Properties of the Toxic Factor in Trichloroethylene-Extracted Soybean Oil Meal

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A bioassay with calves served as a guide in studies of the properties of the toxic factor in trichloroethylene-extracted soybean oil meal, which produces fatal aplastic anemia in cattle. The toxic factor is an organic compound, which is associated with the protein fraction of the soybean oil meal from which it can be liberated by pancreatic digestion in small water-soluble, dialyzable fragments. As it occurs in soybean oil meal, the toxic factor at 65° to 70° C. is stable at pH 1.5, but unstable at pH 12.0. No criterion, other than biological effects, has been found by which the toxic factor could be detected.

EARLIER STUDIES in these laboratories have been mainly concerned with the biological effects of the toxic factor in some specimens of trichloroethylene-extracted soybean oil meal (TCESOM) which produces fatal aplastic anemia in cattle (*18*, *20*). Very little is known about the chemical nature of the toxic factor. The toxic effects of trichloroethylene-extracted soybean oil meal are not due to residual trichloroethylene in the oil meal (*17*, *22*) or to free autoxidation products of trichloroethylene (*16*). As it occurs in trichloroethylene-extracted soybean oil meal, the toxic factor is very stable to aging; no decreased activity was detected in specimens which have been stored at ambient temperatures for several years. It is also stable when the dry meal is autoclaved at 15 pounds pressure up to 30 minutes, but is destroyed by ashing (*15*) or by prolonged hydrolysis with strong acid (*11*). Picken and coworkers (*16*) suggested that the toxic property of trichloroethylene-extracted soybean oil meal may be associated with the protein component of the soybean oil meal and recent studies from the same laboratories (*11*) support this view. Toxic properties have also been detected in meat products processed with trichloroethylene (*19*).

At present, a bioassay with calves (*14*) is the only procedure by which the toxicity of trichloroethylene-extracted soybean oil meal can be evaluated. This procedure has been used as a guide in the development of methods by which the toxic factor can be obtained in soluble and dialyzable form, and to study its stability to treatment with acid, alkali, and proteolytic enzymes.

Experimental

Bioassay The soybean oil meal and the various fractions prepared therefrom were fed to young female Holstein calves purchased on the open market and weighing, initially, from 90 to 100 pounds. The calves were not muzzled, and alfalfa hay was fed *ad libitum*. Control animals were fed $^1/_6$ pound per day per 100 pounds of body weight of nontoxic hexane-extracted soybean oil meal (HEXSOM) or graded amounts of the reference standard—the specimen referred to previously (*14*) as TCESOM-6. (Reference to feeding soybean oil meal or fractions at a certain level indicates the quantity fed per day per 100 pounds of body weight.) The various fractions were fed daily in two equal portions on the basis of their nitrogen content (Kjeldahl) at one or more levels, calculated to furnish the amount of nitrogen present in $^1/_4$, $^1/_6$, $^1/_8$, or $^1/_{20}$ pound of trichloroethylene-extracted soybean oil meal, respectively. The level of feeding was adjusted weekly to the body weight. Liquid concentrates were kept frozen at −15° C. until the daily portions for 1 week's supply were weighed out; the latter were kept at 4° C. until the day of feeding. The various preparations were fed during a 60-day period at the end of which, or shortly thereafter, most of the calves were slaughtered for visual and microscopic examination of various tissues. After the 15th day of each assay, blood counts were made daily to serve as criteria of toxicity (*14*).

Soybean Oil Meal Used for Fractionation Two specimens, having essentially equal toxicity, were used as the starting material. Both were produced on the same day from the same mixed lot of freshly harvested (October 1953) soybeans in a commercial extraction plant using the trichloroethylene-extraction process (*23*). One specimen, TCESOM-6, which has been used as the standard of reference, was run through the toasters during manufacture, as in regular commercial practice; the toasters were not operated in the production of the other specimen, TCESOM-7, in order to obtain a product in which the solubility of the proteins is greater (*21*).

Preparation of Fractions In general, the various fractions were prepared in several batches, which were thoroughly mixed, and, in the case of solids, ground in a hammermill. The procedures outlined below apply to one batch. Unless otherwise indicated, the extractions and precipitations were carried out in a double-walled stainless steel vat; cold water was circulated between the walls to keep the temperature of the extracts at about 14° C. Toluene was added as a preservative to all aqueous media; it was completely removed from the precipitated fractions during the subsequent dehydration with ethyl alcohol or acetone and drying, and from the liquid fractions during concentrations in vacuo at temperatures not exceeding 45° C. In most cases, when a solubilized fraction was assayed, the insoluble residues were also assayed to determine the effects of pH and other factors on the stability of the toxic factor. The yields were calculated as per cent of the starting material used for each fraction.

Table I presents a list of the various fractions made and of the treatments used. The essential details of the procedures used in the preparation of each fraction were the following.

