pathway that is involved in the toxicity. We currently hold that at high levels of dietary methionine, the metabolism of excess methionine follows a pathway similar to that shown for *S*-methyl-L-cysteine (inset, Figure 3), possibly resulting in the production of methyl mercaptan which may be toxic. We are not yet sure, however, about production of methyl mercaptan from methionine in the *S*-methylcysteine-like pathway, but in some exciting preliminary studies, carried out under anaerobic conditions, volatile ^{35}S was obtained from sulfur-labeled methionine.

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Chemistry of the Staphylococcal Enterotoxins

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The staphylococcal enterotoxins are simple, low molecular weight proteins that are relatively heat resistant. They contain one disulfide bridge which can be reduced without affecting the toxicity or antigenicity. At least a part of the tyrosyl and methionine residues and the carboxyl and amino groups can be substituted without adversely affecting the toxicity of the molecule. The individual enterotoxins can be separated into several proteins which are identical in every respect except for their isoelectric points.

The staphylococci produce many biologically active substances which have been studied to various degrees. One group of substances which has received much attention in recent years is the enterotoxins, the causative agents of staphylococcal food poisoning. The enterotoxins are produced by the staphylococci in some foods and in culture media in the laboratory. The ingestion of these substances by humans produces a variety of symptoms, the most common being vomiting and diarrhea in 2–6 hr. The illness is relatively mild, normally lasting only a few hours to 1 day. This illness is not a reportable disease, and its true incidence is unknown since most cases are never seen by a physician and hence go unrecognized. Some have the opinion that staphylococcal poisoning is not of great importance; however, according to Morbidity and Mortality Weekly Reports (1972), 45% of all food-borne disease outbreaks in 1971 was due to staphylococcal food poisoning.

The purification of a protein that caused emesis in monkeys led to the discovery that the staphylococci produce more than one enterotoxin (Bergdoll *et al.*, 1959a), the basis for differentiation being their reaction with specific antibodies. This basis was used in establishing a nomenclature, designating them as enterotoxins A, B, C, etc. (Casman *et al.*, 1963). To date, enterotoxins A (Cas-

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Table I. Some Properties of the Enterotoxins

	Enterotoxin				
	A[a]	B[b]	C_1[c]	C_2[d]	E[e]
Nitrogen content, %	16.2	16.1	16.2	16.0	
Sedimentation coefficient ($S_{20,w}°$), S	3.03	2.89	3.00	2.90	2.60
Molecular weight	27,800 (34,700[f])	28,366[g]	26,000[a] 34,100	34,100	29,600
Isoelectric point, pH	7.26	8.6	8.6	7.0	7.0
Extinction, $E_{1\,cm}^{1\%}$	14.6	14.0	12.1	12.1	12.5

[a] Schantz *et al.* (1972). [b] Schantz *et al.* (1965). [c] Borja and Bergdoll (1967). [d] Avena and Bergdoll (1967). [e] Borja *et al.* (1972). [f] Chu *et al.* (1966). [g] Dayhoff (1972).

man, 1960), B (Bergdoll *et al.*, 1959b), C_1 and C_2 (Bergdoll *et al.*, 1965), D (Casman *et al.*, 1967), and E (Bergdoll *et al.*, 1971) have been identified. It is not known how many enterotoxins the staphylococci produce; however, a large number of enterotoxigenic strains of staphylococci have been isolated that produce an unidentifiable enterotoxin. This fact is of minor importance in a study of the structure and action of the enterotoxin since a sufficient number have been purified for comparative study [A, Chu *et al.* (1966), Schantz *et al.* (1972); B, Bergdoll *et al.* (1959a), Schantz *et al.* (1965); C, Avena and Bergdoll (1967), Borja and Bergdoll (1967); D, Igarashi (1972); E, Borja *et al.* (1972)]. It is, however, of major importance to those concerned with the safety of our foods, since the only reliable methods for the detection of the enterotoxins in foods are the use of the specific antibodies, thus limiting their detection to those that have been identified. For this reason, the identification of new enterotoxins is being pursued in the Food Research Institute Laboratories and in the U. S. Food and Drug laboratories. This is not a satisfactory solution to the problem, but is a necessary course of action until the time a reagent specific for all enterotoxins (both identified and unidentified) becomes available. One of the purposes of our studies is to determine if development of such a reagent for the enterotoxins is possible. This is being approached by studying in as much detail as possible the structure of those enterotoxins that have been purified. There is information available to indicate that the enterotoxins have an area of common structure which may be useful in preparing such a reagent. However, other evidence indicates that it may be possible to readily separate the enterotoxins into two groups, thus requiring two reagents, one specific for each group.

