STUDIES ON THE MECHANISM OF GLUTATHIONE PREVENTION OF CARBON TETRACHLORIDE-INDUCED LIVER INJURY

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Summary.—The prior administration of reduced glutathione (GSH) partially prevents carbon tetrachloride (CCl_4)-induced liver necrosis observed at 24 h after administration of the hepatotoxin. No prevention occurs when observations are made at 72 h. GSH pretreatment does not significantly modify the intensity of the covalent binding of CCl_4 reactive metabolites to microsomal lipids or the intensity of the CCl_4-induced lipid peroxidation process at either 1, 3 or 6 h after poisoning. GSH administration does not significantly prevent CCl_4-induced cytochrome P-450 destruction or glucose 6 phosphatase activity depression. Pretreatment with GSH does not significantly modify the levels of CCl_4 or i.p. administered CCl_4 reaching the liver at 1, 3 or 6 h after intoxication. Pretreatment with GSH significantly prevents CCl_4-induced decreases in body temperature. Results are interpreted as suggesting that GSH prevents CCl_4-induced liver necrosis by changing the liver cell's response to injury rather than by modification of early events of the process such as lipid peroxidation or covalent binding of reactive metabolites.

REDUCED GLUTATHIONE (GSH) is able to ameliorate several manisfestations of acute CCl_4 poisoning which include fatty infiltration (Dianzani and Ugazio, 1973; Torrielli, Gabriel and Dianzani, 1978; Ciccoli *et al.*, 1981), protein synthesis inhibition (Gravela, Gabriel and Ugazio, 1971; Dianzani *et al.*, 1976); polysome breakdown (Gravela and Dianzani, 1970; Dianzani and Ugazio, 1973) and survival (Di Luzio, 1973).

Pretreatment with GSH is also effective in preventing fatty infiltration of the liver of ethanol or phosphorus treated animals (Torrielli *et al.*, 1978; Pani *et al.*, 1972).

In this work, we attempt to elucidate the mechanism of GSH prevention of CCl_4-induced liver injury by testing the effects of GSH on several parameters known to modulate the intensity of CCl_4 effects on the liver.

MATERIALS AND METHODS

Compounds.—$^{14}CCl_4$ (3·4 mCi/mmol) was purchased from New England Nuclear (U.S.A.). All other chemicals used were of analytical grade.

Animals and animal treatment.—Sprague–Dawley male rats (240–280 g) were used. Food was withdrawn 12–14 h before CCl_4 administration, but water was provided *ad libitum*. Reduced glutathione (GSH) at a dose of 2 g/kg was given p.o. in distilled water (400 mg/ml solution) either 30 min before or 6 or 10 h after CCl_4. GSH was solubilized by addition of conc. HCl (pH of the solution $\simeq$ 1). Control animals received the equivalent amount of acidified distilled water. CCl_4 was given i.p. as a 20% (v/v) solution in olive oil at a dose of 5 ml solution/kg (1 ml of pure CCl_4/kg). $^{14}CCl_4$ (3·4 mCi/mmol) was added to this solution to give 16 × 10^6 dpm/ml and injected i.p. at a dose of 5 ml of solution/kg. Control animals received only olive oil. The animals were killed at different times after CCl_4 or $^{14}CCl_4$ by decapitation and bled. Livers were rapidly removed and processed. Whenever blood samples were taken, animals were kept under light ether anaesthesia and

blood was collected from the inferior vena cava into a syringe containing heparin. The rectal temperature of the rats was recorded with a small animal thermistor probe (Telethermometer, Nihon–Köhden).