Fraction A. TCESOM-7, 9 kg., was suspended in 300 liters of dilute acetic acid at pH 4.1. After 12 hours, the supernatant, which contained in various preparations about 5% of the total nitrogen, was siphoned off and discarded. The insoluble residue was resuspended in 300 liters of water and adjusted to pH 11.9 with 10% sodium hydroxide. After 3.5 hours, the upper fairly clear portion of the supernatant was siphoned off and adjusted to pH 4.1 with 10% sulfuric acid. The precipitate thus formed was filtered and

dehydrated with three portions of 95% ethyl alcohol and dried at 60° C. Yield of fraction A, 18.6%; yield of nitrogen, 33.7%; nitrogen content, 14.1%; and ash, 2.15%.

Fraction B. After removal of the supernatant—which yielded fraction A—at pH 11.9, the remaining suspension was collected in glass jars and left to settle for 48 hours at 5° C. The supernatant was siphoned off and adjusted to pH 4.1 with 10% sulfuric acid. The precipitate thus formed was filtered, dehydrated with three portions of 95% ethyl alcohol, and dried at 60° C. Yield of fraction B, 8.7%; yield of nitrogen, 22.9%; nitrogen content, 14.2%; and ash, 1.35%.

Fraction C. TCESOM-7, 9 kg., was suspended in 300 liters of dilute acetic acid at pH 4.1. After 12 hours, the supernatant was siphoned off and discarded. The precipitate was resuspended in 300 liters of water and the pH adjusted to 1.5 with 10% sulfuric acid. The suspension was transferred to glass jars, left to settle for 48 hours at 5° C., and the supernatant was then siphoned off and adjusted to pH 4.1 with 10% sodium hydroxide. The precipitate thus formed was filtered and dehydrated with three portions of 95% ethyl alcohol and dried at 60° C. Yield of fraction C, 6.1%; yield of nitrogen, 10.7%; nitrogen content 13.5%; and ash, 4.1%.

Fraction D. The pH 1.5–insoluble residue remaining after removal of the supernatant, which yielded fraction C., was dehydrated with three portions of 95% ethyl alcohol and dried at 60° C. Yield of fraction D, 55.5%; yield of nitrogen, 73.6%; nitrogen content, 9.6%; and ash, 7.5%.

Fraction E. Prepared from TCESOM-6 by the procedure used for preparation of fraction A. Yield of fraction E, 15.0%; yield of nitrogen, 27.6%; nitrogen content, 14.0%; and ash, 3.85%.

Fraction F. From 36 kg. of TCESOM-7 the alkali-soluble, pH 4.1–insoluble protein was isolated as described for fraction A, but not dehydrated. The protein was suspended in glass jars with 25 liters of water per kg. of protein, adjusted to pH 10.1 with 10% sodium hydroxide, and then to pH 8.0 with acetic acid. After the addition of 15 grams of pancreatin (Parke, Davis & Co.), the mixture was kept at 36° to 38° C. until 50% of the nitrogen remained soluble when the pH was adjusted to 4.1 with acetic acid. The pH of the entire digest was then brought to 4.1 with acetic acid and, after cooling overnight to 4° C., the supernatant was siphoned from the precipitate (the latter yielding fraction G), neutralized with 10% sodium hydroxide to pH 7, concentrated in vacuo, and spray dried. The yield of fraction F was only 3.53 kg. because of a mechanical defect in the drier; yield of nitrogen, 6.24% (not corrected for nitrogen added with enzyme); nitrogen content, 4.95%; and ash, 39.6%.

Fraction G. The pH 4.1–insoluble precipitate obtained during preparation of fraction F was dehydrated with three portions of acetone and dried at 60° C. Yield of fraction G, 10.3%; yield of nitrogen, 18.1%; nitrogen content, 13.7%; and ash, 3.10%.

Fraction H. Prepared from TCESOM-7, like fraction B, but the alkaline suspension was left to settle at 5° C. for 7 days. The supernatant was then siphoned off and adjusted to pH 4.1 with 10% sulfuric acid. The precipitate was dehydrated with three portions of acetone and dried at 60° C. Yield of fraction H, 10.7%; yield of nitrogen, 19.4%; nitrogen content, 14.0%; and ash, 1.70%.

Fraction I. Prepared from 365 pounds of TCESOM-7 in pilot plant equipment of Archer-Daniels-Midland Co., by the procedure used for preparation of fraction A with the following modifications: The alkaline extraction was carried out at pH 11.0 for 15 hours at room temperature; the alkaline extract was separated from the insoluble portion by centrifugation; and the precipitate, formed by adjusting the pH of the alkaline extract to 4.1 with sulfuric acid, was collected by filtration and dried at 70° C. in air. Yield of fraction I, 29.3%; yield of nitrogen, 50.9%; nitrogen content, 13.6%; and ash, 2.3%.

