cates that the stability of riboflavin in alkaline solutions may be influenced by the type of buffer and the concentration of the vitamin.

However, the destruction of nicotinic and folic acids cannot be attributed to the alkaline reaction. According to Hundley (*8*), nicotinic acid is stable on heating with 1 or 2*N* alkali. Folic acid has been kept in solution (protected from light) at pH 9 and 10 for 6 months in the authors' laboratory in the refrigerator without showing any change in potency. This further evidence indicates that alkalinity resulting from ethylene oxide exposure is not the sole factor responsible for the destruction of vitamins.

The influence of moisture in the diets on the destruction of vitamins is now under study. Early in this work it was observed that the presence in the desiccator of a wad of cotton saturated with water during ethylene oxide treatment had no influence on the growth inhibition resulting therefrom. Although the atmosphere may have been saturated with water vapor, there is a possibility that the diet itself had not come into equilibrium with the water.

Discussion

A recent report indicates that when yeast and a rat diet composed of natural products were exposed to ethylene oxide according to one of the methods used commercially, there was no significant destruction of thiamine, riboflavin, nicotinic acid, pantothenic acid, or choline (*11*). There was, however, an indication that some folic acid and pyridoxine were destroyed. The conditions used in the preceding study differ in a number of respects from those used in the present study. The commercial procedures for chemical sterilization with ethylene oxide vary considerably and in some cases approximate those used in the present study.

The work reported in this paper reemphasizes the importance of evaluating the nutritional effects resulting from the treatment of a food with any compound. It is not necessarily enough to study the toxicity of the compound itself, since it may react with substances in the food to produce alterations in nutritional value which would not be apparent in the results obtained from the application of classical toxicological procedures.

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TOXICITY IN OIL MEAL

Bioassay for Toxic Factor in Trichloroethylene-Extracted Soybean Oil Meal

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A bioassay with calves has been developed for estimation of the toxic factor in trichloroethylene-extracted soybean oil meal which is responsible for the production of a fatal aplastic anemia in the bovine. Using the thrombocyte count and the percentage of lymphocytes as the principal and the total leucocyte count and death as supplementary criteria of toxicity, it is possible to differentiate among the biological effects on calves of daily doses of 1/100, 1/40, 1/20, 1/8, 1/6, 1/4, or more pound per day per 100 pounds of body weight of a standard specimen of the toxic meal. Acute or prolonged aplastic anemia of different degrees of severity can be produced in calves by feeding different levels of toxic trichloroethylene-extracted soybean oil meal.

SINCE A SEVERE HEMORRHAGIC DISEASE in cattle was first associated with the feeding of trichloroethylene-extracted soybean oil meal (*17*), this disease has again been encountered in several countries where trichloroethylene was used for the extraction of oil from soybeans [for a review of the literature see (*5*, *11*, *14*)]. During a recent outbreak of this disease in Minnesota (*11*) it was characterized as an aplastic anemia (*11*, *15*) and under controlled experimental conditions the disease has been reproduced in calves (*6*, *9*), heifers (*12*), milch cows (*9*), and horses (*7*) with specimens of trichloroethylene-extracted soybean oil meal of known origin. It has also been clearly established (*8*, *16*, *17*) that the consumption of trichloroethylene is not responsible for the toxic effects of trichloroethylene-extracted soybean oil meal, but the nature of the toxic agent is not known. Until it is identified and can be detected

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by relatively simple tests, the processing of soybeans, and perhaps of other products, with trichloroethylene will entail definite hazards, particularly because many of the conditions required to produce trichloroethylene-extracted soybean oil meal of high or low toxicity are not understood (*12*).

Before proceeding to concentrate the toxic factor in trichloroethylene-extracted soybean oil meal it was clearly necessary to develop an assay that could be used as a guide in the fractionation of toxic specimens. Previous experiments in these laboratories (*12*) with heifers showed that the onset and severity of signs of aplastic anemia of the bovine were affected by the amounts of trichloroethylene-extracted soybean oil meal consumed. This observation suggested that a bioassay based on the time of induction and the severity of signs of aplastic anemia of calves might be feasible. Moreover, the production of controlled degrees of aplastic anemia in any species would be of value for biochemical and other investigations of the hematopoietic processes.

