maximum number of nucleoli per nucleus indicates the number of nucleolar organizing loci, and that an increase in ploidy should therefore result in a doubling of this number.

We conclude that myocardial hypertrophy may be associated with an increased DNA content of myocardial nuclei, but complete gene duplication does not occur; hence, the number of nucleolar organizers does not increase. Although the number of nucleolar organizers appears to be constant, there is increased expression of their function so that the number of nuclei with more than one nucleolus (but less than six) increases.

Summary. The number of nucleoli in myocardial nuclei was determined for human hearts in the weight range of 300–640 g. The variation of nucleolar abundance was significant, but slight, with larger hearts tending to show more nucleoli per nucleus. The maximum number of nucleoli per nucleus was six, regardless of the heart weight. It is concluded that even if the amount of DNA per nucleus increases during the development of myocardial hypertrophy, as suggested by microspectrophotometric studies, the number of potential nucleolar organizers remains fixed at six per nucleus.

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Protection of Brain Metabolism with Glutathione, Glutamate, γ-Aminobutyrate and Succinate* (33713)

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Studies on protective agents in oxygen toxicity experiments led us to believe that a glutathione (GSH)–glutamate–γ-aminobutyrate (GABA)–succinate pathway may serve as a secondary support system in the maintenance of brain energy levels (adenosine triphosphate [ATP] concentration). This "shunt" is shown in Fig. 1. The glutamate–GABA–succinicsemialdehyde–succinate shunt is a well established pathway (1-8) to which no major physiological significance has been attached. The GABA–succinate shunt has been suggested as a means of metabolizing GABA (9, 10). It has also been reported to function as a means of bypassing inhibition of the alpha-ketoglutarate dehydrogenase system of the citric acid cycle by withdrawal of alpha-ketoglutarate from the cycle by transamination with GABA to yield glutamate and reentry of the carbon chain of GABA into the cycle at the succinate level (9, 10). The possible physiological importance of the shunt is seen if one recognizes that succinate markedly stimulates respiration and oxidative phosphorylation.

Krebs *et al.* (11) reported that succinate oxidation can monopolize the respiratory-electron transport chain which is the major source of ATP production. Sanders *et al.* (12, 13) observed significantly higher respiration

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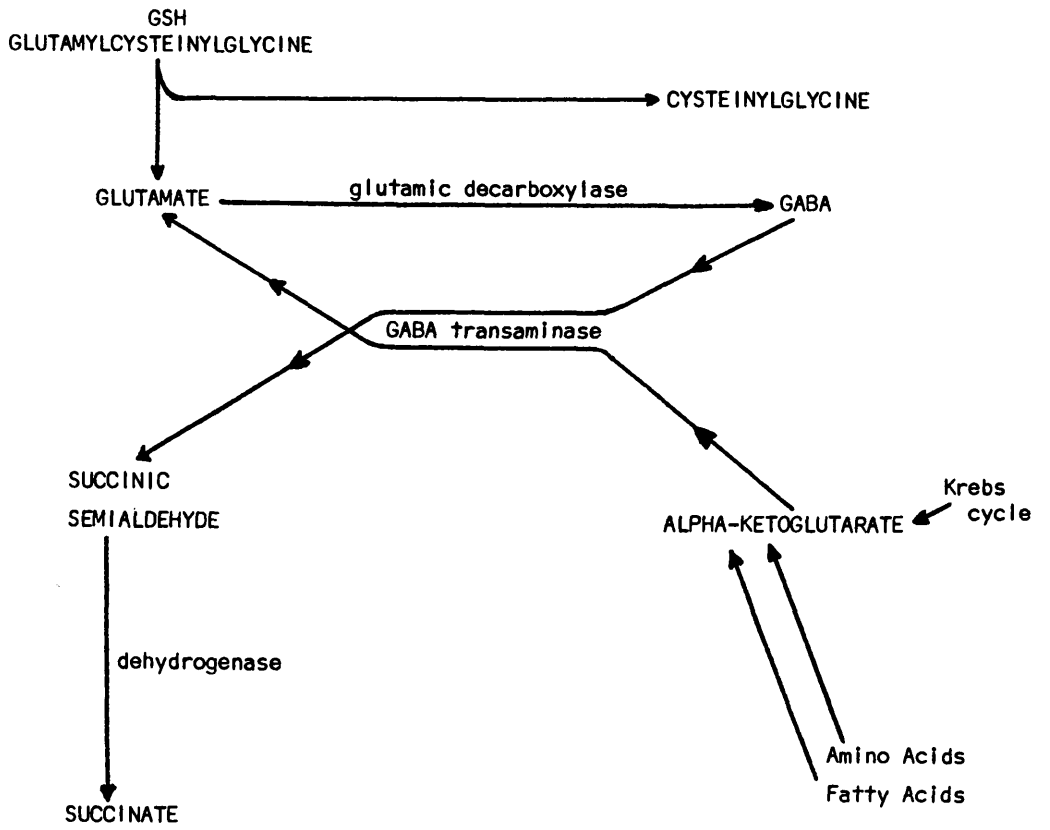