Enzymatic and chemical determinations.—The isolation of the hepatic microsomal fraction and the procedure for determining binding of ^{14}C from [14]CCl4 to microsomal lipids have been previously described by Castro et al. (1972). The extent of the irreversible binding of ^{14}C from [14]CCl4 to microsomal proteins was measured according to the method of Rao and Recknagel (1969). The quantification of the lipid peroxidation *in vivo* was determined by conjugated diene ultraviolet absorption of lipid extracts from microsomal fractions as described by Klaassen and Plaa (1969). The results are expressed as the change in absorbance at 243 nm × 1000 for a solution having 1 mg of microsomal lipid/ml. Glucose 6-phosphatase (G6P-ase) activity in microsomal suspensions was measured at 37° as described by Harper (1963). The liver microsomal cytochrome P-450 (P-450) content was determined as described by Schenkman, Remmer and Estabrook (1967). Procedures for microsome isolation, lipid extraction, separation of lipids into classes by thin-layer chromatography (TLC), as well as obtention and separation of fatty acid methyl esters by gas liquid chromatography (GLC) were as described by Villarruel, Díaz Gómez and Castro (1975). The procedure followed to determine CCl4 content in liver was that of Recknagel and Litteria (1960). The determination of glutathione in liver was performed as described by Ellman (1959). Isocitric acid dehydrogenase activity (ICD) in plasma was measured according to the method of Sterkel et al. (1958). Protein concentrations were estimated by the procedure described by Lowry et al. (1951).

Histological techniques.—Small portions from the left and the central hepatic lobes were fixed in Bouin's solution, embedded in paraffin and stained with haematoxylin and eosin. The specimens were coded to avoid bias and evaluated by two independent observers. To quantify the morphological changes, liver sections were graded for necrosis using an arbitrary scale: + =light (about 20–30% of necrotic cells); + + =moderate (about 50% of necrotic cells); + + + =marked (about 75% of necrotic cells); + + + + =very intense (about 90–100% of necrotic cells). Results are reported as the mean of all the observations made by all observers for a given experimental condition.

Statistics.—The significance of the difference between 2 mean values was assessed by Student's *t* test (Bancroft, 1960). Significance of treatment involving the comparison among 4 mean values was assessed by two-way analysis of variance according to the method of Brownlee (1960). Differences were considered significant at *P* values of 0·05 or less.

RESULTS

Effects of GSH pretreatment on CCl4-induced liver injury assessed at 24 h

Our studies of ICD levels in plasma and of the histologically evidenced degree of necrosis show that the administration of GSH prior to CCl4 or treatment with GSH sometime after it (6 or 10 h) partially prevents CCl4 liver necrosis to a similar extent (Table I). Prevention is no longer histologically observable at 72 h after CCl4 administration.

CCl4 concentrations in liver at different times after its administration to rats pretreated with GSH

Prior oral administration of GSH does not significantly modify the levels of CCl4 reaching the liver at 1, 3 or 6 h after its i.p. administration (Table II).

Effect of GSH pretreatment on CCl4-induced lipid peroxidation

Pretreatment of rats with GSH does not significantly modify the intensity of CCl4-induced lipid peroxidation as measured by the UV hyperconjugation technique, at either 1, 3 or 6 h after administration of the hepatotoxin (Table III).

Effect of GSH pretreatment on the CCl4-induced changes in fatty acid composition of liver microsomal phospholipids 24 h after intoxication

CCl4 administration to rats decreases the arachidonic acid content in liver microsomal phopholipids at 24 h after intoxication (Table IV), while pretreatment with GSH alone significantly increases this parameter (Table IV). The overall effect of GSH administration on CCl4-induced lipid peroxidation is a mild but significant prevention of the process (Table IV). However, arachidonic acid values in microsomal phospholipids from CCl4-treated animals are not significantly

TABLE I.—*Effect of GSH pretreatment on CCl₄-induced liver injury assessed at 24 h*

Treatment*	ICD (units ± s.d.)†	Degree of histologically observable necrosis‡
30 min before CCl₄		
Control	120 ± 40	– – – –
CCl₄	119·000 ± 35·880	+ + + +
GSH	140 ± 70	– – – –
GSH + CCl₄	39·750 ± 30·245§, ‖	+ +
6 h after CCl₄		
Control	80 ± 60	– – – –
CCl₄	108·950 ± 29·400	+ + + +
GSH	110 ± 30	– – – –
GSH + CCl₄	18·200 ± 13·300§, ‖	+ +
10 h after CCl₄		
Control	115 ± 45	– – – –
CCl₄	112·700 ± 21·400	+ + + +
GSH	130 ± 50	– – – –
GSH + CCl₄	25·800 ± 12·800§, ‖	+ +

* Sprague–Dawley male rats (240–280 g) were fasted 12–14 h before CCl₄ administration, which was given i.p. as a 20% (v/v) solution in olive oil at a dose of 5 ml of solution/kg. GSH was given p.o. in distilled water at a dose of 2 g/kg 30 min before or 6 or 10 h after CCl₄, it was solubilized by addition of conc HCl (pH of the solution ≃ 1). Controls received the equivalent amount of olive oil or acidified distilled water. Seven rats per group were used.

