Table III. Recovery Study (Urine) (10-g Sample)

Residue level, ppm	μg of M-analog Added	Found[a]	% recov
0.2	2.0	1.4	70
0.2	2.0	1.2	60
0.5	5.0	2.4	48
0.5	5.0	4.6	92
0.5	5.0	4.0	80
0.5	5.0	5.7	114
1.0	10	6.8	68
1.0	10	7.9	79
1.0	10	11	110
2.0	20	20	100
2.0	20	11	55
5.0	50	59	118
		Av	83%

[a] Corrected for the appropriate blank of untreated control; blank varied from 0.6 to 3.4 μg (0.06 to 0.34 ppm).

0.05 ppm in milk based on a 50-g sample. Because of interferences encountered on untreated urine and tissue samples, smaller sample sizes were analyzed and sensitivities of 0.2 and 0.08 ppm, respectively, were obtained. The procedure is a modification of the M-analog in rumen fluid method of Alicino and Katz (1972); additional clean-up steps were included in our isolation steps to further purify the extract before derivatization. BSA [*N*,*O*-bis(trimethylsilyl)acetamide] was used as the silylation reagent instead of the reagent (Tri-Sil) used by Alicino and Katz. In addition, the gas chromatographic determinations were made using a 6-ft glass column packed with 5% OV-7 on 80/100 mesh Supelcoport. To obtain a highly selective measurement of the desired compound, a sulfur-sensitive flame photometric detector was used. The previous workers used a different GC column (1.5% OV-210 on Gas-Chrom Q) and a flame ionization detector. It was necessary to make these modifications in the published method in order to apply it to this work.

Recoveries of M-analog added to untreated control samples are summarized in Tables I, II, and III. As shown in Table I, methionine, if present in the sample, would not interfere with the analyses. Blanks (positive readings) were encountered on untreated liver and kidney samples. The recovery factors from these tissues have been corrected for the respective blanks. Lower and more erratic recoveries were obtained from 10-g urine samples. These data have also been corrected for blanks (positive readings) obtained on untreated control urine.

Gas chromatographic scans representing control milk, milk fortified at 0.1 ppm, control lean meat, and lean meat fortified at 0.2 ppm are shown in Figures 2 and 3.

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Occurrence of Methylguanidine and Agmatine, Nitrosatable Guanidino Compounds, in Foods

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Among naturally occurring guanidines, nitrosated methylguanidine (MG) has been reported to be strongly mutagenic and carcinogenic and nitrosated agmatine (AG) to be moderately mutagenic. A survey has been conducted on the MG and AG contents of various fresh and processed foods. No appreciable amount or trace amounts of MG could be detected in fresh beef, pork, chicken, and various fish and shellfish; in addition, almost the same low levels of MG were detected in various processed foods, except for the cases of smoked-dried fish products called "Katsuo-bushi" and "Kezuri-bushi" in Japanese, and the MG values for these products ranged from 18 to 178 mg/kg. Comparatively high concentrations of AG were detected in fresh abalone and top-shell muscles ranging from 40 to 200 mg/kg among the fresh foods tested, while fairly high concentrations of AG could be detected in some processed foods in which dried squid was found to contain as high as 650 mg/kg of AG.

Endo et al. (1974a) examined the mutagenicity of various nitrosated guanidine derivatives which are structurally similar to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a well-known mutagen and a potent gastric carcinogen. Among naturally occurring guanidines, nitrosated methylguanidine (MG) has been reported to be the most mutagenic and nitrosated agmatine (AG) to be moderately mutagenic. Mirvish (1971) and Endo and Takahashi (1972) demonstrated that MG was nitrosated under acidic conditions to give methylnitrosocyanamide and methylnitrosourea. The oral and intragastric administrations of these *N*-nitroso compounds have been confirmed to induce gastric and esophagal cancers in experimental animals (Druckrey, 1972; Endo et al., 1974b). MG has long been considered to occur widely in nature, especially in fresh beef and various fish. Concerning the occurrence of MG in foodstuffs, however, most of the studies were conducted in the 1930's and seemingly many problems exist as to the methods of isolation and identification of MG in the test materials. AG, a decarboxylated product of arginine, has been isolated from a few invertebrate sources, notably from the sponge, *Geodia gigas*, from several cephalopods (Baldwin, 1963), and from putrefied material (Hayashi, 1955). No data on the occurrence of AG in fresh foods and various processed foods so far have been available.