Specimens of trichloroethylene-extracted soybean oil meal of known, high toxicity to the bovine and to the horse were administered to many other different species which, however, were found to be relatively resistant to the toxic agent in trichloroethylene-extracted soybean oil meal and therefore, under present conditions, not suitable for development of an assay. This applies to mice, rats, guinea pigs, hamsters, rabbits, dogs (*13*), swine (*3*), goats, sheep (*10*), chickens, including chick embryo explants, turkeys, geese (*4*), *Tribolium confusum*, *L. citrovorum*, *L. arabinosus* 17-5, *L. bulgaricus* 0-9 (*2*), *Tetrahymena pyriformis* W, and T_6 coliphage. In the young calf, however, fatal aplastic anemia can be induced relatively quickly (*8*) with certain specimens of trichloroethylene-extracted soybean oil meal. This animal was therefore selected for development of the bioassay reported herewith.

Experimental

Female Holstein calves, purchased locally and weighing initially about 100 pounds, were used. During a pretrial period of about 2 weeks they were fed mineralized cow's milk. The trial was started when clinical and hematologic observations indicated that the animals were normal.

The same specimen of trichloroethylene-extracted soybean oil meal, identified as TCESOM-6, was used for all experiments on the development of the bioassay. It was produced in October 1953 from 1953-crop soybeans in a commercial plant using the trichloroethylene extraction process (*18*). Control animals were fed hexane-extracted soybean oil meal purchased on the open market, at the same level of intake as trichloroethylene-extracted soybean oil meal or not less than $^1/_6$ pound per 100 pounds of body weight per day. To compensate for differences in initial body weight of the calves and to maintain the same relative intake throughout the assay period, the soybean oil meal was fed at a given level per day per 100 pounds of body weight. (Throughout this paper reference to a certain level of feeding of trichloroethylene-extracted soybean oil meal indicates the dosage per day per 100 pounds of body weight.) Required increments of feed were based on weekly weighings of the animals.

The calves were fed twice daily. For each feeding, one-half of the required daily dose of finely ground trichloroethylene-extracted soybean oil meal was suspended in a small amount of cow's milk and, in most instances, given orally. Complete consumption of the meal was assured by the addition, in several small increments, of more milk until the total amount of milk consumed per feeding was about 5% of the body weight of the calf. The milk was fortified by the addition of a solution which furnished, per pound of milk, 75 mg. of magnesium oxide, 10 mg. of iron citrate, 0.75 mg. of copper sulfate, and 0.57 mg. of manganese sulfate. To a few animals the meal was given through an abomasal fistula (*8*). The purpose of this procedure was to assure quantitative administration of relatively large amounts of trichloroethylene-extracted soybean oil meal and to develop a technique by which unpalatable concentrates might be given. The hematologic response of the calves thus fed was the same as that of calves fed orally. Because of the relatively high toxicity of the available meal and hence the small daily dosage required, constant use of this technique was unnecessary. The calves were muzzled throughout the course of the assay period and kept in pens bedded with wood shavings. Two calves which were not muzzled and received alfalfa hay *ad libitum* in addition to milk and trichloroethylene-extracted soybean oil meal showed essentially the same hematologic response as muzzled calves fed the same amount of trichloroethylene-extracted soybean oil meal. Water was available to the animals at all times.

Measurements of rectal temperatures and examinations for clinical signs were performed daily. Blood was withdrawn at frequent intervals, in most instances daily, from the jugular vein and used for determination, by standard methods (*1*), of hematocrit, hemoglobin, red cell count, total and differential white cell count, and thrombocyte count. From most of the calves bone marrow biopsy specimens were removed at weekly intervals or more often by puncture of a sternebra, the tuber ischii, or the tuber coxae of the pelvic girdle.

