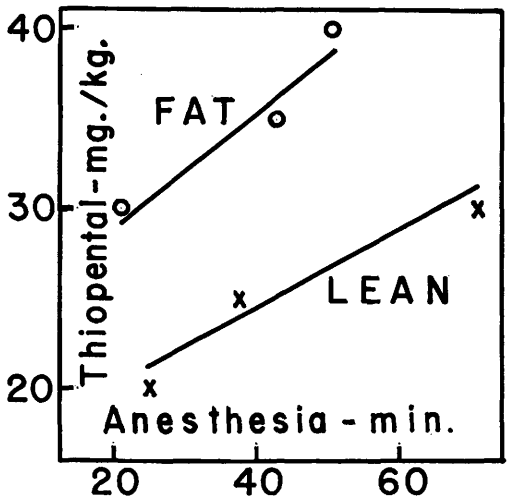

FIG. 1. Relationships between duration of anesthesia and thiopental dosage.

erence to the body fat.

Summary. A 5 g % decrease in body fat of the rat resulted in a 100% increase in length of thiopental anesthesia at a fixed thiopental dosage, or a 40% decrease in the amount of thiopental required for a given duration of anesthesia. In contrast the duration of pentobarbital anesthesia was unaffected by the amount of body fat.

We wish to express our appreciation for the suggestions and criticism received from Dr. Oliver H. Lowry and Dr. Morris Friedkin throughout the course of this investigation.

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Casein and Factor 3 in Dietary Necrotic Liver Degeneration; Concentration of Factor 3 from Casein. (19610)

KLAUS SCHWARZ. (Introduced by Floyd S. Daft.)

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Md.

It is a general experience that standard vit. E-free test diets which contain 10% or more of so-called "vit. free," *i.e.*, alcohol-extracted casein cannot be used for the production of dietary necrotic liver degeneration in rats. The deficiency does develop, however, when the animals are raised on rations containing only 4 to 6% of the "vit. free" casein(1,2). A purified, alkali-treated Hammarsten* casein ("casein VI"), on the other hand, had been used over a period of years at levels of 15 and even 30% for the production of dietary necrotic liver degeneration in experiments which led in 1944 to the detection of the protective activity of vit. E(3,4). It was stated at that time that "the most important condition for the development of the liver damage is to be seen in the nature of the casein used," and that "the occurrence of protective activity in completely dissimilar materials demonstrates that several different substances must exist which have an effect. . . ." (3). Evidence accumulated thereafter for a protective substance which was removed during the preparation of casein VI and which was different from cystine or from vit. E remained unpublished.† Recently, however, we reported the presence of such a factor in certain yeasts(6). The active principle, designated factor 3, prevents the development of dietary necrotic liver degeneration and is found in a wide variety of natural sources.‡

* "Hammarsten" casein (O. Hammarsten 1877, l. c. 7.) is prepared from freshly obtained casein by repeated acid precipitation from dilute alkaline solution and final drying by ethanol and ether.

† See discussion in: Liver Injury, Transactions of the Eighth Conference, Josiah Macy, Jr., Foundation, New York, 1949, p49.

‡ To be published separately.

TABLE I. Protective Effect of Casein and Casein Preparations.

Exp.	Supplement	No. of animals	Dead due to liver necrosis	Avg reciprocal survival time (×100)	Activity, units/g of supplement
A*	0	8	8	2.00 ± .19‖	—
	Crude casein (3%)	9	2	.32 ± .25	9
	"Vit. free" casein a (3%)‡	10	2	.19 ± .12	9.6
B*	0	9	9	2.19 ± .19	—
	"Vit. free" casein b (3%)‡	10	3	.72 ± .41	7.2
	Amino acid mixture (3.6%, ≅ 3% casein)§	10	10	2.03 ± .31	0
C*	0	9	9	2.10 ± .09	—
	Alcohol extr. obtained during casein b production (≅ 5%)	10	10	2.22 ± .13	0
D*	0	9	9	2.74 ± .22	—
	"Vit. free" casein b (3%)‡	10	7	1.19 ± .31	6
	Hammarsten casein prepared from casein b (3%)	10	9	1.98 ± .31	3
E†	0	9	9	2.51 ± .24	—
	Bacto Casitone (2%)‡	10	6	1.1 ± .32	9

* Prophylactic method. † Repletion method (supplementation started 24th day). ‡ Commercial sample. § Composed according to figures given by R. J. Block and D. Bolling, The Amino Acid Composition of Proteins and Foods, C. C. Thomas, Springfield, Ill., Ed. 1945, p. 303. ‖ Mean ± stand. error.