† One unit of enzyme is that required to form 1 nmol NADPH/ml plasma/h at 25°.

‡ + = light (20–30% necrosis), + + = moderate (50% necrosis), + + + = marked (75% necrosis), + + + + = very intense (90–100% necrosis).

§ The P value for the overall effect of GSH on the CCl₄-induced increase in ICD obtained by two-way analysis of variance was $P < 0·001$.

‖ $P < 0·001$ for GSH + CCl₄ vs CCl₄.

TABLE II.—*CCl₄ concentrations in liver at different times after its administration to rats pretreated with GSH*

Treatment*	CCl₄ concentration in liver (μg/g of liver ± s.d.)		
	1 h	3 h	6 h
CCl₄	720 ± 105	580 ± 90	72 ± 66
GSH + CCl₄	690 ± 195†	680 ± 150†	115 ± 80†

* CCl₄ and GSH were administered as indicated in Table 1. The animals were killed 1, 3 or 6 h after the hepatotoxin. Seven rats per group were used.

† $P > 0·1$ for GSH + CCl₄ vs CCl₄.

different from those of the GSH + CCl₄ group ($P > 0·05$).

Effect of GSH pretreatment on the covalent binding of ¹⁴C from ¹⁴CCl₄ to liver microsomal lipids or proteins at 1, 3 or 6 h after CCl₄ administration

Pretreatment with GSH does not significantly modify the covalent binding of ¹⁴C from ¹⁴CCl₄ to liver microsomal lipids or proteins at 1, 3 or 6 h after CCl₄ administration (Table IV).

TABLE III.—*Effect of GSH pretreatment on CCl₄-induced lipid peroxidation at 1, 3 or 6 h after administration*

Treatment*	In vivo lipid peroxidation ± s.d.†,‡		
	1 h	3 h	6 h
Control	152 ± 40	214 ± 41	123 ± 32
CCl₄	290 ± 52§	270 ± 80‖	196 ± 20§
GSH	175 ± 33	213 ± 23	170 ± 27‖
GSH + CCl₄	280 ± 56	301 ± 28	231 ± 37‖

* CCl₄ and GSH 30 min before the hepatotoxin, were administered as indicated in Table I. Five rats per group were used.

† Lipid peroxidation is expressed as absorbance at 243 nm × 1000 of a solution containing 1 mg microsomal lipid/ml.

‡ The P value for the overall effect of GSH on CCl₄-induced lipid peroxidation obtained by two-way analysis of variance was $P > 0·1$ at all the times tested.

§ $P < 0·01$ for CCl₄ vs control at 1 and 6 h.

‖ $P < 0·05$ for CCl₄ vs control at 3 h and for GSH vs control and GSH + CCl₄ vs GSH at 6 h.

TABLE IV.—*Effect of GSH pretreatment on the CCl4-induced changes in fatty acid composition of liver microsomal phospoholipids 24 h after intoxication*

Treatment*	Percent composition of fatty acids†				
	$16:0$	$18:0$	$18:1^{\Delta 9}$	$18:2^{\Delta 9,12}$	$20:4^{\Delta 5,8,11,14}$‡
Control	$42 \cdot 7 \pm 3 \cdot 0$	$40 \cdot 3 \pm 2 \cdot 6$	$6 \cdot 8 \pm 0 \cdot 7$	$7 \cdot 2 \pm 0 \cdot 6$	$3 \cdot 0 \pm 0 \cdot 5$
CCl4	$49 \cdot 8 \pm 3 \cdot 4$¶	$32 \cdot 7 \pm 1 \cdot 5$§	$9 \cdot 4 \pm 0 \cdot 7$‖	$6 \cdot 4 \pm 2 \cdot 3$	$1 \cdot 5 \pm 0 \cdot 9$¶
GSH	$43 \cdot 8 \pm 3 \cdot 7$	$39 \cdot 5 \pm 1 \cdot 5$	$5 \cdot 7 \pm 0 \cdot 2$¶	$7 \cdot 2 \pm 0 \cdot 9$	$5 \cdot 3 \pm 0 \cdot 8$‖
GSH + CCl4	$48 \cdot 8 \pm 1 \cdot 0$‖	$31 \cdot 0 \pm 0 \cdot 7$§	$9 \cdot 0 \pm 0 \cdot 7$‖	$8 \cdot 8 \pm 1 \cdot 3$	$2 \cdot 3 \pm 0 \cdot 6$¶

* CCl4 and GSH 30 min before the hepatotoxin, were administered as indicated in Table I. Five rats per group were used.