Fraction J. TCESOM-6, 9 kg., was suspended in three glass jars in 90 liters of dilute sulfuric acid at pH 1.5 and heated at 65° to 70° C. for 48 hours. After the suspension was cooled to room temperature, the pH was adjusted to 4.1 with 10% sodium hydroxide. The soluble portion—which yielded fraction L—was siphoned off. The residue was washed with dilute sulfuric acid (pH 4.1), dehydrated with three portions of 95% ethyl alcohol, and dried at 60° C. Yield of fraction J, 51.2%; yield of nitrogen, 68.9%; nitrogen content, 10.2%; and ash, 4.10%.

Fraction K. TCESOM-6, 9 kg., was suspended in three glass jars in 90 liters of dilute sodium hydroxide at pH 12.0 and heated at 65° to 70° C. for 48 hours. After the suspension was cooled to room temperature, the pH was adjusted to 4.1 with 10% sulfuric acid. The soluble portion was siphoned off. The insoluble residue was washed with dilute sulfuric acid (pH 4.1), dehydrated with three portions 95% ethyl alcohol, and dried at 60° C. Yield of fraction K, 54.1%; yield of nitrogen, 54.9%; nitrogen content, 7.9%; and ash, 5.0%.

Fraction L. The pH 4.1–soluble material and the ethyl alcohol washings obtained in the preparation of fraction J were neutralized to pH 7.0 with 10% sodium hydroxide and concentrated in vacuo to about 0.1 of the volume. The addition of an equal volume of 95% ethyl alcohol yielded a precipitate, which was dehydrated with more ethyl alcohol, dried at 60° C., and ground. Yield of total nitrogen, 15.7%; nitrogen content, 2.99%; and ash, 48.9%. The alcohol soluble fraction was not assayed.

Fraction M. The pH 4.1–soluble material and the ethyl alcohol washings obtained in the preparation of fraction K were processed as described for fraction L. The alcohol-insoluble portion contained 54.3% ash, 2.43% nitrogen, and gave a yield of 7.19% of the total nitrogen used for the preparation of fraction K. This fraction was not assayed with calves.

Fraction N. Fraction I, 5.4 kg., was suspended in 150 liters of water and adjusted with 10% sodium hydroxide to pH 11.5. The pH was then lowered

Table I. Nature of Fractions Prepared from TCESOM

Fraction	Prepared from	Properties and Treatment
A	TCESOM-7	pH 4.1-insoluble material from extract at pH 11.9 for 3.5 hours
B	TCESOM-7	pH 4.1-insoluble material from extract at pH 11.9 for 48 hours
C	TCESOM-7	pH 4.1-insoluble material from extract at pH 1.5 for 48 hours
D	TCESOM-7	pH 1.5-insoluble residue after extraction at pH 1.5 for 48 hours
E	TCESOM-6	pH 4.1-insoluble material from extract at pH 11.9 for 3.5 hours
F	Fraction A	Soluble at pH 4.1 after 50% digestion with pancreatin
G	Fraction A	Insoluble at pH 4.1 after 50% digestion with pancreatin
H	TCESOM-7	pH 4.1-insoluble material from extract at pH 11.9 for 7 days
I	TCESOM-7	pH 4.1-soluble material from extract at pH 11.0 for 15 hours
J	TCESOM-6	pH 4.1-insoluble material after treatment at pH 1.5 and 65° C. for 48 hours
K	TCESOM-6	pH 4.1-insoluble material after treatment at pH 12 and 65° C. for 48 hours
L	TCESOM-6	pH 4.1-soluble material after treatment at pH 1.5 and 65° C. for 48 hours
M	TCESOM-6	pH 4.1-soluble material after treatment at pH 12 and 65° C. for 48 hours
N	Fraction I	Soluble at pH 4.1 after 75% digestion with pancreatin
N-1	Fraction N	Dialyzable components of fraction N
N-2	Fraction N	Nondialyzable components of fraction N
O	Fraction I	Insoluble at pH 4.1 after 75% digestion with pancreatin
P	TCESOM-6	Soluble at pH 4.8 after 85% pancreatic digestion of TCESOM-6 leached for 24 hours at pH 1.5 and 58° C.
P (control)	HEXSOM	Soluble at pH 4.8 after 85% pancreatic digestion of HEXSOM leached for 24 hours at pH 1.5 and 58° C.
Q	TCESOM-6	Insoluble at pH 4.8 after 85% pancreatic digestion of TCESOM-6 leached for 24 hours at pH 1.5 and 58° C.