TABLE II. Purification of Active Substance from Bacto Casitone.
(Tests by repletion method.)

	Dry wt, mg/g of starting material	Activity u/g of starting material	Activity u/g of dry wt	Approximate conc.
Bacto Casitone	1000	10.5	10.5	1 : 1
Charcoal filtrate*				
Charcoal eluate	356	10.5	29.6	2.8 : 1
Alcohol precipitate from charcoal eluate	261	3.7	14.4	1.4 : 1
Alcohol filtrate	68.4	3.8	56	5.3 : 1
Precipitate from water sol. of alcohol filtrate	12.2	4	320	30 : 1
Water filtrate after separation of precipitate	88†	1.6	27	2.6 : 1

* Not tested. † After neutralization.

In this paper evidence is presented for the occurrence of factor 3 in crude and purified samples of casein and of enzymatic casein digests. The 30-fold concentration of the active substance from an enzymatic casein hydrolysate is described.

Experimental. a) *Animal experiments.* Details concerning the animal experiments were reported previously(5,6). Male Sprague-Dawley rats of the National Institutes of Health colony were used. The animals were weaned at 21 to 24 days of age, at which time their average body weight was 41 g. Litter-matched groups of 10 rats of equal average weight were formed. In each experiment one group was fed the basal ration as a control. The basal diet consisted of 30% torula yeast, 59% sucrose, 5% salts, 5% vit. free lard and a vitamin mixture(5). From this ration the experimental diets were obtained by the addition of supplements at the expense of sucrose. Tests for protective activity were performed either by a prophylactic method (Exp. A-D, Table I), *i.e.*, supplementation was started immediately after weaning, or by a repletion method (Table II), in which case supplementation started after a period of 23 days on the basal diet(6). To express the speed of development of the deficiency, the average of the reciprocal survival time was employed and compared, as described before(3,5). With increasing dietary levels of supplements containing factor 3, the reciprocal survival time decreased. A straight line relationship was observed over a wide range of dosage.‡ Protective effects were evaluated in units (u), one u being defined as the amount of factor 3 required per day per animal to effect a 50% reduction of the average reciprocal survival time as compared to the unsupplemented lit-

termate control group. In other words, one u of factor 3 per day per animal doubled the (virtual) average survival time of the rats. It was found, for example, that 1 u was contained in about 0.1 g of Bacto Casitone.§ It is recognized that the test method used does not represent a perfect biological assay procedure. It permits, however, an estimation of the approximate factor 3 activity of natural materials and preparations therefrom. Repeated tests yielded results consistent within a range of ± 15%.

b) *Concentration procedure.* One thousand g of Bacto Casitone were dissolved in 6 liters of water, the pH was adjusted to 8.7 by the addition of 6 N NaOH, the temperature of the solution was brought to 25°C and 1000 g of charcoal (Darco KB) were added with gentle stirring. The pH dropped below 8 and was brought back to 8.7 by 6 N NaOH before filtration (Buchner funnel). The filtrate was again adjusted to pH 8.7, diluted with 2 liters of water and treated a second time with charcoal as described above. The combined charcoal adsorbates were washed with 2 liters of water with stirring, the pH was kept at 8.5 by the addition of 6 N NaOH. After filtration the charcoal was stirred with 5 liters of ethanol at 50-55°C for 30 minutes. The alcohol elution was repeated. The combined ethanol eluates were evaporated under nitrogen to a sirup which was made up to 1 liter with water ("charcoal eluate"). Nine liters of ethanol were added slowly to the "charcoal eluate" with constant stirring. The precipitate was redissolved in 1 liter of water and precipitated again with 9 liters of ethanol. The mother liquors of the first and second precipitations were combined, cleared from turbidity by filtration and concentrated to a sirup which was taken up in 200 cc of hot water ("alcohol filtrate").