† Animals were killed 24 h after CCl4 administration. Liver microsomal lipids were extracted and separated in classes by TLC. Fatty acid methyl esters from the phospholid fraction were prepared by transesterification and analysed by GLC.

‡ The P value for the overall effect of GSH on CCl4-induced lipid peroxidation obtained by two-way analysis of variance was $P < 0 \cdot 025$.

§ $P < 0 \cdot 001$ for CCl4 vs control and GSH + CCl4 vs control for $18:0$.

‖ $P < 0 \cdot 01$ for GSH + CCl4 vs control for $16:0$, CCl4 vs control and GSH + CCl4 vs control for $18:1$ and GSH vs control for $20:4$.

¶ $P < 0 \cdot 05$ for CCl4 vs control for $16:0$, GSH vs control for $18:1$ and CCl4 vs control for $20:4$ for GSH + CCl4 vs GSH. There is a 50% decrease in $20:4$ in the CCl4 group vs control and a 56·7% in the GSH + CCl4 group vs GSH. The value for $20:4$ in CCl4 group is not significantly different from that in GSH + CCl4 group $P > 0 \cdot 05$.

TABLE V.—*Effect of GSH pretreatment on the covalent binding of ^{14}C from $^{14}CCl_4$ to liver microsomal lipids or proteins at 1, 3 or 6 h after $^{14}CCl_4$ administration*

Treatment*	Irreversible binding of ^{14}C from $^{14}CCl_4$ (dpm/mg lipid ± s.d.)			Irreversible binding of ^{14}C from $^{14}CCl_4$ (dpm/mg protein ± s.d.)		
	1 h	3 h	6 h	1 h	3 h	6 h
$^{14}CCl_4$	44 ± 6	34 ± 3	76 ± 14	12 ± 3	17 ± 2	78 ± 31
GSH + $^{14}CCl_4$	58 ± 13†	29 ± 4†	80 ± 7†	13 ± 2†	15 ± 2†	79 ± 37†

* Male rats fasted for 12–14 h were injected i.p. with $^{14}CCl_4$ (16×10^6 dpm/ml) in 20% CCl4 olive oil solution at a dose of 1 ml CCl4/kg. GSH was administered as indicated in Table I, 30 min before the hepatotoxin. Five animals per group were used.

† $P > 0 \cdot 1$ for GSH + $^{14}CCl_4$ vs $^{14}CCl_4$ for all the times tested.

TABLE VI.—*Effect of GSH pretreatment on the destruction of P-450 and G6P-ase activity depression caused by CCl4 1, 3 or 6 h after its administration*

Treatment*	P-450 content† (nmol/mg protein ± s.d.)			G6P-ase† (μg Pi/15 min/mg protein ± s.d.)		
	1 h	3 h	6 h	1 h	3 h	6 h
Control	$0 \cdot 43 \pm 0 \cdot 05$	$0 \cdot 43 \pm 0 \cdot 07$	$0 \cdot 40 \pm 0 \cdot 13$	73 ± 7	59 ± 6	60 ± 2
CCl4	$0 \cdot 29 \pm 0 \cdot 05$§	$0 \cdot 23 \pm 0 \cdot 03$‡	$0 \cdot 18 \pm 0 \cdot 02$§	52 ± 5§	41 ± 7§	36 ± 6‡
GSH	$0 \cdot 40 \pm 0 \cdot 06$	$0 \cdot 40 \pm 0 \cdot 03$	$0 \cdot 30 \pm 0 \cdot 10$	67 ± 6	60 ± 7	50 ± 7
GSH + CCl4	$0 \cdot 29 \pm 0 \cdot 05$	$0 \cdot 23 \pm 0 \cdot 03$	$0 \cdot 17 \pm 0 \cdot 07$	42 ± 6	39 ± 3	34 ± 4

* CCl4 and GSH 30 min before the hepatotoxin, were administered as indicated in Table I. Five rats per group were used in these experiments.