FIG. 1. Proposed glutathione-glutamate-GABA-succinate shunt.

rates with succinate in brain, liver, and kidney of rats when compared with alpha-ketoglutarate and glutamate. Data shown later indicate that succinate stimulates brain respiration and oxidative phosphorylation in the mouse, rat, guinea pig, rabbit, cat and dog.

The factor limiting the amount of succinate available for metabolism has generally been considered to be the rate at which alpha-ketoglutarate is converted to succinate by enzymes of the Krebs cycle. Roberts (8) showed that conversion of GABA to succinate by GABA transaminase is rapid, and that both glutamic acid decarboxylase and GABA transaminase have pH optima (6.5 and 8.2, respectively) such that small changes in intracellular pH—within the physiological range—could result in large changes in these enzyme activities *in situ* (8, 14, 15). Thus, GABA could serve as a rapidly available source of succinate under physiological stress conditions. The conversion of glutamate to GABA is an established pathway (1–8) and could serve as a source of GABA when required. The conversion of GSH to glutamate (plus cysteinylglycine) has been reported (16). Thus, a possible equilibrium exists between GSH, glutamate, and GABA which could be severely altered if stress phenomena resulted in a decrease in GABA. Many observations reported in the literature support the concept of a kinetic relationship between GSH, glutamate, and GABA during stressful situations. Some of the observations relating changes in GSH or glutamate or GABA with different stress conditions are shown in Table I. The data readily show rapid change of these compounds in tissues subjected to stress.

Sanders *et al.* (17–19) used succinate to protect against high pressure oxygen (HPO) toxicity and showed that normal levels of ATP were maintained with succinate as com-

TABLE I. Variations in GSH, Glutamate, and GABA Levels in Tissues from Animals Subjected to Different Stress Agents.

Stress condition	GSH (%)	Glutamate (%)	GABA (%)	Refs.
Methoxypyridoxine seizures (cat brain)	↓15–41	↓4–60	↓29–82	22
Thiosemicarbazide seizures (rat brain)		↑5	↓30–50	23,24
Epileptogenic foci, spiking lesions (cat brain)	↓53	↓46	↓5	25
Non-spiking lesions (cat brain)	↓25	↓19	↓3	25
HPO (75 psig[a] 100% O_2) (mouse, rat, rabbit, hamster, or guinea pig brain)			↓6–38	10,26

[a] Pounds per square inch gauge.

pared with a decrease of 43% in ATP in controls undergoing identical HPO exposures. GABA was used by Wood and Watson (10) as a protective agent against HPO toxicity, and by Wood *et al.* (20) to protect against thiosemicarbazide induced convulsions. If the proposed GSH–glutamate–GABA–succinate pathway functions to provide a secondary supply of ATP to the brain, then a comparison of efficacy of these compounds, cysteine, and mixtures of cysteine plus succinate or GABA, as protective agents against HPO toxicity, would give circumstantial evidence to support the validity of the hypothesis.