Recently we have developed a method for the determination and identification of some basic guanidino compounds, i.e., MG, AG, and guanidine in foods, and a

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survey has been conducted on the MG and AG contents in various fresh and processed foods.

EXPERIMENTAL SECTION

Food Samples. All fresh foods were collected at the Tokyo Central Wholesale Market, and various processed foods were purchased at food stores in the Tokyo areas.

Reagents. MG and AG were purchased from Sigma Chemical Co. and hexafluoroacetylacetone (HFAA) and other reagents employed in the present study were analytical grade (Wako Pure Chemical Co., Tokyo) and were used without further purification.

Determination of MG and AG in Foods. A well-minced food sample of 20 g was extracted with 4% trichloroacetic acid (TCA; 30 mL, three times), and excess TCA in the extract was removed with diethyl ether (100 mL, three times). After removal of the ether by evaporation in vacuo, the volume of the extract was made up to 20 mL with water. An aliquot of the extract was separated by ion-exchange column chromatography using a weakly cationic resin, Amberlite CG-50 Type 1 (100–200 mesh) pretreated with 0.2 N NaOH (column: 0.9 × 25 cm) according to Stein et al. (1971). Eluate with 1 N NaOH was collected in 4-mL fractions by an automatic fraction collector, and each fraction (1–2 mL portion) was subjected to colorimetric determination with modified Voges-Proskauer color reaction according to Micklus and Stein (1973). Recoveries of MG and AG added to TCA extract over a range 10 to 100 μg were found to be 102 ± 7.2% for MG and 102 ± 10% for AG, respectively.

Identification of MG and AG by Gas Chromatography–Mass Spectrometry. MG and AG separated by ion-exchange chromatography were further identified by gas chromatography–mass spectrometry (GC–MS) after converting the guanidines into hexafluoroacetylacetonates (Kawabata et al., 1977).

Fractions showing a positive color reaction were transferred into a 50-mL separatory funnel containing solid NaCl (1 g/3 mL of eluate). The guanidine was extracted three times with equal volume of H_2O-saturated 1-butanol. The combined butanol extracts were evaporated in vacuo to dryness. The residue was dissolved in a small volume of 80% ethanol, which was then transferred into a hard glass ampule. After the ethanol in the ampule was dried with the nitrogen stream, each 0.1 mL of HFAA and pyridine was added to the ampule, which was then heat-sealed. The HFAA derivatization of MG and AG was conducted at 120 °C for 1 h. After the derivatization, 1 mL of diethyl ether and 3 mL of 3 N HCl were added to the reaction mixture, and this was shaken vigorously and centrifuged. A 2-μL aliquot of the ether layer was employed for the GC–MS analysis. A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer was used to obtain mass spectra of the HFAA derivatives of MG and AG. Separations were carried out on a glass column (2 m × 3 mm i.d.) packed with 3% OV-17 on 60–80 mesh Shimalite W: carrier gas (herium) flow rate, 30 mL/min; the temperatures of the injection port and oven were: the HFAA derivative of MG (MG-HFAA), 250 and 70 °C; the HFAA derivative of AG (AG-HFAA), 250 and 170 °C; separator temperature, 270 °C; ion-source temperature, 270 °C; trap current, 60 μA; electron energy, 70 eV; accelerating voltage, 3.5 kV.

RESULTS AND DISCUSSION

MG and AG were found in some of the samples analyzed. In such cases, the fractions showing a positive color reaction in eluates were further identified by GC–MS in the form of HFAA derivatives. Typical elution patterns of MG and AG by ion-exchange chromatography from