slowly and with vigorous stirring to 8.5 with 10% acetic acid. The suspension was divided into six equal portions, each of which was transferred to a stainless steel can, diluted to 29 liters, and treated with 15 grams of pancreatin (Nutritional Biochemicals, three times U.S.P. potency) suspended in 1 liter of water. The cans were incubated in a water bath at 36° to 40° C. for 66 hours. The pH was maintained between 8.0 and 8.5 by addition of 10% sodium hydroxide 4 hours after the start of the digestion and at 12-hour intervals thereafter. Progress of the digestion was followed by: analysis of pH 4.1–soluble (acetic acid) total nitrogen; measurement of the absorbance at 280 mμ of the deproteinized aliquots (3% final concentration of trichloroacetic acid), and the change in trichloroacetic acid (3%)–soluble amino nitrogen. In 66 hours, the pH 4.1–soluble nitrogen had reached a constant value of about 75% of the total nitrogen. The digests were then cooled, adjusted to pH 4.1 with 20% acetic acid, and, after 12 hours at 5° C., the soluble portion was separated by siphoning and by filtration. The precipitate—which yielded fraction 0—was dehydrated with three portions of 95% ethyl alcohol. The combined filtrates and washings were concentrated in vacuo, neutralized to pH 6.5 with 20% sodium hydroxide, and frozen. Yield, 20.48 kg. of concentrate from 10.8 kg. of fraction I; yield of nitrogen, 64.9%; nitrogen content, 11.5%; total solids, 50.5%; and ash, 12.4%. Ratio of total nitrogen to amino nitrogen was 3.59 to 1.

Fractions N-1 and N-2. Fraction N was separated into dialyzable (N-1) and nondialyzable (N-2) portions by placing about 400 grams of fraction N into bags of 1.5-inch cellophane casing about 26 inches long. Six casings were suspended in a glass jar, containing 25 liters of distilled water, which was agitated gently at room temperature. After 48 hours, the bags were transferred to another jar with distilled water, while a fresh charge of fraction N was dialyzed against the liquid in the first jar. When the bags became too turgid, part of the contents were transferred to a fresh dialyzing bag. Each bag was thus transferred three times, successively, from a more concentrated to a more dilute dialyzing medium at room temperature, and was finally dialyzed in the cold room, first against distilled water in a jar for 24 hours and then, overnight, against running distilled water, which was discarded. All other dialyzates were combined, concentrated in vacuo, and frozen. Fraction N, 12.92 kg., yielded 11.92 kg. of fraction N-1. Yield of nitrogen, 81.3%; solids, 45.8%; nitrogen content, 4.58%; and ash, 12.7%. Ratio of total nitrogen to amino nitrogen was 2.98 to 1.

The nondialyzable portions were collected in glass jars, and permitted to settle; the supernatant was concentrated in vacuo and added to the residue, and the mixture was frozen. Fraction N, 12.92 kg., yielded 12.83 kg. of fraction N-2. Yield of nitrogen, 14.9%; nitrogen content, 0.79%; solids, 7.8%; and ash, 0.5%. Ratio of total nitrogen to amino nitrogen was 6.77 to 1.

Fraction O. The pH 4.1–insoluble material obtained in the preparation of fraction N was dehydrated with 95%

Table II. Changes[a,b] in Thrombocyte and Per Cent Lymphocyte Counts During Assay of TCESOM