The water solution of the alcohol filtrate was kept at +4°C for 24-48 hours. The heavy, creamy precipitate which formed was separated by filtration through a Buchner funnel ("precipitate from water"). This fraction was about 30 times as active as the starting material, as shown below.

§ Difco Laboratories, Detroit, Mich.

Results. Factor 3 activity of casein and preparations. Addition of 3% crude casein and also of various commercial samples of alcohol-extracted "vitamin free" casein at a 3% level to the torula ration greatly inhibited the development of necrotic liver degeneration (Table I, Exp. A, B, and D). The 2 samples of "vit. free" casein of different origin showed an activity similar to that of crude casein. Substitution of an equivalent amount of amino acids, imitating the composition of casein, was without effect (Exp. B).

The alcohol extract obtained during the commercial production of "vit. free" casein was tested and found to be inactive (Exp. C). This shows that factor 3 is not removed to any extent from casein by continuous alcohol extraction.

When "vit. free" casein was purified by the procedure originated by Hammarsten(7), *i.e.*, by solution in alkali and reprecipitation with acid, about half of the "factor 3" activity was eliminated (Exp. D).

Commercial enzymatic casein digests, such as are widely used as ingredients in bacteriological media, showed an activity similar to that of casein, for example, Exp. E, Table I.

It is obvious from these findings that crude and also commercial alcohol-extracted "vit. free" casein contain a component actively protecting against the deficiency. That the effect must be attributed to a factor other than vit. E or cystine, the 2 established protective substances, was derived from the following considerations: "Vit. free" casein does not contain vit. E since such casein is a standard component of rations used for the production of vit. E deficiencies. The quantity of cystine (and methionine), on the other hand, which is supplied by the addition of 3% of casein to the ration is too small to be of consequence. It was found that levels of 0.2% or more of cystine in the diet are required to influence the course of necrotic liver degeneration under our experimental conditions(5).

More direct proof for the existence of factor 3 in casein and for its distinctness from vit. E and from cystine is provided by the fractionation of the active material.

Concentration of factor 3. In enzymatic casein hydrolysates, factor 3 appears to be, at

least in part, present in an easily accessible, low molecular form. Concentration of factor 3 was, therefore, initiated from an enzymatic casein digest as starting material. The animal test was used as assay procedure. The first 3 steps of concentration, described in detail above, have been used repeatedly with only slight variations. Results of a typical run are given in Table II. In order to avoid losing activity in the first step of the concentration process, the material was absorbed on relatively large amounts of charcoal from which the active substance could be removed by repeated treatment with hot ethanol. A loss of activity was not apparent during this process; the charcoal eluate contained about one-third of the original dry weight. Precipitation of inactive impurities, mostly peptides, by ethanol was employed as the next step. Half of the activity remained in solution, the other half was lost in the precipitate, which possibly contains a bound form of factor 3. From the "alcohol filtrate" further purification of factor 3 was provided by the separation of a portion less soluble in water. This product, comprising some organic and inorganic cations, amino acids, and other organic acids shows a sulfur content of less than 0.5%. It is 30 times as active as the starting material and is being used for the further purification of factor 3.

Discussion. The results show that casein contains factor 3. So far no essential difference between the active principle concentrated either from yeast or from casein has been encountered(8). The question, however, whether the active substance from these 2 sources is completely identical remains to be clarified. Both crude and "vit. free" casein compare favorably with the best known sources for factor 3, for example, with yeast K(5). It is apparent that alcohol extraction, the method used for the production of "vit. free" casein, does not remove factor 3. The customary vit. E free diets do not lead to necrotic liver degeneration, evidently because protection is afforded by the amount of factor 3 present in the "vit. free" casein.

