retained sugar in the presence of the product.

The sucrose octadecenylsuccinates were prepared from an experimental polypropylene-derived anhydride (Neut. Equiv.: Calc'd. for $C_{22}H_{38}O_3$: 175.3. Found: 175.5; b.p. 162–220°/0.5–1.0 mm.) and from *n*-octadecenylsuccinic anhydride [2] in 85 and 91% conversions, respectively (Runs 31 and 32).

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[2] Dynacolor Corporation, "acid-free."

The Examination of Fats and Fatty Acids for Toxic Substances

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The chick edema disease factor was found to be present in a number of distillates and residues that were obtained during the production of commercial fatty acids. The raw materials from which the toxic samples were produced included inedible animal tallows, acidulated vegetable oil foots, and oils recovered from tin plate manufacture. The chick edema factor was found to be present in several oleic acids and in a triolein. Twenty stearic acid samples which were examined were nontoxic.

The nonurea adduct-forming fatty acids that were isolated from commercial oleic acids and various distillates and residues from the manufacture of commercial fatty acids were found to be toxic to weanling rats even after hydrogenation. Analysis of the nonurea-adducting monomers that were isolated from a fatty acid by-product distillate indicated the presence of cyclic structures.

THE OCCURRENCE of the chick edema disease factor in a by-product of the manufacture of commercial oleic and stearic acid (1-3) led to the examination of commercial fats and fatty acids for this factor. The chick edema disease is characterized by excessive fluid in the pericardial sac, in the abdominal cavity, and less often by subcutaneous edema. The causative agent in sufficient concentration brings about high mortality, beginning approximately on the third week after addition to the diet. The toxic factor was first found in the unsaponifiable fraction of a fatty acid by-product distillate added to certain lots of feed-grade fat. Purified fractions were physiologically active in fractions of a part per million of the diet. Subsequent investigation of fatty acid production showed that the chick edema factor was often present in the first distillates and residues and in the commercial fatty acids themselves. In addition, these fractions were also found to contain small amounts of fatty acids not forming adducts with urea that were toxic to weanling rats. The occurrence of the chick edema factor in oleic acids and derivatives has been reported by Ames *et al.* (4).[1] It has been isolated in

[1] The Food and Drug Administration's food additive regulations of April 22, 1960 (CFR Title 21, Section 121.86), requires that oleic and stearic acids be "prepared from edible fats and oils, free from chick edema factor."

crystalline form from a feed-grade tallow (5). The crystalline substance was found to contain about 47% chlorine (6). A crystalline halogen-containing material producing chick edema symptoms at 0.1 part per million in the diet has been isolated from a sample of triolein that was toxic to monkeys (7).

Bio-Assay Procedure for Chick Edema Disease Factor

One-day-old, white Leghorn, single-comb cockerels were fed the basal ration described by Friedman *et al.* (2). The samples to be tested were added to the basal ration to give the usual level of 16% (w/w) in the mixed diet. Unsaponifiable extracts were fed when samples were not well tolerated at levels of 16% or higher. The chicks were identified by wing bands, weighed, kept in brooder cages, not more than 10 to a cage, and given feed and water *ad lib.* The animals were weighed, observed for general appearance, and autopsied promptly upon death. After 21 days all surviving chicks were killed and autopsied. The volume of pericardial fluid was determined by aspiration into a calibrated syringe or pipette. The volume of pericardial fluid in normal chicks did not exceed 0.2 ml. Hydropericardium activity was scored as follows: 0–0.2 ml. pericardial fluid, 0; 0.2–0.4 ml., +1; 0.4–1.0 ml., +2; 1.0–2.0 ml., +3; >2.0 ml., +4. A finding of one positive score in a group of chicks was evidence of the presence of the edema disease factor in the sample under test. (A modified bioassay procedure has been studied collaboratively (8) and has been adopted by the Association of Official Agricultural Chemists.)