Methods. Oxygen electrodes were used to measure respiration (12, 13) of brain homogenates and tissue slices from six different species. Succinate respiration was compared with respiration of alpha-ketoglutarate, malate, and isocitrate. These data are shown in Table II.

Male Sprague-Dawley rats (150–200 g), fasted (16–18 hr), were given i.p. injections of either cysteine, glutamate, GABA, succinate, or GSH; or 4 mmoles/kg of cysteine plus 10 mmoles/kg of the preceding compounds, 50 min prior to exposures to 100% oxygen at 5, 7, 9, and 11 atmospheres absolute (ATA). Time to appearance of convulsions was recorded for each animal. These data are shown in Tables III and IV.

In radioactive label experiments, three groups of male Sprague-Dawley rats (150–200 g), fasted (16–18 hr), were given 10 mmoles/kg i.p. injections of uniformly labeled GABA-^{14}C (0.4 *M*). Exposure conditions were: Group 1 (controls), 75 min in air; Group 2, 50 min in air, 25 min in 7 ATA 100% oxygen; Group 3, 50 min in air, 25 min in 4% oxygen plus 96% nitrogen. At the end of the exposure periods, each animal was anesthetized with ether, and the brain was perfused with isotonic saline. The perfusion procedure effectively removed all blood from the brain as indicated by inability to find erythrocytes in the homogenates of the

TABLE II. Comparison of Respiration Rates and ATP Production Rates of Brain Homogenates (Succinate vs Alpha-Ketoglutarate).

Species	Respiration rate ratio (succinate qO_2/alpha-ketoglutarate qO_2) (range)	ATP production rate ratio[a] (succinate/alpha-ketoglutarate) (range)	Increase in ATP production with succinate (%; range)
Mouse	2.19–2.52	1.46–1.68	46–68
Rat	1.82–2.14	1.21–1.43	21–43
Guinea pig	2.14–2.44	1.43–1.63	43–63
Rabbit	2.44–3.69	1.63–2.46	63–146
Cat	3.31–5.00	2.21–3.33	121–233
Dog	6.28–7.17	4.19–4.78	319–378

[a] This takes into account differences in P/O ratios for succinate and alpha-ketoglutarate, and is obtained by multiplying column one by ⅔.

TABLE III. Comparison of Protection against Oxygen Toxicity of Different Substrates at 5 ATA 100% O_2.*

Dosage (mmoles/kg)	Time to convulsions (min; mean ± SD)				
	Cysteine	GABA	Glutamate	Succinate	GSH
4	106.9 ± 31.4 (23)	98.7 ± 27.8 (12)	81.5 ± 52.8 (12)	76.7 ± 38.6 (12)	198.7 ± 45.6 (19)
10	Lethal	124.4 ± 36.5 (12)	130.0 ± 37 (15)	138.6 ± 36.3 (14)	222.5 ± 39.4 (23)
12	Lethal			208.0 ± 43.0 (8)	289.6 ± 27.6 (8)
	Mixture		Mixture		Mixture
4 and 10	Cysteine and GABA	167.3 ± 32.9 (8)	Cysteine and glutamate	171 ± 45 (13)	Cysteine and succinate 213 ± 44 (9)
4 and 12					Cysteine and succinate 271 ± 51 (15)

* Controls (isotonic saline) = 62 ± 31.6 min.

brain. Following perfusion, the brain was removed and a (5:1) homogenate (v/w) prepared with ice-cold water. After the addition of perchloric acid to a final concentration of 10% v/v, the homogenate was spun at 20,000*g* for 20 min and the supernatant was decanted. This supernatant was neutralized with K_2CO_3 and the supernate was decanted after centrifugation at 5000*g* for 10 min. The neutralized, acid-soluble fraction from the homogenate was frozen and dried, then resuspended in 0.4 ml of water.