When the calves became moribund or after an assay period of about 60 days, they were slaughtered and examined for gross lesions, tissues were removed for microscopic study, and blood and bone marrow specimens were collected for

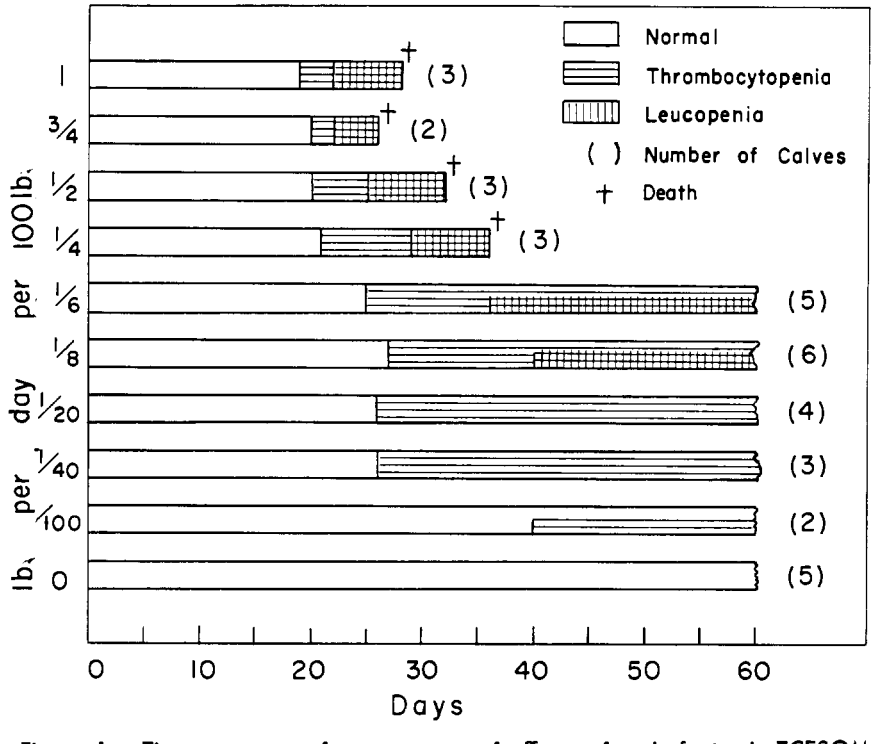

Figure 1. Time sequence of appearance of effects of toxic factor in TCESOM

In groups represented by bars, in which the symbol does not cover the whole width of the bar, not all calves showed this symptom.