The enterotoxins are simple, relatively low molecular weight proteins that are hygroscopic and easily soluble in water and salt solutions. The enterotoxins in the active state are resistant to proteolytic enzymes such as trypsin, chymotrypsin, renin, and papain. Pepsin destroys their activity at a pH of about 2 (Bergdoll, 1970), but is ineffective at higher pH values. The enterotoxins are considered to be quite heat resistant because boiling of crude solutions for 30 min does not destroy all of the activity. The time required to inactivate enterotoxin A depends upon the amount of enterotoxin present in the sample (Denny *et al.*, 1971; Hilker *et al.*, 1968); the larger the amount of the toxin, the longer the time required to reduce the content to less than 1 μg/ml, as determined by the single gel-diffusion tube test. For example, 21 μg of crude enterotoxin A/ml in Veronal buffer, pH 7.2, required heating at 100° for 130 min to reduce the enterotoxin content to less than 1 μg/ml. It would be expected that the amount present in foods (1–10 μg/100 g) would require much less time to bring the enterotoxin level below the danger point. The enterotoxin can be inactivated by processes used in canning foods commercially (Denny *et al.*, 1966), although pasteurization or spray drying of milk as currently practiced does not inactivate enterotoxin B (Read and Bradshaw, 1966).

Table II. Amino Acid Composition of the Enterotoxins in g/100 g of Protein

	Enterotoxin				
Amino acid	A[a]	B[b]	C_1[c]	C_2[c]	E[d]
Lysine	11.26	14.85	14.43	13.99	10.83
Histidine	3.16	2.34	2.91	2.87	3.04
Arginine	4.02	2.69	1.71	1.75	4.50
Aspartic acid	15.53	18.13	17.85	18.38	15.10
Threonine	5.96	4.50	5.31	5.80	6.36
Serine	2.99	4.05	4.58	4.81	4.72
Glutamic acid	12.36	9.45	8.95	8.93	12.15
Proline	1.35	2.11	2.16	2.23	1.93
Glycine	2.96	1.78	2.99	2.90	4.10
Alanine	1.94	1.32	1.85	1.61	2.38
Half-cystine	0.66	0.68	0.79	0.74	0.81
Valine	4.93	5.66	6.50	5.87	4.36
Methionine	0.96	3.52	3.20	3.60	0.45
Isoleucine	4.11	3.53	4.09	4.02	4.30
Leucine	9.78	6.86	6.54	6.13	10.08
Tyrosine	10.63	11.50	9.80	10.27	9.79
Phenylalanine	4.31	6.23	5.35	5.25	4.47
Tryptophan	1.46	0.95	0.99	0.84	1.51
Amide NH_3	1.80	1.66	1.71	1.62	1.66
Total	98.37	100.15	100.00	99.99	100.88

[a] Schantz *et al.* (1972). [b] Bergdoll *et al.* (1965). [c] Huang *et al.* (1967). [d] Borja *et al.* (1972).

The molecular weights reported in the literature differ and only the value for enterotoxin B has been decided definitely. Its actual molecular weight is 28,366 (Dayhoff, 1972), as determined from the amino acid sequence of the enterotoxin (Huang and Bergdoll, 1970). (The figure of 28,496 given in this reference is due to a miscalculation.) The latest reported values (Table I) are lower than those reported earlier. These differences indicate that it is difficult to determine accurately the molecular weights of proteins, the staphylococcal enterotoxins in particular, and that only when the sequence is known can the true molecular weight be calculated. It should be noted that Schantz *et al.* (1972) reported lower values for enterotoxin A and C than had been reported from the Food Research Institute and a value for enterotoxin B within 3% of the value obtained from the sequence data. These data were obtained by sedimentation equilibrium (Schachman and Edelstein, 1966), whereas the other values were obtained from sedimentation and diffusion data. Huang (unpublished data) calculated the molecular weights from the half-cystine values and obtained values very near to the actual value for B (29,000 *vs.* 28,366) and those reported for A and C by Schantz *et al.* (1972). It does appear that the molecular weights of all of the enterotoxins are within 26,000–30,000, which is what one would expect in the case of a common group of proteins.