Thin-layer chromatography was performed on aliquots of the acid-soluble cytoplasmic sap fractions with Mallinckrodt silica gel-7G Chromaplates and an ether:formic acid:water (7:2:1) solvent. After drying, the chromaplates were developed with ninhydrin and bromphenol blue indicators. The ^{14}C content of succinate, fumarate, and malate, and the total aliquot were determined by liquid scintillation counting techniques (Table V).

Results and Discussion. The data comparing succinate vs alpha-ketoglutarate respiration and ATP production rates in brain homogenates are shown in Table II. Similar results were obtained with brain slices. The ratios of succinate respiration: malate respiration, or succinate respiration:isocitrate respiration, or succinate respiration:(malate + isocitrate + alpha-ketoglutarate) respiration, and subsequent ATP production ratios, are even higher than the succinate:alpha-ketoglutarate ratios. Thus, if a source of succinate were readily available to the brain, there would be a marked increase in respiration and ATP production in the six species studies.

At 4 mmoles/kg, GSH is a much better "protectant" against convulsions than the

TABLE IV. Convulsion Time for Different Substrates at 5, 7, 9, and 11 ATA 100% O_2.

Substrate and dosage (mmoles/kg)	Oxygen pressure (ATA): (min; mean ± SD) 5	7	9	11
Controls (isotonic saline)	62 ± 31.6 (80)	16.9 ± 4.0 (10)	7.4 ± 3.0 (17)	4.4 ± 0.9 (9)
Cysteine, 4	106.9 ± 31.4 (23)	23.6 ± 6.8 (8)	10.6 ± 1.8 (8)	6.1 ± 1.3 (8)
GABA, 10	124 ± 36.5 (12)	18.8 ± 6.2 (6)	8.4 ± 3.2 (8)	5.8 ± 2.7 (6)
Succinate, 10	138.6 ± 36.3 (14)	53.2 ± 20.4 (9)	18.7 ± 5.3 (15)	12.6 ± 3.8 (8)
GSH, 4	198.7 ± 46 (19)	22.3 ± 6.8 (10)	10.5 ± 3.8 (9)	5.7 ± 2.3 (10)
Cysteine, 4 and succinate, 10	213 ± 44 (9)	80.2 ± 20 (6)	37.4 ± 7.9 (7)	20.6 ± 4.4 (8)

TABLE V. ^{14}C Content in (Succinate + Fumarate + Malate) a % of Total ^{14}C in Brain Cytoplasmic Sap.

	% of total ^{14}C[a]	
Controls (air exposure) (75 min)	20.1 ± 5.9 (6)	
Air (50 min) + 7 ATA 100% O_2 (25 min)	36.8 ± 5.8 (6)	$p < 0.01$
Air (50 min) + 1 ATA 4% O_2–96% N_2 (25 min)	37.8 ±10.2 (6)	$p < 0.01$

[a] Mean ± SD.

other compounds (Table III). This, however, may not be attributed only to sulfhydryl group protection (i.e., SH groups are readily oxidizable, and comparable levels of sulfhydryl protection could be obtained with 4 mmoles/kg of cysteine). At this dosage, GSH protection is approximately twice that of cysteine. GABA, glutamate, and succinate are not significantly different from controls at the 4 mmoles/kg dosage. The ability of GSH to protect at 4 mmoles/kg is interpreted to be due to sulfhydryl group protection from the cysteine molecule, and from the glutamic molecule of GSH shunting to succinate to provide substrate for ATP production. As each molecule of glutamate is shunted via GABA to succinic semialdehyde, there is a molecule of glutamate formed via GABA transaminase and alpha-ketoglutarate. Thus a replacement of this source of succinate continues as long as a supply of alpha-ketoglutarate is maintained. At 4 mmoles/kg, GABA, glutamate, and succinate are considered to have been utilized by the tissues between the time of injection and the early stages of HPO exposure—since they give little, if any, protection. In contrast, when 10 mmoles/kg is used, GABA, glutamate, and succinate all give approximately the same protection. The protection obtained, however, with 10 mmoles/kg of GSH, though much better than GABA, glutamate, and succinate, is not significantly different from that obtained with 4 mmoles/kg of GSH.