			Thrombocyte Counts Interval, Days of Assay					Lymphocyte Counts Interval, Days of Assay				
Calf No.	Soybean Oil Meal or Fraction	Pounds[c] Fed/Day/-100 Lb.	Through 20 base line[a]	21–30	31–40	41–50	51–60+	Through 20 base line[a]	21–30	31–40	41–50	51–60+
					% of Base Line[b]					% of Base Line[b]		
Mean of 8	HEXSOM	1/6	892	91.5	86.8	80.4	75.1	59.9	100	104	102	96.8
Std. E.M.			±48.2	±2.1	±1.8	±3.4	±3.6	±2.2	±3.2	±3.5	±5.0	±2.5
1482	TCESOM-6	1/4	544	22.1	6.1	(Dead 33rd day)		56.0	139	173	...	...
1372	D	1/4	1029	25.4	2.2	0.9	(Dead 44th day)	51.8	129	189	192	...
1451	J	1/4	763	20.2	5.0	(Dead 36th day)		54.7	155	177	...	...
1452	K	1/4	648	67.7	60.0	58.8	70.5	64.7	94.7	109	97.1	96.7
1503	P	1/4	1131	31.6	8.0	7.2	2.0	60.3	120	152	143	152
Mean of 3	TCESOM-6	1/6	1210	31.6	10.5	17.0	9.3	65.5	126	144	137	141
1330	A	1/6	657	12.2	15.1	(Dead 35th day)		64.4	116	143	...	...
1331	B	1/6	821	41.9	4.5	3.4	2.2	60.2	105	147	157	162
1340	C	1/6	965	28.7	2.2	(Dead 33rd day)		67.4	131	145	...	...
1369	D	1/6	1080	39.4	11.3	8.3	12.5	69.8	98.4	134	135	120
1368	E	1/6	962	17.8		(Dead 29th day)		70.5	130	...	...	...
1410	H	1/6	839	47.1	12.3	38.9	27.6	46.7	135	204	185	164
1432	I	1/6	864	30.0	4.7	8.1	9.2	71.9	111	135	135	132
1492	N	1/6	769	28.7	7.8	(Dead 31st day)		70.5	125	139	...	...
1502	P	1/6	1081	51.0	15.4	9.2	13.4	46.7	116	167	189	185
1556	TCESOM-6	1/8	1069	45.6	19.4	35.9	16.0	51.2	142	178	139	184
1483	L	1/6 11 days 1/8 49 days	671	74.3	87.7	85.1	63.3	68.7	104	106	101	106
1568	N-1	1/8	1432	47.2	19.9	34.5	18.2	58.5	139	155	122	141
1493	O	1/8	844	53.4	41.7	41.4	41.6	56.9	119	134	141	134
1504	Q	1/8	1200	39.4	15.2	20.5	17.2	52.4	115	156	161	164
Mean of 3	TCESOM-6	1/20	928	75.1	65.4	53.2	53.8	71.2	105	104	102	102
1367	A	1/20	1009	79.6	40.2	39.5	32.5	47.2	109	149	161	163
1370	C	1/20	974	57.0	27.3	37.4	25.6	75.7	99.3	121	118	104
1371	D	1/20	450	38.9	13.3	25.1	23.2	79.3	105	113	109	93.4
1380	E	1/20	921	74.0	52.1	64.4	60.6	71.5	104	111	84.4	93.8
1399	F	1/20	948	83.5	52.5	54.4	45.7	73.8	107	102	112	103
1400	G	1/20	992	57.4	52.9	41.1	43.8	65.4	104	95.4	118	101
1409	H	1/20	973	78.6	60.6	42.9	36.5	59.6	84.8	102	111	126
1429	I	1/20	1047	57.4	25.0	33.1	40.1	66.8	109	121	113	106
1450	J	1/20	677	62.2	59.2	54.8	49.5	71.2	98.7	110	109	107
1453	K	1/20	455	55.8	72.9	66.4	68.9	57.6	117	124	101	110
1494	N	1/20	1060	78.1	50.1	52.3	34.3	53.9	109	132	135	126
1570	N-1	1/20	1243	82.1	57.0	43.6	44.7	60.9	121	119	120	121
1569	N-2	1/20	879	82.5	68.6	66.1	62.2	62.5	112	120	109	104
1501	P	1/20	989	91.2	52.7	46.3	42.1	65.2	70.4	94.5	114	110

[a] The base line values are the means of at least 10 counts made through the 20th day of the assay, given as thrombocytes $\times 10^{-3}$ per cu. mm. of blood and as the percentage of lymphocytes of the total white count.

[b] The values given for the five 10-day intervals after the 20th day of the assay are the means of daily counts calculated as per cent of the base line values for the same animal or group.

[c] For all fractions the amount required to furnish the quantity of nitrogen contained in the stated amount of soybean oil meal from which fractions were prepared.

ethyl alcohol and dried at 60° C. Yield of fraction O, 24.5% by weight; yield of nitrogen, 20.7%; nitrogen content, 11.5%; and ash, 3.8%.

Fraction P. Preliminary experiments showed that pancreatic digestion of TCESOM-6 solubilized only relatively small amounts of nitrogen from the toasted specimen. Because most of our supply of the toxic meal is in this form and because the toxic factor is stable at pH 1.5 (fraction J), the following procedure was devised to increase the yield. TCESOM-6, 10.8 kg., was suspended in 180 liters of dilute hydrochloric acid at pH 1.5 and heated, in glass jars, at 58° to 62° C. for 24 hours. After the suspension was cooled, the pH was adjusted to 4.1 with 10% sodium hydroxide, the precipitate was allowed to settle for 8 hours, and the supernatant was siphoned off and discarded. The residue was washed twice by sedimentation with dilute acetic acid, pH 4.1. These treatments removed 14.9% of the total nitrogen in TCESOM-6. The combined residues were solubilized in dilute sodium hydroxide at pH 11.8; then, slowly and with vigorous stirring, they were adjusted to pH 8.5. The mixture was divided equally between six stainless steel cans, diluted, and digested with pancreatin as described for fraction N. In 66 hours, the pH 4.1–soluble nitrogen had reached a constant value of 85% of the total nitrogen. After the mixture was cooled, the pH was adjusted to 4.8 with 20% acetic acid. The soluble portion was filtered by gravity and the precipitates—which yielded fraction Q—were dehydrated with three portions of 95% ethyl alcohol. The combined filtrates and ethyl alcohol washings were concentrated in vacuo, neutralized to pH 7.0, and frozen. Yield, 34.5 kg. of concentrate from 32.4 kg. of TCESOM-6; yield of nitrogen, 57.0%; nitrogen content, 6.0%; solids, 47.6%; and ash, 11.3%. Ratio of total nitrogen to amino nitrogen was 3.93 to 1.