† The P value for the overall effect of the GSH pretreatment on the CCl4-induced loss of P-450 and G6P-ase activity by two-way analysis of variance was $P > 0 \cdot 1$ at all the times tested.

‡ $P <$ for CCl4 vs control for P-450 at 3 h and for G6P-ase at 6 h.

§ $P <$ for CCl4 vs control for P-450 at 1 and 6 h and for G6P-ase at 1 and 3 h.

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TABLE VII.—*Effect of GSH pretreatment on the CCl₄-induced decrease of body temperature at different times after administration*

Treatment*	Body temperature (°$\pm$s.d.)†				
	1 h	3 h	6 h	10 h	24 h
Control	$36 \cdot 1 \pm 1 \cdot 1$	$35 \cdot 5 \pm 0 \cdot 1$	$35 \cdot 4 \pm 0 \cdot 5$	$35 \cdot 6 \pm 0 \cdot 4$	$34 \cdot 7 \pm 0 \cdot 3$
CCl₄	$33 \cdot 3 \pm 0 \cdot 5\ddagger$	$33 \cdot 8 \pm 0 \cdot 5\ddagger$	$35 \cdot 2 \pm 0 \cdot 6$	$35 \cdot 9 \pm 0 \cdot 6$	$33 \cdot 0 \pm 0 \cdot 8\S$
GSH	$35 \cdot 2 \pm 0 \cdot 5$	$34 \cdot 9 \pm 0 \cdot 8$	$36 \cdot 0 \pm 0 \cdot 6$	$35 \cdot 1 \pm 0 \cdot 6$	$34 \cdot 2 \pm 0 \cdot 6$
GSH+CCl₄	$33 \cdot 5 \pm 0 \cdot 9$	$34 \cdot 1 \pm 0 \cdot 5$	$35 \cdot 0 \pm 0 \cdot 4$	$35 \cdot 6 \pm 0 \cdot 8$	$33 \cdot 8 \pm 0 \cdot 3$

* CCl₄ and GSH were administered as indicated in Table I. Five rats per group were used in these experiments.

† The P value for the overall effect of GSH on the CCl₄-induced decrease in body temperature obtained by two-way analysis of variance was $P < 0 \cdot 05$ at 3, 6 and 24 h.

‡ $P < 0 \cdot 001$ for CCl₄ vs control at 1 and 3 h.

§ $P < 0 \cdot 01$ for CCl₄ vs control at 24 h.

Effect of GSH pretreatment on the destruction of P-450 and G6P-ase activity depression caused by CCl₄ 1, 3 or 6 h after administration

Pretreatment with GSH does not significantly prevent the CCl₄-induced destruction of P-450 content or the depression of G6P-ase activity caused by the hepatotoxin at 1, 3 or 6 h after administration (Table VI).

Effect of GSH pretreatment on the CCl₄-induced decrease of body temperature at different times after administration

CCl₄ administration decreases body temperature of rats during the whole period of intoxication (Table VII). The pretreatment of the animals with GSH significantly prevented the CCl₄-induced decreases in body temperature at 3, 6 or 24 h after CCl₄ administration (Table VII).

GSH levels in liver at 1 or 3 h after its administration to rats

One hour after oral administration, GSH levels are significantly higher than in control rats (controls $9 \cdot 3 \pm 0 \cdot 9$ μg/g; treated $12 \cdot 1 \pm 1 \cdot 3$ μg/g, $P < 0 \cdot 01$, 134% of control). At 3 h after administration of GSH, the liver content was not significantly different in control and treated animals (control: $9 \cdot 3 \pm 1 \cdot 0$ μg/g; treated: $10 \cdot 8 \pm 1 \cdot 5$ μg/g, $P > 0 \cdot 05$).