Weanling Rat Bio-Assay for Urea Filtrate Toxicity

Weanling rats, not more than 21 days old, weighing 30–40 g. were divided into groups so that there were not less than four animals per group with the same number of males and females in each group. Where possible, a litter-mate of the same sex was present in each group. Each animal was kept in an individual cage during the course of the test with food and water *ad lib.* Body weight and food intake were recorded daily. The samples were fed by tube, using a blunted No. 18 hypodermic needle 2–3 in. long. Each animal in the group was fed 0.4 ml. of the test sample on the first and second days of the test period. One group, designated as the negative control group, was also force-fed fresh vegetable oil in the same amount. All doses were fed at the same time of day, and the animals were observed for a period of seven days after the first dose was given. Toxicities were scored as follows: death of animal 1–2 days after initial feeding, +4; death in 3 days, +3; death in 4–5 days, +2; death in 6–7 days or 10-g. weight loss without death, +1.

Isolation of Unsaponifiables and Urea Filtrate Acids for Toxicity Studies

a) *Animal and Vegetable Fats, Oleic Acids, and Derivatives of Oleic Acid.* The 400-g. samples were refluxed with 950 ml. of alcohol and 200 ml. of 50% (w/w) KOH for 1.5 hrs. and extracted six times with ethyl ether in 6-liter separatory funnels. The ether extracts were washed free of soaps with water and 0.5 *N* KOH, dried with sodium sulfate, and freed of ether. The unsaponifiable material was weighed and stored under nitrogen at −15°C. The fatty acids were regenerated by acidification with concentrated HCl, extracted with ethyl ether, dried with sodium sulfate, and freed of solvent. Ethyl esters were prepared by refluxing 200 g. of acids for 2.5 hrs. with nine volumes of anhydrous ethyl alcohol containing approximately 1.5% H_2SO_4. The esters were obtained from the esterification mixture by dilution with an equal volume of H_2O, followed by extraction with ethyl ether-light petroleum ether (1 + 1). The extract was carefully washed with 0.5 *N* KOH and H_2O, then dried with sodium sulfate and freed of solvent.

Urea filtrate esters were isolated as follows: 200 g. of ethyl esters and 400 ml. of 90% ethanol were added to a 4-qt. stainless steel mixing bowl, the mixture was warmed to 50°C., and 800 g. of urea (passed through a No. 20 sieve just before use) were added with continuous stirring to minimize the formation of lumps. Stirring was continued for 1 min. after the addition of urea, and the mixture was kept at 50°C. for 30 min., with stirring every 3–4 min., then covered with aluminum foil, and kept over-night at room temperature. The mixture was transferred to a 241-mm. i.d. Büchner funnel (Coors porcelain, size No. 6) holding a 24-cm. filter paper (Whatman No. 4 or equivalent, previously washed with a small portion of urea-saturated 90% ethanol) and filtered. The precipitate was washed four times with 400-ml. portions of urea-saturated 90% ethanol by stirring the contents of the funnel to form a slurry and then applying vacuum each time. The filtrate was diluted with an equal volume of water, acidified with HCl, and extracted four times with petroleum ether-ethyl ether (1 + 1). The filtrate esters were stored under nitrogen at −15°C.

b) *Stearic Acids.* Urea filtrate substances were isolated by treatment of 1,000-g. portions of the sample directly with 2,000 ml. of 90% ethanol and 4,000 g. of urea as with the esters. Unsaponifiables were extracted from the isolated filtrate material as with the acids in (a). The urea filtrate acids were esterified with 10–12 volumes of anhydrous ethanol that contained 1% H_2SO_4. The esters were isolated as described above and stored under nitrogen at −15°C.

Determination of Urea Filtrate Acids from Fats and Fatty Acids as the Ethyl Esters

Unsaponifiables were extracted from 25 g. of sample by the A.O.A.C. method by using ethyl ether (9). Ethyl esters were prepared from the regenerated fatty acids by refluxing 20 g. of dry acids for 2.5 hrs. with 10 volumes of anhydrous ethyl alcohol that contained 1.5% H_2SO_4.