Acid precipitation of casein from alkaline solution ("Hammarsten" procedure(7)) partly removed factor 3. This might explain why "casein VI" diets could be used for the induction of the deficiency in the older experiments. At that time "casein VI" had been prepared from a repeatedly reprecipitated "Hammarsten" casein by additional treatment with alkali in the heat and subsequent acid precipitation(3). For the preparation of casein VI, the purity of the starting material appears to be a decisive factor. Several attempts to produce casein VI from alcohol-extracted "vit. free" casein were unsuccessful(8-10).

A potential discrepancy might be seen between the protective effect elicited by the addition of 3% casein to the torula diet and the older findings that diets containing 4, 6, or even 10% casein produced the deficiency. This might be explainable on the basis of differences in the factor 3 contents of casein samples employed at various times. Whether other reasons are involved, however, has to be clarified by further experimentation. It is obvious, however, that the protective effect of casein on dietary necrotic liver degeneration is not due simply to the improvement of the protein supply; it is of a more specific nature.

Summary. 1. Samples of crude and "vit. free," alcohol-extracted casein, and also enzymatic casein digests, were found to contain considerable amounts of factor 3, which protects against dietary necrotic liver degeneration. This explains why the deficiency does not develop on customary vit. E free casein diets. 2. A method is described for the concentration of factor 3 from an enzymatic casein hydrolysate, affording a 30-fold purification in 3 steps, with an over-all recovery of about 38%. This concentrate is being used for the further purification of factor 3.

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Adenosine Triphosphatase Activity of the Vascular System.* (19611)

C. JELLEFF CARR, FREDERICK K. BELL, AND JOHN C. KRANTZ, JR.
(With the technical assistance of Mary Frances Byrd.)
From the Department of Pharmacology, University of Maryland, School of Medicine, Baltimore, Md.

The contractile mechanism which specializes skeletal muscle for quick movement is presumably the same as that which enables smooth muscle to maintain its tonus state. It has been shown that the energy-rich phosphate bonds of adenosine triphosphate are the sole immediate source of energy for skeletal muscle contraction. In addition, evidence points to the fact that ATP plays a central role in the maintenance of smooth muscle tone and in the initiation of its contraction. Dubois and Potter(1) devised a method to determine the amount of adenosine triphosphatase (ATPase), the enzyme which is responsible for ATP hydrolysis, in various tissues. Krantz, Carr and Bryant(2) showed that the acutely-acting vasodilators of the nitrite-nitrate series inactivated ATPases present in the aorta of rabbits. The interference with enzyme activity occurred with therapeutic levels of the vasodilators. Thus it appears possible that these vasodilators may exert their action through the ATP-ATPase mechanism as the target enzyme system in arterial tissue.

Owing to this observation we considered it of interest to study the ATPase activity of various segments of the vascular bed. Furthermore, the normal variations within a single species of animal appeared important. In addition, the variation of the vascular ATPase activity of different species was determined, and the effect of age upon the enzyme concentration in the vascular wall was estimated.

* The expense of this investigation was defrayed in part by a grant from the National Institutes of Health.

Method. The principal studies were carried out on male mongrel dogs approximately in middle life. The animals were maintained on a balanced laboratory ration for a period of several months until a good nutritional state was established. The animals were anesthetized with an intravenous dose of pentobarbital sodium, and the vessels removed immediately after anesthesia ensued. It was previously determined that this barbiturate did not affect the ATPase activity of the vessels. All determinations were carried out within one-half hour after tissue removal. It was determined that during this period the ATPase activity did not undergo diminution. This same procedure with suitable modifications, to adapt it to the various species, was employed in all animals studied, omitting barbiturate anesthesia. Rats were used for the studies concerning the influence of age on the enzyme owing to the ease of obtaining animals of definite age. The ATPase activity of the tissues was determined by the method of Bell, Carr and Krantz(3). This method employs the tissue incubation of DuBois and Potter, the precipitation of the phosphate as magnesium ammonium phosphate recommended by Griswold(4), and the use of ammonium sulfate as suggested by Lowry and Lopez(5). The recovery of phosphate from tissue by the method is approximately 90%. The reproducibility of the method on a single specimen of tissue is shown by a standard deviation of 0.18 unit and a coefficient of variability of 3%. The values are expressed in ATPase units as adopted by DuBois and Potter, *i.e.*, the number of gam-