DISCUSSION

This study shows that in addition to preventing several manifestations of CCl₄-induced liver injury such as fatty unfiltration, protein synthesis inhibition, polysome breakdown and survival (Gravela and Dianzani, 1970; Gravela et al., 1971; Dianzani and Ugazio, 1973; Dianzani et al., 1976; Di Luzio, 1973), GSH also partially prevents CCl₄-induced liver necrosis when given 30 min before CCl₄. This preventive effect is not due to interference with the process of CCl₄ activation to reactive metabolites or the covalent binding of them to cellular constituents such as proteins or lipids. This is important because several workers consider that the covalent binding of CCl₄ to reactive metabolites is related to CCl₄-induced liver injury (McLean, 1967; Castro et al., 1973; Díaz Gómez et al., 1975; Bernacchi et al., 1980; Gillette et al., 1974). Further, GSH preventive action cannot be attributed to inhibition of lipid peroxidation, as GSH does not prevent the appearance of the characteristic UV changes due to lipid peroxidation, revealed by the diene hyperconjugation technique we employed to measure the intensity of this process at 1, 3 or 6 h after CCl₄ administration. These observations are in agreement with the previous limited observations quoted by Dianzani (1970). This is particularly important, because GSH is known to powerfully inhibit lipid peroxidation in vitro (McCay et al., 1976; Burk, 1982), apparently by prevention of the initiation of lipid peroxidation rather than by the reduction of lipid peroxides to lipid

alcohols, as previously thought (McCay et al., 1981). The mechanism of antioxidant effect implies that GSH also prevents arachidonic acid decreases occurring during lipid peroxidation in vitro (McCay et al., 1981). In the experiments on CCl4 poisoned animals, this was shown not to be the case, since CCl4 administration decreased by 50% the arachidonic acid content of liver microsomal phospholipids from control rats and by 56·7% that from GSH pre-treated animals. These results on arachidonic acid content of microsomal lipids are further evidence that GSH preventive effects cannot be attributed to inhibition of lipid peroxidation. This is relevant because several authors have considered CCl4-induced peroxidation a key factor for CCl4-induced liver necrosis (Recknagel, 1967; Recknagel and Glende, 1973; Slater, 1966).

Furthermore, protection cannot be attributed to lower levels of CCl4 reaching the liver in GSH-treated animals as was the case with other chemicals (Marchand et al., 1970, 1971). Neither can protection by GSH be explained in terms of lowering of body temperature, as postulated for the preventive effects of other chemicals in rats (Marzi et al., 1980) or experimental conditions (Larson and Plaa, 1965), because neither GSH alone decreases body temperature in rats nor is this parameter in GSH + CCl4-treated animals lower than that of CCl4 poisoned rats. Moreover the GSH + CCl4 group of rats has a body temperature not significantly different from that of control animals. To exclude a temperature-mediated effect is very helpful in understanding the mechanism of the preventive effects of GSH because, as it also occurred in our experiments, temperature only delays the occurrence of CCl4-induced necrosis at 24 h, while at 72 h damage is fully observable (Larson and Plaa, 1965; Marzi et al., 1980). In the case of GSH, the observed delay should be attributed to some other effect on the cell of a reversible nature, which gradually diminishes after 24 h.

This effect, as we have already discussed, is not related to interference with events occurring at early stages of the intoxication process (e.g. lipid peroxidation or covalent binding) since GSH still retains all its preventive potential when given as late as 6 or 10 h after CCl4 administration. At this time of the intoxication process, most of the lipid peroxidation or covalent binding has already occurred and some cells are either dying or irreversibly damaged (Recknagel, 1967; Smuckler, 1968; Smuckler and Arcasoy, 1969). Consequently, GSH protective effects are apparently due to a modification of the cell's response to injury giving it an increased chance of survival. How GSH achieves this task is not easy to understand. The first problem is that according to current views on GSH metabolism and function, GSH is transported out of the liver to plasma and that no extracellular GSH is absorbed by the epithelial liver cell (Griffith and Meister, 1979; Meister, 1981). In our studies, however, we observed a transient increase in GSH content at 1 h which rapidly decreased to control levels at 3 h. One way of explaining this apparently controversial observation in terms of current views in the field is to envisage that when the large GSH dose we administered reaches circulation, it slows down transport out of the liver and this provokes a temporary increase in the GSH intracellular levels until cellular regulatory systems make the proper adjustments.

Another problem, the major one, is to understand what GSH does to the cell to improve chances of survival. This might be the major problem to face in future studies, particularly since GSH has many known different roles in cell physiology and biochemistry and one envisages that many of them remain to be discovered (Meister, 1981; Reed and Beatty, 1980; Kosower and Kosower, 1978).

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