Urea filtrate esters were determined as follows: Twenty ml. of 90% ethanol were added to a 10-g. sample in a 250-ml. beaker, and the mixture was warmed to 50°C. in a water bath. Forty g. of urea (passed through No. 30 sieve before use) were added slowly, with constant stirring to prevent the formation of lumps. Stirring was continued for 1 min. after the addition of urea and every 3 to 4 min. during the next 30 min. that the sample was kept at 50°C. The beaker was removed from the bath, and, after standing at room temperature for 30 min., the contents were stirred for an additional 2 min. to break up any lumps that may have formed. After standing over-

TABLE I

Examination of Samples from Commercial Splitting and Distillation of Inedible Animal Tallow

Sample	% of original split fat	Unsap., %	Polymers, %	Test for steroidal hydrocarbons, $A_{625\,m\mu}$	Urea filtrate monomers, %	Weanling Rat Toxicity			Chick edema toxicity, 10% level
						Urea filtrate monomers	Urea filtrate monomers hydrogenated	Polymers	
1. 1st distillate, continuous still	80	0.35	0.3	0.015	0.65	+3	+3	.....	± (+1, ½% unsap.)
2. 1st residue, continuous still	20	6.60	21.1	0.585	5.62	+3	+2	+3	+1
3. 2nd distillate, continuous still	12	2.40	0.3	0.640	2.70	+4	+2	.....	+1
4. 2nd residue, continuous still	8	8.65	17.3	1.35	4.62	+4	+3	neg.	+2
5. Distillate, batch still	5	14.8	13.2	2	8.77	+3	+3	neg.	+4

night in a vacuum desiccator under nitrogen, the mixture was transferred to a sintered glass (medium porosity) funnel in a filtering flask. The beaker was washed with 35 ml. of urea-saturated 90% alcohol, the washings were added to the funnel, the contents of the funnel were slurried, and vacuum was applied until the filtrate flow rate slowed to about 1 drop/sec. The adduct was washed three more times in this manner. Since the wash solvent decomposes a small amount of adduct, a "wash blank" was determined by repeating the washing steps above; the combined four washings were kept separate from the combined initial washings and filtrate. Each filtrate was evaporated in the steam bath to about 60 ml. and transferred to a 500-ml. separator, using a total of 150 ml. of H_2O and 100 ml. of redistilled petroleum ether-ethyl ether (1 + 1). Two ml. of concentrated HCl were added, and the sample was extracted four times, using 75-ml. portions of mixed solvent for the last three extractions. The combined extracts were washed free of acid and dried with sodium sulfate. The solvent was removed, and the sample was dried to constant weight by heating the flask with constant swirling on the steam bath while attached to a vacuum pump until an internal pressure of 2–3 mm. Hg. was reached. The flask was cooled 30 min. and weighed. A reagent blank was also determined. The "wash blank" and reagent blank were subtracted from the weight of initial urea filtrate esters.

The details of the development of this procedure will be reported elsewhere (10).

Other Methods

The relative amounts of steroidal hydrocarbons present in the samples examined were determined by a color test (1). Gas chromatograms of various samples were obtained before and after hydrogenation, using a Perkin-Elmer Vapor Fractometer Model 154C, with a diethylene glycol-glutarate polyester substrate on a silane-coated Chromosorb W. (Johns-Manville 80–100 mesh) support, hot wire detectors, and an oven temperature of 200°C. Samples were hydrogenated at room temperature for 40 min. in a low pressure Parr reaction apparatus (Parr Instrument Company, Moline, Ill.) at pressures of 50 p.s.i. of hydrogen. One part of 10% palladium on charcoal catalyst was used to 10 parts of sample dissolved in 10 volumes of 95% ethanol. Unsaponifiable matter and Wijs iodine values were determined by the official A.O.A.C. methods (9), peroxide values and oxirane oxygen according to the official A.O.C.S. methods (11), carbonyl oxygen by the method of Knight and Swern (12), hydroxyl contents, using acetic anhydride in pyridine as the acetylating reagent (13), molecular weights by the cryoscopic method of Gay (14). Monomers and polymers were isolated by molecular distillation. The derived ethyl esters were fractionated in a micromolecular still (15) or were distilled in a 2-in. Rota Film molecular still (A.F. Smith Company, Rochester, N.Y.).

Results

The chick edema factor was present in the distillates and still residues obtained from several manufacturers of commercial fatty acids. Results of the examination of a series of samples obtained by the processing of what was described as an inedible tallow by one manufacturer are given in Table I. Chick edema toxicity was observed in all samples, including the initial distillate. The results indicated that the sterol color test and content of polymers and unsaponifiables could not be used as an indication of the presence of the edema factor. The samples contained urea filtrate monomers, which were toxic to weanling rats both before and after hydrogenation. Only one of the polymer fractions isolated from the samples was toxic to weanling rats. In contrast, urea filtrates obtained from the ethyl esters of fresh cottonseed and corn oils and beef and pork fats were not toxic to weanling rats. Also urea adducts from the toxic samples were not toxic to weanling rats.