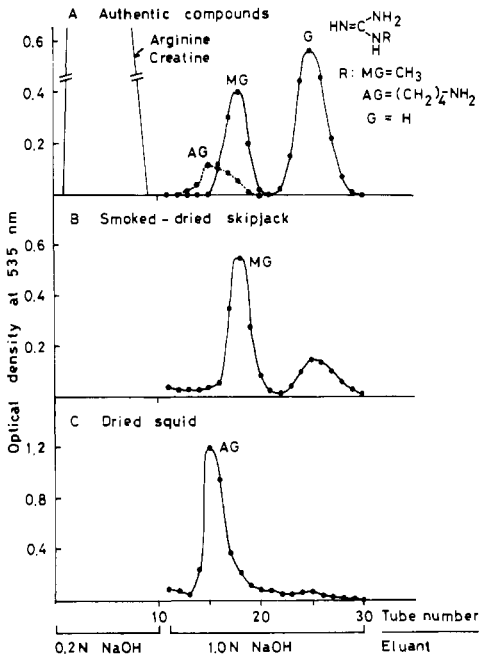

Figure 1. Elution patterns of methylguanidine, agmatine, and guanidine separated by ion-exchange column chromatography: (A) from authentic compounds; (B) from the TCA extract of smoked-dried skipjack; and (C) from the TCA extract of dried squid.

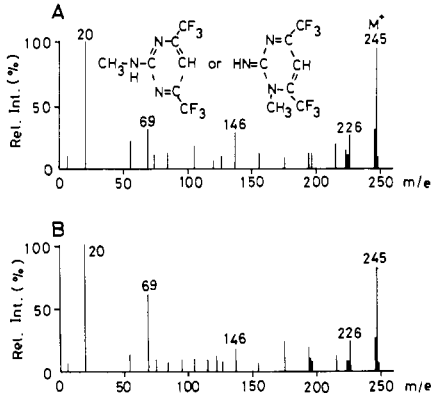

Figure 2. Mass spectra of the hexafluoroacetylacetone derivative of methylguanidine: (A) from authentic MG and the hypothesized structure; and (B) from MG isolated from the smoked-dried skipjack shown in Figure 1 (B).

authentic compounds and those from some foods are shown in Figure 1. GC–MS spectra of the MG-HFAA and AG-HFAA from authentic compounds and those from some foods are shown in Figures 2 and 3.

Recently, Fujinaka et al. (1976) have reported the MG contents in various foods, and they employed a combination of column chromatography using microcrystalline cellulose and ion-exchange resin, paper chromatography, gas chromatography, and gas chromatography–mass spectrometry. Methods used in the present study were apparently simpler than those employed by Fujinaka et al.; in addition, it was found that both MG and AG were easily derivatized with HFAA, while AG could not be derivatized with acetylacetone which was employed by Fujinaka et al. (Kawabata et al., 1977).

MG and AG contents in various fresh foods are shown in Table I. As shown in Table I, no appreciable amount or trace amounts of MG ranging from no detection to 2.5 mg/kg, could be detected in the samples tested so far (minimum detection limit of MG was 0.25 mg/kg). MG has long been considered to occur naturally in various foods

Table I. Methylguanidine and Agmatine Contents in Fresh Fish, Shellfish, and Meats

Common names (Japanese)	Scientific names	Content, mg/kg MG	Content, mg/kg AG
Fish			
Broadbill swordfish (Mekajiki)	*Xiphias gladius*	ND[a]	1.3
Chubmackerel (Masaba)	*Pneumatophorus japonicus japonicus*	ND	3.4, ND
Chum or dog salmon (Sake)	*Onchorhynchus keta*	ND	7.1, ND
Cutlass fish (Tachi-uo)	*Trichiurus lepturus*	ND	7.0, ND
Flounder (Magarei)	*Limanda herzensteini*	ND, 1.9	1.0
Flyingfish (Tobi-uo)	*Progmichtys agoo*	ND, 0.5	6.9
Horse mackerel (Maaji)	*Trachurus japonicus*	ND	6.6
Pacific halibut (Ohyo)	*Hippoglossus stenolepis*	ND, 0.3	17.7
Pacific saury (Sanma)	*Cololabis saira*	ND	2.4
Rainbow trout (Niji-masu)	*Salmo gairdnerii irideus*	ND	3.5
Sardine (Ma-iwashi)	*Sardcnops melanosticta*	ND, 0.5	5.8
Shark (Nezumi-zame)	*Lamma ditropis*	1.8	12.2
Skipjack (Katsuo)	*Katsuwonus pelamis*	ND	3.1
Spanish mackerel (Ushi-sawara)	*Scomeromorus sinensis*	1.0	3.1
Yellowtail (Buri)	*Seriola quinqueradiata*	ND, ND	ND, ND
Shellfish			
Abalone, muscle (Awabi)	*Haliotis sieboldii*	ND	40
Abalone, viscera		ND	69
Top-shell, muscle (Sazae)	*Turbo (Batillus) cornutes*	ND	200
Top-shell, viscera		ND	224
Arkshell (Aka-gai)	*Anadara broughtonii*	ND	2.4
Scallop (Hotate-gai)	*Patinopecten yessoensis*	ND	ND
Shortneck clam (Asari)	*Tapes (Amygdalc) japonicus*	ND	ND
Livestock and poultry			
Beef		ND, 1.5	2.2, 2.8
Chicken		ND, 2.6	9.8, 2.9
Pork		ND, 0.8	ND, 2.1

