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A Hitherto Unrecognized Factor Against Dietary Necrotic Liver Degeneration in American Yeast (Factor 3) (19240)

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Most American yeasts, in contrast to many European yeasts, do not induce necrotic liver degeneration when used as protein source in purified, vit. E free rations for rats. However, the deficiency can be obtained readily with American grown torula yeast(1), and it can be prevented either by cystine or by vit. E (2). In earlier work some evidence had been accumulated for the involvement of a third unidentified factor in the prevention of the defect(3). It had been noted particularly that the addition of a few percent crude casein, or ordinary "vitamin free", alcohol extracted casein to necrosis producing diets inhibited the damage,* whereas purified, alkali-treated casein "Hammarsten" did not influence the development and had even been used extensively as a main dietary component for the production of the deficiency(4).

It is shown in the following experiments that American yeasts G† and K.‡ which do not produce the defect(1) have a considerable protective activity when added in small amounts to torula diets. It is concluded from simple fractionation experiments with yeast K that this effect is not due to the presence of vit. E or cystine but that protection is brought about by a third, unidentified principle which has been designated "factor 3."

Experimental. Details concerning the induction of the deficiency have been described (1). Weanling Sprague-Dawley rats of the National Institutes of Health strain were used exclusively. Animals in this series were males except for a few groups of mixed sex as indicated. Each group started with 10 animals. Groups in one experiment consisted of equally distributed litter-mates of even average weight. The basal ration, as reported previously, contained 30% torula yeast, 59% sucrose, 5% salts, 5% vit. free lard and a vitamin mixture(1). From this formula the different experimental diets were derived by adding supplements at the expense of torula or of sucrose. Tests for protective activity were performed in two ways: (A) Prophylactic method (Table I, exp. A, B and C). The supplement was given from the beginning of the experiment. (B) Repletion method (Table II). The animals were kept on the basal diet, in sets of 5 litter mates to a cage, for a preliminary period of 23 days. At the 24th day they were separated and supplementation was started. The repletion method was adopted as a standard procedure, since it requires less space and less supplemented material and yields results in a shorter experimental time. It is based on two observations: Very few rats succumb to dietary necrotic

* To be published separately.

† Cultured, primary dried yeast, Anheuser-Busch, Inc., St. Louis, Mo.

‡ Debittered, dried brewer's yeast, Anheuser-Busch, Inc.

TABLE I. Different Levels of Torula Yeast and Inhibitory Effect of Yeast G and Yeast K on Dietary Necrotic Liver Degeneration (Prophylactic Method).

Exp.	Torula yeast, %	Yeast G, %	Yeast K, %	No. of animals	Dead due to liver degeneration	Avg of reciprocal survival times (×100)
A (different levels of torula yeast)						
	20	0	0	8	8	2.20§ ± .15‡
	30	0	0	8	8	2.43 ± .25
	40	0	0	8	8	2.15 ± .13
B*	30	0	0	9	9	2.48 ± .10
	40	0	0	6	6	1.87 ± .25
	30	10	0	9	3	.66 ± .25
	30	0	10	9	0	.0 ± .0
C†	20	0	0	10	10	2.22 ± .04
	20	0	10	10	1	.13 ± .12

* Terminated after 130 days. † Terminated after 90 days. ‡ Stand. dev. of mean. § Avg survival time = 47 days.

TABLE II. Inhibitory Effect of Yeast K, and Fractions Thereof (Repletion Method).

Exp.	Torula yeast, %	Supplement, %	No. of animals	Dead due to liver degeneration	Avg of reciprocal survival times (×100)
D*	30	—	9	9	2.40 ± .18‡
	27	Yeast K 3	10	2	.58 ± .35
	25	" 5	8	0	.0 ± .0
E†	30	—	9	9	2.17 ± .19
	30	" extract** ≅ 5	9	8	2.00 ± .32
	30	" residue** ≅ 5	10	1	.21 ± .23
F*	30§	—	9	9	2.40 ± .18
	25	" residue** ≅ 5	10	2	.54 ± .32
	25	Residue hydrolysate†† ≅ 5	10	1	.19 ± .18

* Terminated after 72 days. † Terminated after 75 days. ‡ Stand. dev. of mean. § Same control group as in Exp. D.

** One part yeast K boiled for 20 min with 5 parts of water, filtered by suction, procedure repeated. The residue was extracted twice by boiling 20 min with alcohol. Water and alcohol extracts combined, evaporated to dryness and dissolved in 2 parts water: "yeast K extracts." Residue of extraction dried at air until free from alcohol: "yeast K residue."

†† One part of "yeast K residue" hydrolyzed by boiling with 10 parts of 4 N sulfuric acid 18 hr, SO_4 was removed by Ba hydroxide. Ba sulfate filtered off and washed 3 times with boiling water. Combined filtrates evaporated to dryness, taken up in 2 parts of hot water and stored at +4°C several days. Precipitate formed, containing some cystine, removed by filtration. Filtrate, consisting of soluble portion of hydrolysate, was "residue hydrolysate."

liver degeneration before the twenty-fourth day, and substances active against the defect, like vit. E, afford protection even when supplementation is started at this time(5). For evaluation of the results the average of the reciprocal of the survival times was calculated for each experimental group and compared to littermate controls(1).