A number of samples derived from vegetable oil foots were examined. Acidulated fatty acids and primary distillates and residues from coconut, corn, and soybean oil foots showed no chick edema activity. One sample of acidulated cottonseed oil foots and the split distillate and residue were inactive. Another cottonseed oil sample showed chick edema activity (+1) in the primary residue after distillation.

A sample described by the manufacturer as hydrolyzed palm oil recovered from tin plate processing during 1959 was also examined (Table II). Only the still residue, representing about 15% of the feedstock, showed chick edema activity. Similar results were obtained with another recovered "palm oil" of similar composition. Only the still residue, representing 26% of the feedstock, showed chick edema toxicity when it was fed at a level of 16% in the diet. When the unsaponifiables, extracted from the feedstock and distillate, were fed at a level equivalent to 175% of the original sample, all the chicks died within one week. However no definite chick edema symptoms were observed.

Samples of hydrogenated and unhydrogenated batch still distillates from another manufacturer were examined. The raw material was reported to be hydro-

TABLE II

Examination of Palm Oil Fatty Acids Recovered from Manufacture of Tin Plate

	Split undistilled	Distillate	Residue
Unsaponifiables, %	3.23	0.76	21.0
Urea filtrate, %	3.57	4.37	38.5
Steroidal hydrocarbon test, $A_{625\,m\mu}$	0.010	0.045	0.280
Chick edema toxicity	neg.	neg.	+4
Weanling rat toxicity, urea filtrate	+3	neg.	+4
Fatty acid composition:			
C_{14}	2.3	1.9	.....
C_{16}	51.2	52.9	.....
C_{18}	2.1	1.1	.....
C_{18} monoene	38.5	38.1	.....
C_{18} diene	6.0	7.0	.....

lyzed inedible tallow. Hydrogenation of the tallow decreased the iodine number from 50 to 0.56. The steroidal hydrocarbon absorbance decreased from 0.235 to 0.035, presumably because of saturation of cholestadiene and related compounds. However unhydrogenated and hydrogenated tallows had the same order of chick edema toxicity (+1.0 and +0.5, respectively).

A fatty acid by-product distillate was used as a source of material in studies on the isolation of the edema factor. Repeated column chromatography of the unsaponifiable fraction resulted in the preparation of toxic concentrates with UV spectra of substituted naphthalenes (2,7). Concentrates of the toxic factor are active in fractions of a part per million in the diet. The urea-adducting fatty acids from this material (molecular weight of methyl esters 288) were nutritionally equal to those from fresh fat. The urea filtrate acids isolated from the by-product (molecular weight of methyl esters 323) were toxic to weanling rats, showing the same toxicity before and after hydrogenation. Residual unsaturation after hydrogenation indicated the presence of a structure with substituents in the vicinity of the double bonds. Treatment of the urea filtrate monomers, according to the aromatization procedure of Scholfield and Cowan (16), indicated the presence of cyclic structures. The absorption curve of the oxidized brominated-dehydrobrominated material in the 270–280 mμ region was similar to that of phthalic anhydride.

A sample of ethyl ester urea filtrate was chromatographed on six parts of alumina (Fisher Scientific Company, A540). Eight liters of petroleum ether eluate and six liters of ethyl ether eluate were collected. The polymeric material remaining on the column was removed by ethyl ether extraction of an acidified slurry of the column material. The petroleum ether eluate was hydrogenated and again chromatographed on alumina. The results (Table III) indicate that the toxicity was not correlated with the degree of oxidation. It is noteworthy that the low-pressure hydrogenation did not saturate the toxic material. Gas chromatograms of the urea-adduct esters and the monomeric urea filtrate esters before and after hydrogenation are shown in Figure 1. About