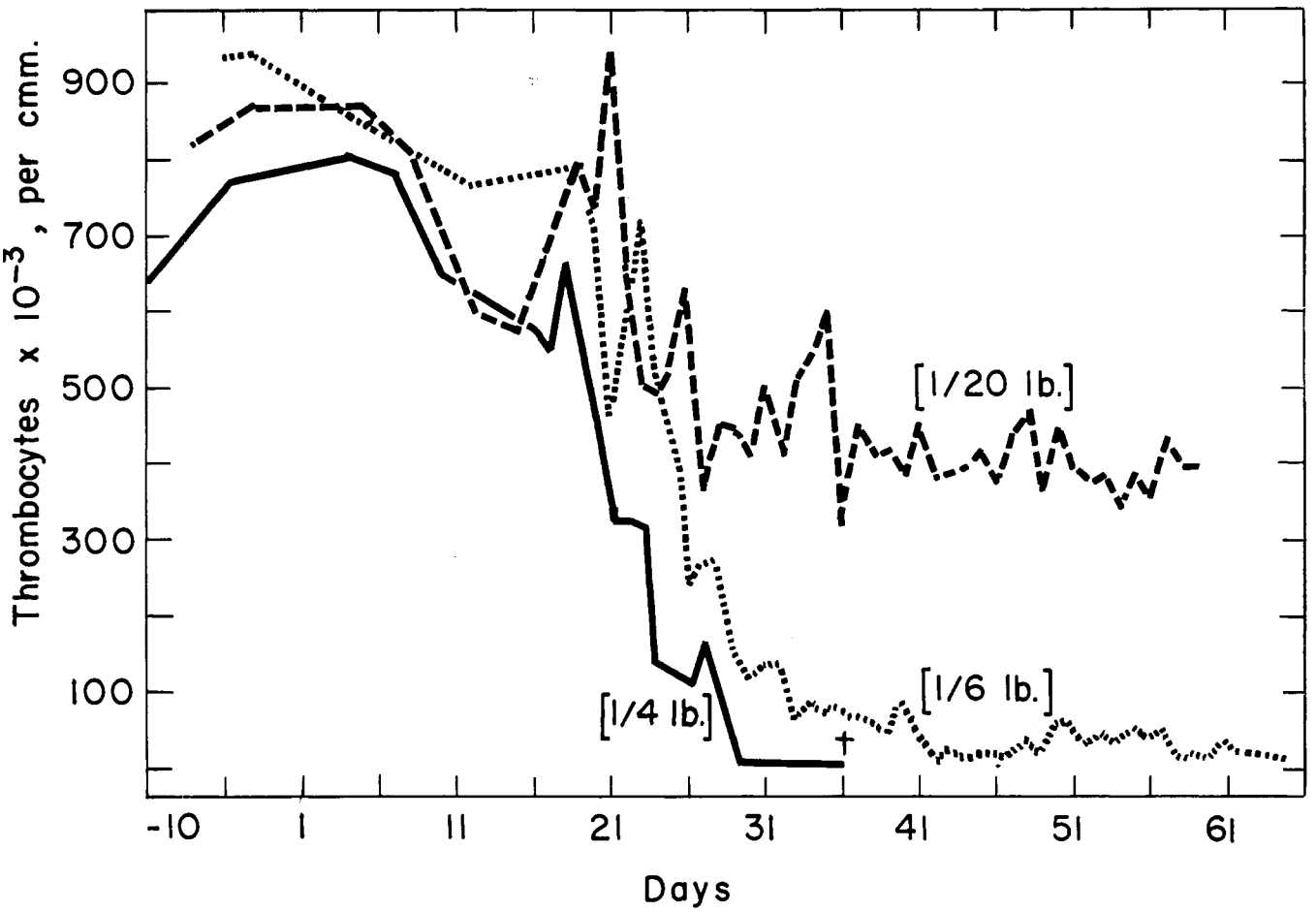

Figure 2. Individual records of thrombocyte counts of three calves fed different levels of TCESOM

[- - - lb.] per day per 100 lb.

biochemical investigations. The results of these studies will be reported in another paper.

Results and Discussion

Criteria for Evaluation of Toxicity. Experience with these and other calves fed trichloroethylene-extracted soybean oil meal has indicated that the most useful criteria for differentiating between the biological effects of feeding different levels of this product are the establishment and progress of thrombocytopenia, leucopenia, the development of a relative lymphocytosis, and the occurrence of death. Other indexes of toxicity such as increased clotting time, increased bleeding time of the blood, decreased clot retraction, hemorrhages on the visible mucous membranes, pyrexia, and changes in the serum albumin–globulin ratio occur mainly during the terminal stages of the intoxication and offer no advantage over the other criteria referred to above. Extensive cytologic studies of stained smears of bone marrow biopsy specimens have shown that such examinations are not suited either for the detection of incipient signs of aplastic anemia or for differentiation of the various levels of intake of trichloroethylene-extracted soybean oil meal which are not lethal in the assay period.

Evaluation of Response to Toxic Trichloroethylene-Extracted Soybean Oil Meal. The time required for the onset of various symptoms of trichloroethylene-extracted soybean oil meal intoxication is shown in Figure 1. On about the 20th day of the trial or shortly thereafter there occurred a marked decrease of the thrombocyte count in all calves fed $^1/_{40}$ pound of trichloroethylene-extracted soybean oil meal or more. At high levels of intake of trichloroethylene-extracted soybean oil meal this was always followed within a few days by a leucopenia and the establishment of a relative lymphocytosis (the latter is not indicated in Figure 1). All calves receiving $^1/_4$ pound or more of the standard specimen of trichloroethylene-extracted soybean oil meal died before the 40th day of the trial, whereas all calves receiving $^1/_6$ pound or less survived the 60-day assay period in good condition in spite of severe blood dyscrasia at the $^1/_6$- and $^1/_8$-pound levels. Death of the calves, therefore, serves to differentiate roughly between an acute form of aplastic anemia and a more prolonged form. Although the calves fed $^1/_4$ pound of trichloroethylene-extracted soybean oil meal died a few days later than those fed higher levels, the assay is relatively insensitive when such amounts of a very toxic specimen are fed.