[a] ND means not detected, and the minimum detection limit of MG was 0.25 mg/kg, and that of AG was 0.5 mg/kg.

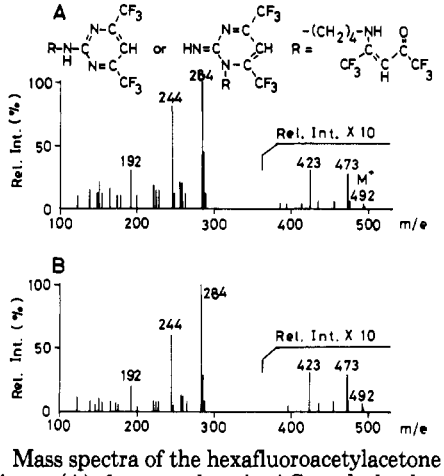

Figure 3. Mass spectra of the hexafluoroacetylacetone derivative of agmatine: (A) from authentic AG and the hypothesized structure; and (B) from AG isolated from the dried squid shown in Figure 1 (C).

such as fresh beef (60 mg/kg, Komarrow, 1929; 323 mg/kg, Kapeller-Adler and Krael, 1930a), fish-rays (120 mg/kg, Kapeller-Adler and Krael, 1930b), cod (297 mg/kg, Kapeller-Adler and Krael, 1930b), sardines (17 mg/kg, Sasaki, 1938), and shark (1900 mg/kg, Kapeller-Adler and Krael, 1930b).

In order to clarify the reasons for the discrepancies between the high values reported by the earlier workers and the low ones obtained in the present study, we examined the procedures employed by the earlier workers. It was already known in the 1930's that mercuric oxide and other mercuric salts, which were frequently used as precipitants of nitrogenous bases in biological materials, could catalyze the oxidation of either creatine or creatinine to give MG as an artifact (Baldwin, 1963; van Thoai, 1965). As shown in Table II, it has become clear that some other metallic salts in addition to mercuric chloride can catalyze

Table II. Formation of Methylguanidine from Creatine and Creatinine in the Presence of Metallic Precipitants

Precipitant[a]	Yield of MG from, % Creatine	Yield of MG from, % Creatinine
Phosphotungstic acid and 5% H_2SO_4	0.02	0.13
$AgNO_3$ in acidic solution	0.04	0.17
Excess $AgNO_3$ and $Ba(OH)_2$	1.88	2.60
$HgCl_2$ and 2.5% H_2SO_4	0.21	1.05

[a] The precipitants and conditions were the same as shown in the isolation procedures of MG and other nitrogenous bases by Komarrow (1929) and Sasaki (1938).

the oxidation of creatine and creatinine to form MG. Among these metallic compounds, silver nitrate ($AgNO_3$) under alkaline conditions gave the highest yield of MG. Komarrow (1929) and Sasaki (1938) employed these metallic salts as precipitants in the isolation process of MG. Evidently, the MG values reported by them must include the artifact derived from creatine and creatinine. To the contrary, Kapeller-Adler and Krael (1930a,b), who reported the presence of 1900 mg/kg of MG in shark muscle, did not use such metallic precipitants to isolate MG, but they obtained their MG values indirectly by estimating the methylamine generated by the hydrolysis of an alcohol-soluble extract under alkaline conditions. It is, therefore, unlikely that MG occurs naturally in fresh foods in high concentrations as previously reported.

