of estrone should not influence the pseudo-estrus of vitamin A deficiency.

Summary. Impairment in ability to inactivate estrone accompanies dietary hepatic injury and can be rectified by the addition of lipotropic factors such as methionine or protein digests.

Fivefold increase of the basal vitamin B supplements was ineffective.

The author is greatly indebted to Mrs. C. K. Gilkey for technical assistance.

15185

Studies on Toxicity Complement-Fixing and Immunogenic Activity of Typhus-Infected Yolk Sacs.

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The use of the yolk sac of the developing chick embryo for cultivating typhus rickettsiæ[1] led to the development of vaccines prepared from infected yolk sacs by phenol precipitation[2] and ether extraction[3,4] and to the demonstration of a labile toxic substance in yolk sac cultures of typhus rickettsiæ which was lethal for mice and which was associated with the rickettsiæ themselves.[5,6]

It has been shown that both the complement-fixing activity and the rickettsial content of typhus vaccines were related to the time of harvest of the constituent yolk sacs with reference to the peak of embryo deaths.[7] However, it was also observed that the presence of numerous rickettsiæ did not necessarily indicate toxicity[6] and that the antibody response of guinea pigs, commonly used as a measure of antigenicity, is subject to considerable variations.[8] It was of interest, therefore, to compare complement-fixing activity with the toxicity of yolk sac suspensions and with the antigenicity and rickettsial content of typhus vaccines.

Methods. Eggs in the 7th day of embryonic development were inoculated into the yolk sac with the Breinl strain of epidemic typhus according to the method of Cox.[1] The eggs were candled daily and all yolk sacs harvested were frozen immediately and stored at −70°C until used. All vaccines were prepared by the ether extraction method[4] using a standard procedure (*cf.* [7]).

Complement-fixation tests were done according to the method of Bengtson.[9] Vaccines were titrated with 2 units of hyperimmune guinea pig serum and the titer was recorded as the highest dilution of vaccine showing complete (4+) fixation.

Toxicity titrations were performed as follows: 0.5 ml of serial 2-fold dilutions of the various yolk sac suspensions was injected into

* On temporary leave from the Division of Microbiology, The Squibb Institute for Medical Research.

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