In the more prolonged form of aplastic anemia, as observed in calves fed from $^1/_{40}$ to $^1/_6$ pound of trichloroethylene-extracted soybean oil meal, there were qualitative and quantitative differences in the severity of the blood dyscrasia. In all of these calves the thrombocyte count also decreased abruptly shortly after the 20th day. However, the severity and progress of the thrombocytopenia were dependent on the amounts of trichloroethylene-extracted soybean oil meal fed. As shown in Figure 2, with $^1/_6$ pound the decrease in blood platelets was very rapid and proceeded until extremely low levels of about 100,000 or less per cu. mm. were attained and maintained. On the basis of the thrombocyte count alone, the response to $^1/_4$ or $^1/_6$ pound could not be differentiated but the occurrence of death at the higher level permits such differentiation. When $^1/_{20}$ pound was fed, however, the thrombocyte count, after an initial rapid decrease, became somewhat stabilized at a level of about 400,000 per cu. mm. (Figure 2). Feeding of $^1/_8$ pound led to an average thrombocyte count, after 30 days, which was intermediate between the values ob-

tained with $^1/_{20}$ and $^1/_6$ pound. The severity of the thrombocytopenia can therefore serve as one criterion by which the effects of feeding different levels of trichloroethylene-extracted soybean oil meal may be differentiated. Only one of the two calves fed $^1/_{100}$ pound of trichloroethylene-extracted soybean oil meal developed thrombocytopenia within the assay period.

Determinations of the total and particularly the differential leucocyte counts also furnish useful information. While the former is subject to great daily variations not only between individuals but sometimes also in the same animal, the occurrence of a leucopenia was usually observed in calves fed $^1/_8$ and $^1/_6$ pound of trichloroethylene-extracted soybean oil meal although sometimes only for periods of 5 to 7 days. As a result of the predominant decrease of cells of the neutrophilic series, a marked relative lymphocytosis developed gradually in calves fed $^1/_{20}$ pound of trichloroethylene-extracted soybean oil meal or more. The extent of the relative lymphocytosis and the thrombocytopenia, considered jointly, furnish apparently the most useful information for differentiating between different degrees of prolonged intoxication by trichloroethylene-extracted soybean oil meal under the conditions of this assay.

Table I. Base-Line Values of Different Groups (through 20 days)

TCESOM[a] Fed, Lb./Day/100 Lb.	Thrombocytes × 10^{-3} per Cu. Mm.			Lymphocytes		
	Count	±Std. e.m.[b]	Samples	%	±Std. e.m.[b]	Samples
0	715	13.8	52	62.6	1.29	47
1/100	845	20.1	33	57.9	1.62	33
1/40	861	15.3	41	64.1	1.46	40
1/20	799	20.5	46	56.2	1.49	46
1/8	889	17.0	75	57.1	1.29	74
1/6	771	25.2	27	60.9	1.21	27
Mean of all groups	820		274	59.5		267

[a] Trichloroethylene-extracted soybean oil meal.
[b] Std. error of mean.

Attempts to use hematologic data for quantitative evaluation of a toxic compound must take into account individual variations and apparently normal daily fluctuations of the counts. This applies particularly when the experimental animals are not from uniform stock. The data in Table I show that the mean thrombocyte and relative lymphocyte counts obtained during the pretrial period and during the first 20 days of the assay—that is, before the effects of intoxication by trichloroethylene-extracted soybean oil meal became apparent—were not uniform in all groups.