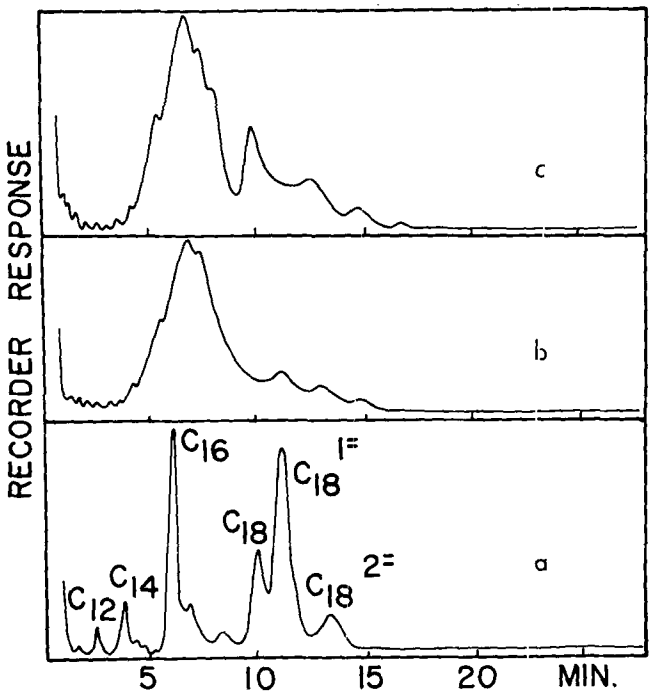

FIG. 1. Gas chromatograms of a) ethyl ester urea adduct, b) urea filtrate, and c) hydrogenated urea filtrate from a fatty acid by-product distillate.

TABLE III

Chromatographic Fractionation of By-product Ethyl Ester Filtrate

Sample No.	Description	Appearance	% of total fatty acids	Iodine value (Wijs)	Carbonyl oxygen, %	Hydroxyl oxygen, %	Weanling rat toxicity
1	By-product filtrate	Dark brown liquid	14.0	60	0.18	0.25	+3
2	Petroleum ether eluate of (1)	Light yellow liquid	11.8	53	0.09	0.43	+3
3	Ethyl ether eluate of (1)	Dark brown liquid	1.7	63	0.82	1.43	+2
4	Residual polymers of (1)	Dark brown viscous liquid	0.5	.....	.....	.....	.....
5	Petroleum ether eluate (2) after hydrogenation	Water-white liquid	10.9	40	0.03	0.33	+3

TABLE IV

Commercial Oleic Acids

Sample No.	Unsap., %	Peroxide value	Oxirane oxygen, %	Iodine value (Wijs)	Steroidal hydrocarbon test $A_{625\,m\mu}$	Urea filtrate, %	Weanling rat toxicity urea filtrate esters		Chick edema toxicity	
							Unhydrogenated	Hydrogenated	Heart fluid incidence	Numerical group score
1	0.44	2	0.01	91.3	0.050	0.54	+3	+3	0/10	0
2	0.22	0	0.01	87.6	0.076	0.60	+3	+3	2/10	+0.2
3	0.29	8	0.00	87.6	0.175	0.77	+2	+1	10/10	+3.1
4	0.33	11	0.01	92.2	0.325	1.01	+1	neg.	0/10	0
5	0.30	4	0.02	94.0	0.068	0.45	+3	+3	0/10	0
6	0.35	6	0.06	91.6	0.085	0.61	+3	neg.	0/10	0
7	0.25	2	0.14	94.0	0.105	0.55	+3	+3	0/10	0
8	0.41	3	0.00	92.6	0.095	0.64	+4	+3	1/10	+0.1
9	0.28	3	0.01	93.2	0.065	0.67	+1	neg.	0/10	0

TABLE V

Fatty Acid Composition of Commercial Oleic Acids by Gas Chromatography

Sample numbers	1[a]	2	3[b]	4[c]	5[d]	6	7	8	9
Components	%	%	%	%	%	%	%	%	%
C_{12}	0.1	0.4	0.2	0	0.2	0	0	0.2	0
C_{14}	2.8	3.0	4.0	1.0	3.1	0.6	0.7	3.7	3.2
C_{14} Monoene	2.7	3.0	3.0	0.8	2.8	0.1	0.4	3.2	2.1
C_{16}	4.8	4.8	2.8	3.3	4.0	4.5	1.6	3.3	2.7
C_{16} Monoene	12.5	12.9	12.6	6.8	12.3	5.9	5.7	13.0	11.3
C_{17} Monoene	1.7	1.9	1.4	1.4	1.8	0.2	0.7	1.8	0.8
C_{18}	0.4	0.4	0.8	1.0	0	0.3	0	0	0
C_{18} Monoene	70.5	71.0	73.2	73.8	68.6	75.8	77.8	70.9	74.7
C_{18} Diene	2.2	2.6	1.9	8.2	4.6	12.6	6.5	3.6	5.2
C_{18} Triene	1.2	0	0	3.4	1.8	0	0	0.3	0

[a]Also 0.3% C_{15} monoene and 0.9% C_{21}.
[b]Also 0.3% C_{15} monoene.
[c]Also 0.8% C_{20} monoene.
[d]Also 0.1% C_{10}.