Fraction P (Control). This was prepared from hexane-extracted soybean oil meal by the same procedure as used for fraction P. Yield 25.4 kg. from 21.6 kg. of hexane-extracted soybean oil meal, yield of nitrogen 66.3%, nitrogen content 4.2%, and ash 9.7%.

Fraction Q. The pH 4.8–insoluble material from fraction P was dehydrated with three portions of 95% ethyl alcohol and dried at 60° C. Yield of fraction Q, 18.9%; yield of nitrogen, 14.7%; nitrogen content, 5.52%; and ash, 5.1%.

Chemical Tests In attempts to find criteria other than the biological activity by which the toxic factor might be recognized, trichloroethylene-extracted soybean oil meal and hexane-extracted soybean oil meal and some of the fractions derived therefrom were investigated with respect to:

1. Amino acids present. TCESOM-6 or hexane-extracted soybean oil meal or the alkali-soluble proteins prepared therefrom (fraction A) were hydrolyzed with 0.05*N* hydrochloric acid in the presence of a cation exchange resin (Dowex-50), according to Paulson and Deatherage (*13*), for 70 hours or with boiling 5*N* barium hydroxide for 8 hours. The progress of the acid hydrolysis was followed by the increase in amino nitrogen (*4*). After complete hydrolysis, suitable aliquots were used for two dimensional paper chromatography by the procedure of Levy and Chung (*8*).

Chromatograms sprayed with ninhydrin or platinic iodide (*25*) were also prepared by the same method from: fraction N described above; fraction N after digestion for 48 hours with an extract prepared from calf intestinal mucosa (*6*), the digest was dialyzed against distilled water and the dialyzate concentrated in vacuo prior to chromatography; and the precipitates, which separated from 1-butanol extracts, prepared according to Dakin (*2*) from fraction P and from fraction N before and after digestion with extracts of calf intestinal mucosa. In this instance, ammonium hydroxide was used instead of sodium hydroxide for the preparation of fraction N.

2. Rate and extent of hydrolysis during incubation at 37° C. for 48 hours of 250 mg. of the alkali-soluble protein with 10 mg. of commercial pancreatin or of a special preparation of pancreatic enzymes of bovine origin or with Ficin (Merck & Co.). At intervals, aliquots of the digests were deproteinized with trichloroacetic acid (final concentration, 3%) and the soluble portion was analyzed for total nitrogen (Kjeldahl), increase in products of hydrolysis as measured by the Lowry-Folin reagent (*9*), amino nitrogen (*4*), and the increase in absorbance at 280 mμ (*7*). The ultraviolet absorption spectrum of the deproteinized digests was also measured.

3. Tryptophan and tyrosine as determined in the unhydrolyzed, alkali-soluble proteins by the method of Dickman and Crockett (*3*).

Results and Discussion

Bioassay The principal data from which the results of the bioassay were evaluated are summarized in Table II. The following properties of the toxic factor support the conclusion that it is associated with the protein fraction of the soybean oil meal. In this form:

1. It is not soluble in water at pH 4.1 (fractions A, B, C, D, E, G, H) or in 95% ethyl alcohol (fractions A, B, C, D, K) or in acetone (fractions G and H).

2. It is soluble in dilute aqueous alkali (fractions A, E, and I) or in dilute aqueous acid (fraction C) and it can be precipitated from such solutions at pH 4.1.

3. By pancreatic digestion of the crude protein fraction, the toxic factor becomes soluble at pH 4.1 to 4.8 and dialyzable at neutrality without much loss of activity (fractions F, N, and P). The survival for more than 60 days of calf 1503, fed fraction P at a level equivalent to 0.25 pound of TCESOM-6, might indicate loss of activity in this fraction. Between the 30th and 38th day, her lymphocyte count rose to 97% of the total white count, and she had the pyrexia which usually precedes death. For unknown reasons she survived, in precarious condition, until the 75th day. The response of calves 1501 and 1502, which received fraction P at lower levels, demonstrates, however, that the fraction retained much of the toxicity of TCESOM-6.

4. The starting materials and the toxic fractions, when fed at the same daily intake of nitrogen, elicited in most instances essentially the same biological response. Exceptions to this were calves 1330, 1340, 1368, and 1496, which became moribund within 35 days as a result of consuming fractions A, C, E, and N, respectively, at levels equivalent to the nitrogen in 1/6 pound of TCESOM-6. In a previous study (*14*) and in this series of experiments, no calves fed 1/6 pound of TCESOM-6 died within the 60-day assay period, although some became moribund shortly thereafter. The toxicity of these fractions, per unit weight of nitrogen, appears to have been somewhat increased; this conclusion is supported by the degree of thrombocytopenia which developed in calves 1367, 1370, and 1494, which were fed fractions A, C, and N at a lower level. This could be the result of removal of nonprotein nitrogen in the fractionation or removal of more extensive absorption of the toxic factor from the intestinal tract.