To evaluate subsequent changes in blood counts from the base-line values it is necessary to let each animal, or each group of similarly treated animals, serve as its own control. If the mean thrombocyte and relative lymphocyte counts through the first 20 days are taken as base-line values of 100 and the

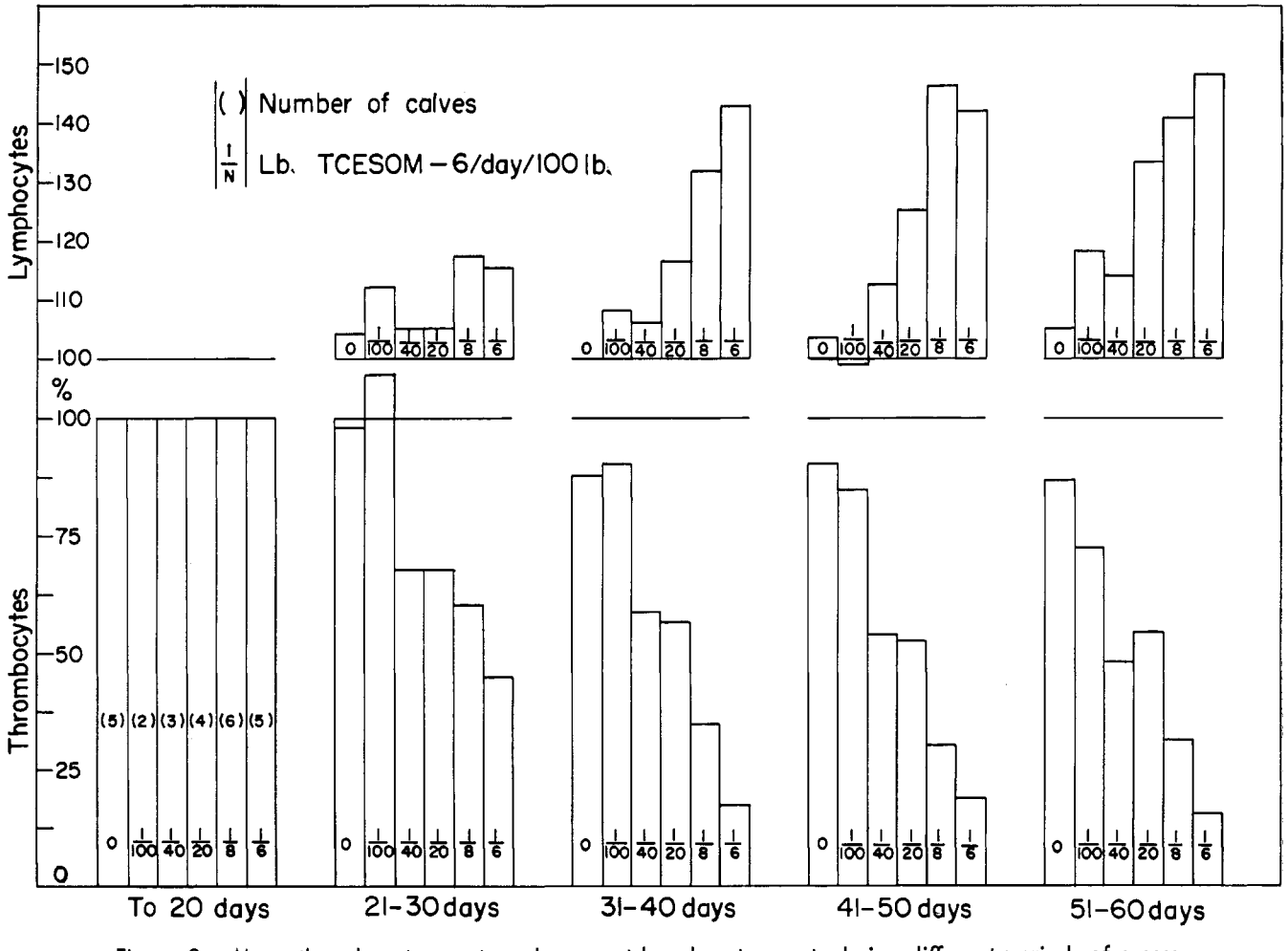

Figure 3. Mean thrombocyte counts and per cent lymphocyte counts during different periods of assay