80% of these filtrate esters were classified as "abnormal" constituents. The abnormal constituents are defined as those compounds not having gas chromatographic retention-times corresponding to those of normal fatty acids.

A subacute oral toxicity study with rats showed that the filtrate fatty acids caused growth depression, anemia (hypochromic), and increased liver, kidney, and heart weights. The plasma alkaline phosphatase values were increased 90% and 250% over the controls at 1% and 5% dietary feeding levels, respectively (17).

A number of commercial oleic and stearic acids were examined for chick edema toxicity as well as weanling rat toxicity. The oleic acids were collected in late 1959 from food-manufacturing plants. Several of the oleic acids showed varying degrees of chick edema toxicity (Table IV). Most of the urea filtrates isolated were lethal to weanling rats. Generally the toxicity of the filtrates was not changed by hydrogenation. The fatty acid composition of the oleic acids is given in Table V. The oleic acid (C_{18} monoene) content varied from 69 to 78%, palmitoleic (C_{16} monoene) from 6 to 13%, and linoleic (C_{18} diene) from 2 to 13%. Gas chromatographic analyses of the urea filtrates isolated from the oleic acid samples indicated that approximately half of the filtrates were abnormal material, distributed mostly in the C_{16}, C_{18}, and C_{20} regions. Gas chromatograms of the total esters and of the filtrate material from one of the commercial oleic acids are shown in Figure 2. Both chick edema and weanling rats toxicity were found in this sample. Approximately 50% of abnormal material was estimated to be present in the urea filtrate, comprising at least six components.

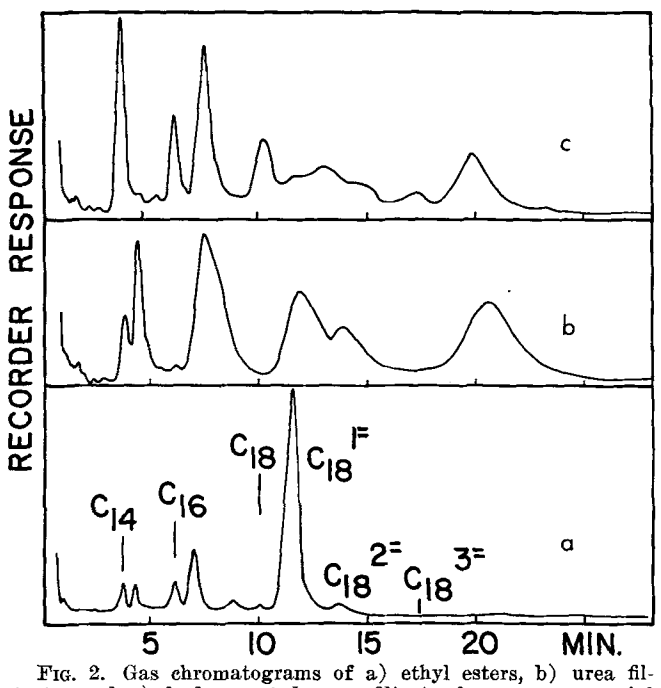

FIG. 2. Gas chromatograms of a) ethyl esters, b) urea filtrate, and c) hydrogenated urea filtrate from a commercial oleic acid.

Nine commercial derivatives of oleic acid, including glyceryl mono-oleate, propylene glycol mono-oleate, glyceryl oleostearate, polyoxyethylene sorbitan monooleate and triolein were examined for chick edema toxicity. Eight of the samples were inactive. One of two triolein samples were very toxic (+4).

Neither chick edema toxicity nor weanling rat-toxic filtrate acids were found in a group of 20 stearic acids that were examined.

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