Creatine and creatinine are known to occur in vertebrate muscles in fairly high concentrations, and these substances may be converted to MG by oxidation via guanidinoglyoxylic acid (creatone). We consume in our diet a variety of processed foods including dried meat and fish products, and it is of great importance to evaluate the MG levels in

Table III. Methylguanidine and Agmatine Contents in Various Processed Foods

Names (Japanese and scientific names)	Content, mg/kg MG	AG
Smoked-dried fish products		
Smoked-dried skipjack (Katsuo-bushi)	93.6, 178, 21.2, 56.0, 37.9, 18.1	ND[a]
Mixture of sliced products of chubmackerel and horse mackerel (Kezuri-bushi)	37.5, 44.4, 46.5	ND
Sliced product of sardines (Kezuri-bushi)	37.8	ND
Salted and dried fish products		
Salted-dried horse mackerel	ND[a]	ND
Salted-dried chubmackerel	ND	ND
Salted-dried round herring (*Etrumeus micropus*)	ND	217
Seasoned-dried round herring (Urumeiwashi-no-mirinboshi)	ND	ND
Salted-dried "Shishamo" (*Spirinchus lancepatus*)	1.9	0.5
Dried anchovy larvae (Tatami-iwashi)	1.6	ND
Boiled-dried anchovy (*Engraulis japonicus*)	1.4	ND
Boiled-semidried anchovy larvae (Shirasu-boshi)	ND	ND
Dried common filefish (*Stephanolepis cirrhifer*)	ND	112
Salted cod (*Gadus morrhua macrocephalus*)	ND	ND
Salted salmon (*Onchorhnchus keta*)	ND	ND
Salted saury (*Cololabis saira*)	ND	ND
Salted steenstrup squid (Surume, *Ommastrephes sloani pacificus*)	ND	650
Smoked fish and shellfish:		
Smoked cod	ND	3.0
Smoked salmon (*Onchorhynchus nerka*)	ND	1.7
Smoked octopus (*Octopus vulgare*)	ND	1.6
Smoked squid (*Ommastrephes sloani pacificus*)	ND	ND
Smoked scallop	ND	44.8
Canned fish		
Chubmackerel, boiled	ND	4.4
Chubmackerel, seasoned with "Miso"	ND	8.4
Sardine, seasoned with soysauce (Iwashi-no yamatoni)	ND	ND
Tuna (albacore), flakes seasoned with soysauce	ND	ND
Miscellaneous fish products		
Alaska pollack roe, fresh (Nama-tarako)	ND	72.5
Alaska pollack roe, salted (Tarako)	ND	ND, 1.6
Fish-ham	ND, 1.0	ND
Fish sausage	ND	ND
"Kamaboko" (fishjelly product)	ND	ND
Cured meat products:		
Bacon	ND, 0.5	9.8, ND
Boneless ham	ND, 0.5	ND, ND
Canned Frankfurt sausage	ND	ND
Canned Wiener sausage	ND	ND
Dry sausage	ND	4.6, ND
Japanese pressed ham	ND	ND
Pork ham	ND	ND, 9.7
Smoked-boiled loin	ND	ND
Soft salami	ND	ND, 4.7
Wiener sausage	ND	ND

[a] ND means "not detected", and the minimum detection limit of MG was 0.25 mg/kg, and that of AG, 0.5 mg/kg.

such products from the standpoint of public health. A survey has been conducted on the MG and AG contents of various processed food collected in the Tokyo areas, and the results are shown in Table III. As can be seen in the table, no appreciable amount or trace amounts of MG could be detected in these products, except for the cases of smoked-dried skipjack (*Katsuwonus pelamis*), "Katsuo-bushi" in Japanese, and the thin-sliced products prepared from smoked-dried mackerel (*Pneumatophorus japonicus japonicus*), sardines (*Sardinops melanosticta*), and horse mackerel (*Trachurus japonicus*), called "Kezuri-bushi". The MG levels for "Katsuo-bushi" ranged from 18.1 to 178 mg/kg, and for "Kezuri-bushi" from 37.5 to 46.5 mg/kg. These values were almost comparable to those reported by Fujinaka et al. (1976). Both "Katsuo-bushi" and "Kezuri-bushi" are traditional seasoning materials in Japan. Recently, however, the household consumption of these seasonings has markedly decreased, because such synthetic seasonings as sodium glutamate, sodium inocinate, etc. have become very popular. No valid data on the per capita consumption of these smoked-dried fish products is available at present; however, it is presumed that in ordinary home cooking the amount of these products consumed per day per person may be as low as 1 to 2 g, which corresponds to less than 0.2 mg of MG. In addition, the reaction rate of the vitro nitrosation of MG to give methylnitrosourea has been reported to be very slow (Mirvish, 1971). Therefore, it would seem unreasonable to claim that high incidence of gastric cancer in Japan is related to MG derived from these fish products.