All values are referred to a base-line count of 100, which represents the mean observed in each group during the pretrial period and the first 20 days of the assay.

mean counts in subsequent 10-day periods are calculated as per cent of the base-line values, a relationship between the intake of toxic trichloroethylene-extracted soybean oil meal and the severity of the blood dyscrasia becomes apparent. Examination of the data in Figure 3 shows that it is possible, through the thrombocyte count, to differentiate among the biological effects produced by $^1/_6$, $^1/_8$, $^1/_{20}$, and $^1/_{100}$ pound of the standard specimen of trichloroethylene-extracted soybean oil meal. When the progressive development of the relative lymphocytosis is considered in conjunction with the thrombocyte counts, a differentiation of the effects of $^1/_{20}$ and $^1/_{40}$ pound can also be made.

The data illustrated in Figure 3 show clearly that through feeding of graded levels of trichloroethylene-extracted soybean oil meal a prolonged type of aplastic anemia of different degrees of severity can be induced and that this, in turn, can be used to assay the toxicity of the various specimens of trichloroethylene-extracted soybean oil meal. In the application of this assay as a guide for the preparation of concentrates of the toxic factor, the level of feeding should be so chosen that the hematologic response corresponds to that produced by $^1/_6$ to about $^1/_{40}$ pound of the standard specimen. When larger amounts are fed, a reliable differentiation between various quantities producing an acute aplastic anemia can probably not be made (see Figure 1).

Necropsy findings were of little value as criteria for the assay. In the animals which were slaughtered in moribund condition the distribution and severity of the hemorrhages were very variable and bore no distant relationship to the intake of trichloroethylene-extracted soybean oil meal. In those animals which survived the 60-day assay period distinct hemorrhagic lesions were found only in calves receiving $^1/_6$ or $^1/_8$ pound of trichloroethylene-extracted soybean oil meal, and these were often limited to petechial hemorrhages. Similar considerations apply to the examination of stained smears of bone marrow biopsy specimens. In the animals fed $^1/_6$ pound of trichloroethylene-extracted soybean oil meal or more there was often marked depression of the neutrophilic series of cells and in those that became moribund a marked predominance of the lymphoid elements was observed during the terminal stages. Such examinations, however, did not afford criteria of toxicity which could not be established equally well or better by the examination of the peripheral blood.

A total consumption of less than 1 pound of trichloroethylene-extracted soybean oil meal is sufficient to produce signs of aplastic anemia in calves, and the total quantity of trichloroethylene-extracted soybean oil meal consumed is not related to the time of onset of the first signs of aplastic anemia. There is at present no valid explanation why, with the specimen used, an interval of about 20 days was invariably required before aplastic anemia developed; the extent to which different amounts of trichloroethylene-extracted soybean oil meal are digested and absorbed is unknown but it may have a bearing on this problem, particularly when concentrates of the toxic factor are assayed.

With specimens of other trichloroethylene-extracted products the first signs of thrombocytopenia in calves have been observed as late as 80 days after the start of feeding. The observations made in the present studies, when $^1/_{100}$ pound of trichloroethylene-extracted soybean oil meal was fed, do therefore not appear to represent the lowest level of sensitivity of the calf for a response to the toxic factor. For the purpose of a practical bioassay, however, it would not be profitable to extend the assay for longer periods and the responses to very low doses of the toxic factor might be difficult to interpret.

The control animals fed hexane-extracted soybean oil meal remained normal throughout the period of assay. With increasing age of the calves there was a gradual, slight decrease of the thrombocyte count (see Figure 3), which should not be confused with the thrombocytopenia induced by trichloroethylene-extracted soybean oil meal. In the control group two animals were also encountered which had temporary episodes of relative lymphocytosis. This was not of sufficient severity or duration, however, to affect appreciably the mean values illustrated in Figure 3.

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