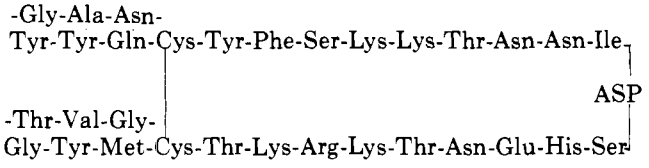
The enterotoxins are single polypeptide chains which contain relatively large amounts of lysine, aspartic, and glutamic acids and tyrosine (Table II; Bergdoll, 1972). The enterotoxins appear to contain only two residues of half-cystine and one or two residues of tryptophan. Although data from four enterotoxins may be insufficient to draw conclusions, some differences and similarities can be observed. The amino acid compositions of enterotoxins A and E are quite similar while the compositions of B and the C's are similar. For example, the methionine content of A and E is much less than for B and the C's, 1–2 residues *vs.* 8 residues, respectively. There are differences also in the lysine, aspartic and glutamic acids and leucine contents. The C-terminal residue for enterotoxin A is serine (Bergdoll, 1970), lysine for B (Bergdoll *et al.*, 1965; Spero

Table III. The Portion of Enterotoxin B Molecule Which Contains the Half-Cystine Residues

-Lys-	Asp-	Leu-	Ala-	Asp-	Lys-	Tyr-	Lys-	Asp-	Lys-
71	72	73	74	75	76	77	78	79	80
Tyr-	Val-	Asp-	Val-	Phe-	Gly-	Ala-	Asn-	Tyr-	Tyr-
81	82	83	84	85	86	87	88	89	90
Gln-	Cys-	Tyr-	Phe-	Ser-	Lys-	Lys-	Thr-	Asn-	Asn-
91	92	93	94	95	96	97	98	99	100
Ile-	Asp-	Ser-	His-	Glu-	Asn-	Thr-	Lys-	Arg-	Lys-
101	102	103	104	105	106	107	108	109	110
Thr-	Cys-	Met-	Tyr-	Gly-	Gly-	Val-	Thr-	Gly-	His-
111	112	113	114	115	116	117	118	119	120
Gly-	Asn-	Asn-	Gln-	Leu-	Asp-	Lys-	Tyr-	Tyr-	Arg-
121	122	123	124	125	126	127	128	129	130

et al., 1965), glycine for C_1 and C_2 (Huang *et al.*, 1967), and threonine for E (Borja *et al.*, 1972). The N-terminal residue for enterotoxin B, C_1, and C_2 is glutamic acid and is serine for E. Initially, the N-terminal residue for enterotoxin A was determined to be alanine (Bergdoll, 1970), but the purified enterotoxin obtained from the high-producing mutant strain of Friedman and Howard (1971) contained no detectable N-terminal amino acid residue (Schantz *et al.*, 1972). The difference has not been clarified, but the amino acid sequence studies on A that are now in progress should help to resolve this problem.

The amino acid sequence studies of enterotoxin B revealed the presence of 239 amino acid residues in this enterotoxin (Table II; Huang and Bergdoll, 1970). There are only two half-cystine residues present and these are relatively close to the center of the chain at positions 92 and 112 (Table III). Since there are no free -SH groups in enterotoxin B, the two half-cystines are joined into one cystine residue which requires folding of the chain in some fashion, as indicated below.



However, the disulfide bridge can be reduced and the -SH groups alkylated with either iodoacetamide or iodoacetate without any appreciable effect on the physical or emetic properties of the toxin molecule (Dalidowicz *et al.*, 1966). Apparently, the breaking of this linkage does not result in an unfolding of the chain since viscosity and sedimentation values remain essentially unchanged. The structure surrounding the disulfide bridge may be common to all of the enterotoxins since only two half-cystines have been found in any of the enterotoxins whose amino acid composition has been determined. Preliminary data from the amino acid sequence studies of enterotoxin A indicate that at least part of the sequence involving one of the half-cystine residues is the same as for enterotoxin B.