As to the occurrence of AG in fresh foods, no appreciable amount or very low levels of AG, ranging from no detection to 18 mg/kg, could be detected in the fresh fish, shellfish, and meat samples tested, except for the cases of certain shellfish, i.e., abalone and top-shell (minimum detection

limit was 0.5 mg/kg). It was found that the AG level of abalone muscle was 40 mg/kg and its viscera, 69 mg/kg, and that of top-shell muscle was 200 mg/kg and its viscera, 224 mg/kg. In contrast, the AG levels of some processed foods were found to be fairly high, viz., salted-dried round herring contained 217 mg/kg, dried common filefish, 112 mg/kg, and salted steenstrap squid, as high as 650 mg/kg. The remaining processed foods contained almost no appreciable amount or very small amounts of AG.

AG has been isolated from a few invertebrate sources, notably from the sponge, *Geodia gigas*, and from several cephalopods (Baldwin, 1963). Present study revealed that relatively high concentrations of AG could be detected in fresh abalone and top-shell muscles ranging from 40 to 200 mg/kg. Both shellfish belong to gastropods which contain large amounts of arginine instead of creatine as phosphagen. The mode of AG formation from arginine in the shellfish remains unsolved; perhaps it might be formed by the action of an arginine decarboxylate in the muscle. It is shown that certain bacteria can decarboxylate arginine to form AG. Interestingly, the processed foods containing fairly high concentrations of AG did not give any sign of deterioration.

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Comparison of Chemical and Biological Methods for Determination of Thiamin in Foods

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The thiamin content of green beans was determined by the fluorometric thiochrome procedure and rat bioassay to determine the correlation between the chemical thiamin assay and the biologically available thiamin. Several methods of sample preparation for fluorometric analysis were examined to maximize the removal of interfering compounds. Preparative chromatography using Decalso ion-exchanger columns was found to provide lower apparent thiamin values than either direct analysis or preextraction with isobutanol of the sample extract of all rat bioassay diets analyzed. No significant difference was observed in the apparent thiamin content of green beans between the extract purification methods. These results indicate the necessity of preliminary testing of extract purification effects on each product assayed to ensure accuracy of routine chemical analysis. The rat bioassay was evaluated by growth, feed conversion efficiency, and urinary thiamin creatinine ratio. Estimates of available thiamin were equal by all three indicators. Comparison of the fluorometric and biological assay data indicated total bioavailability of thiamin in green beans.

Accurate chemical determination of the vitamin content of foods is of little value unless a correlation can be determined between the chemical assay value and the biologically available fraction of the vitamin in the food. Data on the bioavailability of vitamins is important for the evaluation of the adequacy of dietary intakes.

For the determination of total thiamin in foods, chemical procedures have been based on the oxidation of thiamin in a food extract to thiochrome, extraction of the thiochrome into isobutanol, and the subsequent fluorometric measurement of the thiochrome (Pyke, 1937).

Automated methods for thiamin analysis have been developed for foods (Khoury, 1966; Kirk, 1974; Pelletier and Madere, 1975, 1977) which improve the precision and shorten the analysis time. Chromatographic cleanup of the sample extract using the Decalso cation-exchange chromatographic procedure has been used for the removal of potentially interfering compounds from the sample extract (Freed, 1966; Strohecker and Henning, 1965; AOAC, 1975). Although previously reported to be a major source of imprecision in the thiamin assay (Jowett, 1940; Harris and Wang, 1941; Betchel and Hollenbeck, 1958), the Decalso purification procedure has been recently modified to greatly reduce this variability (Pippen and

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