Results. Variation of dietary levels of torula yeast. The influence of various levels of torula yeast on the development of dietary necrotic liver degeneration was studied, since different dietary levels of this material were involved in some of the experiments, and also because the question has been raised as to whether the presence of a "toxic" substance in some yeasts, and its absence in others, would explain the differences between yeasts of various sources in this respect(6,7). There was no significant difference between groups with 20, 30, or 40% torula in the diet (Table I, exp. A, see also controls in exp. B-F).

Inhibition by yeast G and yeast K, prophylactic method. On the 30% torula ration the incidence of dietary necrotic liver degeneration was 100%, the usual average survival time was 45.5 days(1). When 10% yeast G was added from the beginning of the experiment, 6 out of 9 animals survived (Table I, Exp. B). A more pronounced effect

was caused by 10% yeast K, which afforded complete protection. Survivors of the experiment were killed after 130 days; their livers were found to be normal. When 10% yeast K§ was added to the 20% torula diet (Table I, Exp. C) only one animal died, at the 78th day, and the other 9 displayed normal livers after 90 days. A control group on the 20% torula ration died from liver degeneration within the usual length of time.

Inhibition by yeast K, repletion method. Subsequent tests showed that almost complete protection against dietary necrotic liver degeneration was provided when 3% or 5% of yeast K was added at the expense of torula, beginning on the 24th day, after a depletion period of 23 days on the standard torula ration (Table II, Exp. D).

Elimination of cystine and vit. E. For reasons which have been discussed extensively by various authors attempting to interpret the difference between necrosis inducing yeasts and others(7-9) the protective efficacy of yeasts G and K could hardly be ascribed to cystine or tocopherol, the known protective agents. The amount of cystine in yeast is small. Cystine determinations were performed in the protein fraction obtained after removal of interfering substances by extraction with boiling water and alcohol, using the method of Sullivan *et al.*(10). After hydrolysis .6% cystine was found in the torula protein fraction, the corresponding values for yeast G and K were .7% and .6%. Replacement of a small fraction of torula yeast in the diet by yeast G or yeast K protein therefore causes no significant change in the overall cystine content. Tocopherol does not seem to be a normal component of dried brewer's yeast which has been used extensively as a standard ingredient of vitamin E free rations (11-13). Customary chemical assay methods can not be applied to yeast since yeast fat contains interfering substances(5).

The fact that the protective quality of yeast G and K could not be attributed to cystine or vit. E was established by fractionation of the active principle from yeast K.

§ The following description is restricted to experiments with yeast K, the more effective material.

TABLE III. Substances Inactive Against Dietary Necrotic Liver Degeneration Induced by Torula Diet (Prophylactic Method).

Supplement	Doses*		No. of rats	Dead due to necr. l.d.
Thiamine	4	mg %	10†	10
Riboflavin	25		10†	10
Pyridoxine	20		10†	10
Nicotinamide	20		10†	10
Ca pantothenate	4		10	10
Inositol	10		10†	10
Choline chloride	100		10	10
Biotin	10	γ %	10†	10
Folic acid	100		10†	10
	100	γ d.s.‖	10†	10
Vit. B_{12}	.1		6†	6
	.25		9	9
Vit. B_{12} + folic acid	.25+100		8	8
Citr. factor‡	2		9	9
Citr. factor§	2		9	9
Vit. K	.9	mg %	10	10
Ascorbic acid	.5	%	10†	10
Xanthine	.5		10†	10
Alpha-amino ethanol	.5		9	9

* % means % in dict. † Group contained 50% females. ‡ "Leucovorin" Lederle, kindly supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y. § Started on 8th day of experiment. ‖ d.s. = daily subcutaneously.

Fractionation experiments with yeast K. A simple procedure was devised which would separate cystine, and also tocopherol if present. Yeast K when boiled twice with water and twice with ethanol left an insoluble residue amounting to 60 to 65% of the starting material. This consisted mainly of protein. Tocopherol and its esters would have been removed by the alcohol extraction. The protein residue had the full protective activity of yeast K (Table II, exp. E & F). This material was hydrolyzed with hydrochloric or with sulfuric acid. After neutralization and concentration of the hydrolysate, a little cystine and considerable tyrosine precipitated out. The remaining solution did not give a detectable reaction for cystine, which indicated that the cystine content was less than .1% of the dry weight. The preparation had full activity against dietary necrotic liver degeneration when added to torula diets in an amount equivalent to 5% yeast K.

Ineffective substances. The commonly known vitamins and some other substances

were tested against dietary necrotic liver degeneration and had no appreciable activity (Table III). In some of these tests the supplement seemed to shorten the life span, in others a slight prolongation of the survival time could be observed. However, in all of these groups no animal survived. It seems noteworthy that xanthine which is known to protect against chloroform liver damage(14), and which was found to inhibit dietary liver degeneration caused by casein VI diets(4), had no activity. This might be explained by the presence of high amounts of nucleic acid in yeast which furnish an appreciable quantity of xanthine during the metabolic breakdown of adenine and guanine. Alpha-amino ethanol, which protects against L-penicillamine intoxication(15), was also ineffective.