The toxic properties may be fairly uniformly distributed throughout the major portions of protein molecules, as the toxicity per unit of total nitrogen was essentially the same in the acid or alkaline extracts and in the nonextractable residues. Hence, enzymatic proteolysis was given preference over fractionation of proteins in attempts to achieve concentration of the toxic factor. The toxic property remains associated with small fragments of the proteins in which the average ratio of total to amino nitrogen is as low as 3 to 1 (fraction N-1). Hence there must be a chemical difference between fragments of this, or even a smaller size, obtained from trichloroethylene-extracted soybean oil meal and hexane-extracted soybean oil meal, respectively. Limited experiments suggest, however, that as pancreatic digestion approaches completion, the fragments, which remained nondialyzable (fraction N-2, calf 1569) and which had an average ratio of total

nitrogen to amino nitrogen of 6.8 to 1, retained a smaller proportion of the toxicity than the smaller fragments in fraction N-1.

As it occurs in trichloroethylene-extracted soybean oil meal, the toxic factor is not appreciably inactivated at pH 1.5 at 65° to 70° C. for 48 hours (fraction J), and it can still be precipitated at pH 4.1 after such treatment (fraction L had very low activity). Similarly it is not appreciably inactivated at pH 11 for 15 hours at room temperature (fraction I) or for 7 days at 5° C. (fraction H). However, extensive destruction occurred by treatment at pH 12 at 65° to 70° C. for 48 hours (fraction K, particularly calf 1452). The effect of weak alkali (pH 8 to 8.5 for 66 hours) on the stability of the toxic factor was not sufficient to destroy the activity in pancreatic digests.

The response of the calves to hexane-extracted soybean oil meal and to graded amounts of TCESOM-6 was essentially the same as that found previously (*14*) with one exception—the increase in the lymphocyte percentage of the calves fed 0.05 pound of TCESOM-6 was negligible. This may be because the calves fed $^1/_{20}$ pound of TCESOM-6, as well as other calves (No. 1370, 1380, 1399, 1400, 1424, 1415) fed different fractions at an equivalent level of nitrogen, had a relatively high lymphocyte percentage during the period (through 20 days) which was used as a base line for evaluation of the subsequent hematological response. Other calves (1367, 1409, 1494, and 1570), however, with lower initial lymphocyte percentages showed the subsequent increase that was previously observed concurrently with the marked decrease in thrombocyte count (*14*). Also, there were several calves (particularly 1504, 1568, and 1570, and one calf fed $^1/_6$ pound of TCESOM-6) with an unusually high and a few (1371, 1453 and 1482) with low initial average thrombocyte counts; these conditions, however, did not alter the hematologic response to the toxic factor.

The conclusions that the toxic factor in trichloroethylene-extracted soybean oil meal is insoluble at pH 4.1, soluble in dilute alkali, and associated with the protein fraction of the toxic meal are in accord with those reached independently (*11*). Very recently, McKinney and associates (*12*) announced that *S*-(dichlorovinyl)-L-cysteine produces in calves a syndrome typical of trichloroethylene-extracted soybean oil meal toxicity and suggested that this compound "may be related in structure to a part of, or may possibly be, the toxic principle of" trichloroethylene-extracted soybean oil meal.

Chemical Studies None of the procedures which were applied to either trichloroethylene - extracted soybean oil meal, hexane-extracted soybean oil meal, or fractions prepared therefrom provided any basis for differentiating toxic from nontoxic specimens. The chromatographic patterns of the amino acids obtained by acid hydrolysis of trichloroethylene-extracted soybean oil meal, hexane-extracted soybean oil meal, or of the alkali-soluble proteins prepared therefrom were identical (*24*) with respect to distribution and intensity of color of 18 spots—including that of an unidentified component. The alkaline hydrolyzates also failed to reveal any difference between toxic and nontoxic products. The tryptophan content of the alkali-soluble proteins of TCESOM-6, TCESOM-7, and hexane-extracted soybean oil meal was 1.68, 1.60, and 1.54%, respectively, and the tyrosine content was 5.71, 5.22, and 5.95%, respectively.

The rate and extent of hydrolysis of the soybean proteins with commercial pancreatin or specially prepared bovine pancreatin was essentially the same. The use of these enzymes or of Ficin and the criteria by which hydrolysis was measured provided no basis for differentiating between toxic or nontoxic soybean meals or the alkali-soluble proteins derived therefrom.

The failure to observe differences by the various tests applied could be because the toxic factor, as it occurs in trichloroethylene-extracted soybean oil meal or its various fractions, is not affected by the reagents used or that its concentration is too small to permit—without preliminary concentration—its detection by these tests in the presence of other components of the proteins.