Acetylation of enterotoxin B and C with acetylimidazole and nitration with tetranitromethane revealed that 5–6 "free" tyrosyl residues were present (Borja, 1969; Borja and Bergdoll, 1969; Chu, 1968). These modifications have little or no effect on the immunological or toxic properties of the enterotoxin molecule. In 8 *M* urea, approximately ten tyrosyl residues were acetylated with an approximate 20% inhibition in the antigen–antibody reaction. In 5 *M* guanidine hydrochloride, the enterotoxins undergo considerable unfolding, which is indicated by a large change in the intrinsic viscosity and the acetylation of all the tyrosyl residues. Complete acetylation of the tyrosyl residues results in essentially all loss of activity. Information obtained about the electrostatic interaction factor indicates the enterotoxins to exist as very compact, unhydrated molecules over a wide pH range. The observation of a conformational change around pH 11.5 where the dissociation of tyrosine is time-dependent, however, indicates that the abnormal tyrosine groups may play an important role in the structural features of the toxin, such as interaction with other side chain groups to form an internal core to maintain the rigid structure. It is concluded that since the enterotoxin cannot refold to the original conformation after more than 14 tyrosyl residues have been modified, then, like most other biologically active proteins, the structure integrity for this toxin is essential for the biological activity.

Modification of the methionine residues in enterotoxin B with iodoacetic acid and hydrogen peroxide (Chu and Bergdoll, 1969) revealed that four of the eight residues reacted at a faster rate. Immunochemical and fluorometric analyses of the modified toxin indicated a change in conformation when more than six methionine residues are modified. The emetic activity of the toxin was also lost when six methionine residues reacted.

All 33 of the carboxyl groups in enterotoxin B were modified with glycine methyl ester in the presence of water-soluble carbodiimide in the presence of 6 *M* guanidine hydrochloride (Chu and Crary, 1969). In the absence of a denaturing reagent, the 21 to 24 residues which reacted had only a slight effect on the antigen–antibody reaction and fluorescence properties and little or no effect on emetic activity. The emetic activity was lost when 30 or more carboxyl groups were modified. Expansion of the molecule was observed after the modification.

Guanidination of the free amino groups of enterotoxin B with 3,5-dimethyl-1-guanylpyrazole converted 31–32 of the 33 ϵ-amino groups and 30% of the N-terminal residue (Spero *et al.*, 1971). The substitution of these groups produced no gross change in the conformation or in the biological activity although the enterotoxin was markedly reduced in solubility. These authors concluded that the lysine residues were not involved in either the antigenic or toxic sites. Chu *et al.* (1969) reported that guanidination of 30 lysine residues with *O*-methylisourea did not alter the emetic activity or the antigen–antibody reaction. Spero *et al.* (1971) found that guanidination of 32 of the 33 lysine residues with the same reagent resulted in a loss of about 90% of the emetic activity of the enterotoxin and gave a line of only partial identity with the antitoxin. These authors suggest that the guanidination of one less lysine residue with 3,5-dimethyl-1-guanylpyrazole may account for the difference in the activities of the guanidinated derivatives. These derivatives were not appreciably altered in physical properties, as judged by sedimentation coefficient, intrinsic viscosity, and Stoke's radius. These investigators as well as Chu *et al.* (1969) found that removal of the ϵ-amino groups or neutralizing the positive charge in some way, such as acetylation, results in inactivation of the toxin. Serious structural changes occurred with loss in activity, indicating that the positive charges are essential for maintaining the confirmation and the activity of the toxin. Although Chu *et al.* (1969) suggested that the amino groups may play a role in the emetic activity and in the antigen–antibody reaction, it is more likely that their main function is to maintain the native conformation which is essential to the biological activity.

An attempt was made to locate the 5–6 "free" tyrosyl residues in enterotoxin B by nitration with subsequent peptide mapping of the tryptic and cyanogen bromide peptides (Keller, 1972). Although not all of the nitrated residues were located, the results show that the C-terminal part of the enterotoxin molecule is buried and one of the half-cystine residues may be at least partially buried.