Discussion. The results of this investigation demonstrate clearly that yeast G and especially yeast K exert a strong protective effect against dietary necrotic liver degeneration when added in relatively small amounts to torula diets. It is evident that this activity is due to a substance (or substances) different from vit. E and from cystine. The possibility has to be left open that factor 3 is an active derivative of either cystine or of tocopherol. Factor 3 is water-soluble and stable against acid hydrolysis. In yeast it is firmly bound, presumably to protein. It is not identical with any of the better known water soluble vitamins. Whether factor 3 is an ordinary non-catalytic building stone of cell matter or whether it is of vitamin nature remains to be settled.

Dietary necrotic liver degeneration was originally thought to be a cystine deficiency (16-18). When the preventive role of vitamin E was elucidated(19) it became evident that, at least on yeast diets, either cystine or vitamin E alone prevented the damage, and it was concluded that necrotic liver degeneration in this instance was due to a simultaneous deficit in both of these factors. The dietary interchangeability was interpreted as indicating a metabolic interrelation between these substances(2). This conception can now be extended; under our experimental conditions a simultaneous lack of 3 factors—cystine, vit. E and factor 3—is a prerequisite for the development of dietary necrotic liver degeneration, and each one of them alone can protect.

The possibility has been discussed that a hypothetical toxic component, which would have to be present in some yeasts and absent in others, could be the reason for the discrepancy between yeasts of different degeneration-inducing capacities(6,7). Evidence in favor of this postulate could not be detected in the present experiments. The deficiency can be induced by a large variety of different rations without any yeast, as pointed out before(1), and the speed of the development of the liver damage does not increase with increasing dietary levels of torula. The discovery of factor 3 in yeasts which do not induce the damage provides a natural and adequate explanation for the differences in regard to this deficiency.

Dietary insufficiency of vit. E can manifest itself by a great variety of pathological lesions according to species and to dietary conditions used (general edema or fatal encephalomalacia in chicken, muscle dystrophy in guinea pigs, rabbits, goats, calves, and also ducks and turkeys, necrotic liver degeneration or general edema or slowly developing genital lesions in the rat, etc.‖ The question arises whether different levels of factor 3 in various rations are the reason for the multiplicity of these vit. E deficiency diseases, since it appears from a comparison of the results of this investigation with earlier work that the appearance of the avitaminosis in rats is decisively influenced by the absence or presence of this factor, dietary necrotic liver degeneration killing the animal within several weeks in the first case, the disturbance of gestative functions developing more slowly in the second.

Summary. (1) It is shown that yeast G (cultured, primary dried yeast) and yeast K

‖ These defects, including dietary necrotic liver degeneration, are much more severe and probably more specific than the slowly developing, well-known deficiency symptoms in the genital tract. Tocopherol should not be considered to be merely an antisterility factor, its functions being essential for brain, liver, muscles, including the heart, and probably for other organs.

(debittered, dried brewers yeast), which do not induce liver degeneration, exert a strong protective influence when added to diets causing the disease. It has been demonstrated by fractionation experiments that this effect is not due to vit. E or cystine, but due to a third unidentified factor, designated factor 3, which has been concentrated from yeast K. (2) The presence of factor 3 explains why dietary necrotic liver degeneration cannot be produced with American yeasts G and K. Factor 3 is water soluble, stable against acid hydrolysis, and is not identical with any one of the presently well-known vitamins.

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Free Amino-Acids in Rat Brain During Insulin Shock. (19241)

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It has been reported(1) that after insulin administration, the rat's brain content of free glutamic acid lessens, free amino acid nitrogen is slightly diminished, but no changes occur either in glutamine or ammonia. The explanation suggested was that during the severe conditions accompanying hypoglycemia, glutamic acid is oxidized, and that ammonia is in some way metabolized or liberated from the brain, without any change taking place in other free amino acids. Nevertheless, the authors considered it necessary to submit this last assertion to the test of experiment, particularly in connection with amino acids closely related to glutamic acid in tissue metabolism, such as aspartic and γ-aminobutyric acids, the latter recently found in brain as a decarboxylation product of glutamic acid(2,3).

Experimental. Nine groups, each with 6 to 8 young male albino rats each weighing 100 to 150 g were used. Rats of groups 1-7 received no food (water *ad libitum*) during 24 hours, and then, to half the rats, subgroup B, 350 IU/k of Lilly's insulin were injected intraperitoneally, the other subgroup A, being kept as controls. Starvation conditions were further maintained, in the subsequent period, during which insulin shock developed in subgroups B, and some of their members died. Rats of groups 8 and 9 were not starved before their subgroups B were injected with 60 IU/k insulin, thus maintaining them under conditions similar to those in Dawson's ex-