Miscellaneous Observations One calf was fed $^1/_6$ pound of hexane-extracted soybean oil meal which had been extracted at 60° C. with triethylamine, a compound which is used as a stabilizer in commercial extraction-grade trichloroethylene (*10*). The air-dried, insoluble residue was then washed three times with 95% ethyl alcohol containing 4% acetic acid, to remove residues of triethylamine which had caused a calf to refuse the meal not treated in this fashion. No evidence of thrombocytopenia was obtained in this animal. As a control, one calf was fed $^1/_6$ pound of TCESOM-6 which had been washed three times with 95% ethyl alcohol containing 4% acetic acid. This animal developed the same degree of thrombocytopenia and relative lymphocytosis as the control animals fed the same amount of TCESOM-6. The toxic factor is, therefore, not formed by the stabilizer present in commercial trichloroethylene.

As several of the fractions had relatively high concentrations of salts, one calf was fed 68 grams of sodium sulfate decahydrate in addition to 0.05 pound of TCESOM-6. The presence of the salt in the diet did not alter the course of the blood dyscrasia.

One calf was fed a pancreatic digest prepared from hexane-extracted soybean oil meal by the method used for preparation of fraction P, in amounts equivalent to the nitrogen in $^1/_6$ pound hexane-extracted soybean oil meal. This animal died accidentally through strangulation on the 37th day of the trial. To that time, however, her blood counts were in the normal range and gave no evidence that products of pancreatic digestion of nontoxic soybean oil meal induce a blood dyscrasia in calves.

In birds, the feeding of β-aminopropionitrile induces a hemorrhagic syndrome through development of aortic disecting aneurysm (*1*). Garbutt and Strong (*5*) could not detect the presence of this compound in soybeans by their chemical procedure, and our analyses of trichloroethylene-extracted soybean oil meal are in accord with this finding. Nevertheless, one calf was fed 600 mg. of mono-β-aminopropionitrile fumarate per day per 100 pounds for 60 days. Her blood counts remained in the normal range throughout the trial, and no abnormalities were observed in this animal.

Acknowledgment

The specimens of toxic soybean oil meal were generously supplied by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. We are indebted to E. K. Clocker and his staff of the Archer-Daniels-Midland Co., Minneapolis, Minn., for their work in preparing Fraction I; to S. T. Coulter and C. M. Stine of the Department of Dairy Husbandry, University of Minnesota, for use of the vacuum pan and the spray drier; to C. E. Graham of the Wilson Laboratories, Chicago, Ill., for the specially prepared beef pancreatin; and to D. V. Frost of the Abbott Laboratories, North Chicago, Ill., for the supply of β-aminopropionitrile fumarate. We are indebted to Shohachi Wada for chromatographic studies of the protein hydrolyzates; to Philip Klubes for determinations of β-aminopropionitrile, tyrosine, and tryptophan; and to Yolanda Sto. Domingo for the observation on the proteolysis of the soybean proteins. M. E. Bergeland, F. R. Bilkovich, and D. D. Joel gave competent and devoted assistance with the bioassays.

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Received for review May 23, 1957. Accepted August 22, 1957. Paper No. 3775, Scientific Journal Series, Minnesota Agricultural Experiment Station. This work was supported in part by Contract No. AT(11-1)-364 with the U. S. Atomic Energy Commission.

PASSION FRUIT BY-PRODUCTS

Nutritive Values and Utility of Passion Fruit By-Products

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Passion fruit rinds and seeds, by-products of the juice industry, present a serious disposal problem. Experiments involving milch cows, wethers, and rats were conducted to determine the nutritive value of passion fruit by-products as animal feeds. Milk production, feed efficiency, digestion test, and growth data were used as criteria of evaluation. The oil from passion fruit seed was chemically and physically characterized. The by-products were satisfactory for supplementing or supplanting the carbonaceous feedstuffs for dairy cows. The seed oil can also be used to supply the fat requirements of animals.

COMMERCIAL PROCESSING of juice from the passion fruit (*Passiflora edulis flavicarpa*) (Figure 1) for nectar, sherbet, punches, and other food products has been developed during the past years in Hawaii (*16*, *17*). The fragrantly aromatic and pleasingly tart juice is marketed primarily as a frozen product. Commercial vineyard acreage has grown from practically nothing to approximately 1000 acres (*1*) within the past 3 years. Scott (*15*), after surveying the United States (mainland) market potential, estimated that juice from 5000 acres can be marketed annually.

Approximately one third of the weight of the fruit is juice. The rest is composed of about 90% rind and 10% seeds. The processing of fruit from 5000 acres, with an average yield of 10 tons per acre, will result in an annual production of about 60,000,000 pounds of rind and one ninth as much of seeds. The quantity of by-products presents an economic as well as a disposal problem. Thus, some satisfactory solution for the utilization of the residues is needed by the industry. Sherman, Cook, and Nichols (*17*) explored the possibility of extracting pectin from the rind but found that the market for this product can be supplied more economically by other sources. A preliminary investigation on the utilization of the rind as a feed constituent showed considerable promise (*12*).

Passion fruit rind is high in carbohydrates, low in ether-extractable material, and fair in crude protein. It