In recent years, isoelectric focusing has been used in the

purification of proteins. When it was applied to the enterotoxins, several proteins with differing isoelectric points but with identical antigen–antibody reactions were obtained (Chang and Dickie, 1971a,b; Chang *et al.*, 1971a; Dickie *et al.*, 1972; Schantz *et al.*, 1972). Refocusing of each protein gave only proteins of lower isoelectric points, although Metzger *et al.* (1972) reported that in their experiments, the major protein from an enterotoxin B preparation was stable when refocused. The general consensus is that the differences in the proteins are in the number of amide groups in the molecule. Chang and Dickie's (1971a) investigations indicate that the removal of amide groups can take place during the isoelectric focusing, while the work of Metzger *et al.* (1972) suggests the difference in amide groups may arise during fermentation or in procedures used in the purification. Bergdoll (1956) reported the fractionation of enterotoxin B into at least three fractions with identical antigen–antibody reactions by chromatography on the ion-exchange resin, Amberlite XE-64. Rechromatography of these fractions indicated that they were distinctly different, indicating that they were formed during the production of the enterotoxin. Chang *et al.* (1971b) also separated enterotoxin B into three fractions that gave identical antigen–antibody reactions by use of hydroxyl apatite chromatography.

Baird-Parker and Joseph (1964) showed that purified enterotoxin B could be separated into two fractions in starch gel electrophoresis which appeared to be identical. Schantz *et al.* (1965) separated enterotoxin B into two fractions by electrophoresis in starch gel using 0.02 *M* borate buffer at pH 8.6. Rerunning of the slower moving material gave one band, while rerunning of the faster moving material gave two bands. The two materials were identical in toxicity in monkeys, in their reaction with the specific antibody, and in amino acid composition. The results of the various types of experiments indicate that slightly different materials can be formed during enterotoxin production and that it is also possible to change the molecule during electrophoresis. The evidence indicates the differences can be explained on the variation in the number of amide groups in the different proteins.

As was stated earlier, the enterotoxins are identified on the basis of their reactions with specific antibodies. Initially, the only observable difference between enterotoxins C_1 and C_2 was thought to be in their isoelectric points (Table I) (Bergdoll *et al.*, 1965), but on close examination it was apparent from spur formation in Ouchterlony plates that the reaction of the enterotoxins with the heterologous antibodies is not complete. The major antigenic sites are the same for both enterotoxins, but each apparently has a specific minor antigenic site. During the identification of enterotoxin E, it was discovered that this enterotoxin was neutralized by enterotoxin A antiserum but not by B or C antisera. Further investigations showed that under certain conditions, cross reactions of enterotoxin A with E antiserum and E with A antiserum did occur (Bergdoll *et al.*, 1971). These reactions showed that enterotoxins A and E had major heterologous antigenic sites but had a common minor antigenic site.

Gruber and Wright (1969) showed by the ammonium sulfate coprecipitation of Farr (1958) that enterotoxins B and C contained similar antigenic determinant groups. At the time of the identification of enterotoxin C, no cross reaction between the two enterotoxins was noted by antigen–antibody precipitation techniques (Bergdoll *et al.*, 1965), but evidence was available which indicated that enterotoxin B antiserum neutralized enterotoxin C. In this case, the enterotoxin C was treated with the B antiserum before intravenous injection into rhesus monkeys. Further experiments in our laboratories indicated that enterotoxin C will no longer give a precipitate with its specific antibody after the toxin is treated with enterotoxin B antiserum and *vice versa*.

Information available about the enterotoxins shows that they are a closely related group of proteins with a common toxic action. We believe that the site responsible for the toxic action is a common one in all of the enterotoxins and is not involved in the antigenicity of the toxins. Further studies on the chemistry of the enterotoxins should be very helpful in relating structure to antigenicity.

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Vitamin B_6 Antagonists of Natural Origin

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Vitamin B_6 deficiencies resulting from the ingestion of natural antagonists fed at the natural level are rarely observed. The only well documented case is in chickens from the ingestion of linseed meal which contains linatine, a hydrazino peptide. D-Cycloserine, a cyclic hydroxylamine derivative with antibiotic properties, also produces B_6 deficiencies when administered at therapeutic levels. Other potential antagonists include L-canaline, obtained by the enzymatic degradation of canavanine (*Canavalia* sp.), 4-hydroxymethylphenylhydrazine, a constituent of agaritine (*Agaricus* sp.) and methylhydrazine produced from gyromitrin (*Gyromitra esculenta*). Several dietary substances produce a stress on the vitamin B_6 supply or induce neurological disorders that respond to pyridoxine therapy including L-dopa, choline, mimosine, β-cyanoalanine, high level of dietary protein, and thermally processed high protein foods.

Compounds having antivitamin B_6 activity have been used for experimental and chemotherapeutic purposes for many years. For the most part, these have been synthetic compounds that are either structural analogs of the B_6 vitamers or carbonyl trapping agents. Of the many structural analogs studied, 4-deoxypyridoxine is perhaps the most potent and widely used agent for *in vivo* studies with higher animals, especially when used in conjunction with a vitamin B_6-deficient diet. Under these conditions, classical vitamin B_6 deficiency symptoms can be induced which respond dramatically to pyridoxine (PD).

There are many reports in the literature of the induction of vitamin B_6 deficiency through ingestion of hydrazines, either by accidental poisoning or by use of drugs. Convulsions associated with therapeutic doses of isonicotinyl hydrazide are readily relieved or prevented by timely administration of PD. Hydrazines, hydroxylamines, and semicarbazides of the type RNH_2 are all capable of forming stable hydrazones, oximes, and semicarbazones, respectively, with pyridoxal (PL) and pyridoxal phosphate (PLP). Derivatives of carbonyl trapping agents, both hydrazines and hydroxylamines, have been isolated from food and feedstuffs obtained from diverse genera. Although the carbonyl trapping agents generally occur in nature in the form of unreactive derivatives, enzymes having hydrolase or transferase activity capable of releasing the reactive carbonyl trapping agent are widely distributed in nature, including the digestive tract of higher animals. The release of these agents by digestive processes offers the possibility for inactivation of a portion of the vitamin B_6 in the metabolic pool.

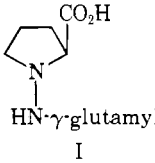
Other factors which may place a stress on the vitamin B_6 supply are the conditions of processing, especially thermal processing of high protein foods, and a high protein diet. Although these latter conditions are not true examples of *in vivo* vitamin antagonism, the results are much the same—clinical conditions which are relieved by administered PD.

The existence of a vitamin B_6 antagonism is sometimes suggested by certain drugs and neurotoxins which affect the central nervous system and produce convulsions similar to those induced by B_6 deficiency. In some of these cases, partial relief is obtained by use of PD, even though evidence for a vitamin B_6 deficiency is not substantiated by laboratory tests.

Recent reviews have discussed some of the conditions which may alter nutritional requirements for vitamin B_6 (Brown, 1972) and methods for the biochemical assessment of the nutritional status of vitamin B_6 (Sauberlich *et al.*, 1972). For the present article, a natural vitamin antagonist is defined as a substance of natural origin which raises the vitamin B_6 requirement or induces an adverse physiological condition which responds favorably to vitamin B_6 therapy. Included are substances having chemical characteristics of vitamin B_6 antagonists which are produced from inactive precursors by means of simple enzymic transformations which might be encountered in higher animals. The general topic of vitamin B_6 antimetabolites was reviewed by Rosen *et al.* (1964).

CARBONYL TRAPPING AGENTS

Linatine. The only clear example of a vitamin B_6 deficiency resulting from a natural feedstuff or food was given by Kratzer *et al.* (1954), who found that the poor growth shown by chickens on a diet of linseed meal was counteracted by the addition of PD to the diet. Subsequent studies by Klosterman *et al.* (1967) showed that linseed meal contains about 100 ppm of linatine (I). Hydrolysis of linatine produced 1-amino-D-proline (DAP) (II), an asymmetrically substituted secondary hydrazine which is probably responsible for the *in vivo* toxicity of linseed meal diets. DAP condenses readily with the carbonyl of PL and PLP to form stable hydrazones.



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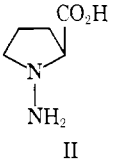

II

Although first observed in the flax seed, linatine has been found to occur in all parts of the immature flax plant, *Linum usitatissimum* (Nugent, 1971). Acute doses

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