If the GSH–succinate shunt is the method by which the GSH protection is obtained, then mixtures of cysteine plus succinate should equal the GSH protection at 10 mmoles/kg. Such is the case, as seen in Table III. Similarly, when the dosage of GSH is increased to 12 mmoles/kg, the increase in protection above the 10 mmoles/kg GSH dosage is approximately equalled by increasing the succinate component of the cysteine plus succinate mixture, to 12 mmoles/kg. The difference between cysteine plus GABA, cysteine plus glutamate, and cysteine plus succinate is believed to be differences in rates of transfer across the blood–brain barrier between succinate and GABA and glutamate.

The observation by Gershman *et al.* (21) that oxidized glutathione gave protection against oxygen toxicity in mice, but to a lesser degree than GSH, may be explained by the glutathione to succinate shunt—since the glutamic molecule could be split from oxidized glutathione to give protection via the glutamate–GABA–succinate shunt, without the sulfhydryl protection from cysteine. The comparison of GSH to GSSG protection (191/148) at 6 ATA 100% oxygen from the data of Gershman *et al.* (21) is analogous—if the shunt hypothesis is correct—to comparison of GSH to succinate protection (222/139) at 5 ATA 100% oxygen in our experiments. The closeness of the two ratios support the shunt concept.

A further test of the GSH–succinate shunt would be to subject animals to greater stress by increasing oxygen pressures so that the time required to shunt from GSH to glutamate and/or glutamate to GABA and/or GABA to succinate may be too long to maintain the amount of succinate required. Under such conditions, succinate would be expected to be the best single protectant.

With increased oxygen pressure, succinate protection greatly exceeded GABA protection and GSH protection at 7, 9, and 11 ATA (Table IV). The 4 mmoles/kg GSH protection is not significantly different from the cysteine protection at these pressures. The

cysteine plus succinate protection was greater than succinate or GSH. The cysteine sulfhydryl protection appears to be additive to the succinate protection. With increasing stress of high pressure oxygen, the best single protective agent is succinate, which may be immediately utilized in ATP production without having to pass through intermediate steps. These data support the concept that succinate is ultimately the compound that yields HPO protection obtained with GSH, glutamate, or GABA.

Wood *et al.* (20) showed that GABA has a protective effect in delaying thiosemicarbazide (10 mg/kg) induced convulsions in rats. We increased the amount of thiosemicarbazide (TSC) to 17.5 mg/kg, and compared the protective action of 10 mmoles/kg of 0.4 *M* succinate or GABA or of equal volumes of isotonic saline (controls) given i.p., 50 min prior to intramuscular injection of the TSC. Time to convulsions after TSC injection was recorded. At the 17.5 mg/kg dosage of TSC, succinate injected animals had an average convulsion time of 123.4 ± 26 min (N = 15) vs 89.4 ± 15.3 min (N = 10) for GABA; vs 81.2 ± 11.1 min (N = 19) for controls. Thus, with the increasing stress of higher TSC dosage, GABA protection became insignificant while succinate protection was still present—similar to the results with increasing HPO pressure. Again, the concept that succinate is the ultimate protective agent in the GSH–glutamate–GABA–succinate pathway is supported.

If the GSH–succinate shunt serves to supply ATP, any stress which would result in decreased brain ATP concentration should result in a rapid shunting of GABA to succinate and subsequently to fumarate and malate. Brain cytoplasmic sap content of succinate-^{14}C, fumarate-^{14}C, and malate-^{14}C was determined in the animals given GABA-^{14}C, and exposed to either hyperoxia, hypoxia or air. The sum of the ^{14}C content of succinate plus fumarate plus malate was expressed as a percentage of total ^{14}C content and is shown in Table V. Both the 25-min HPO exposure and the 25-min hypoxia exposure resulted in marked increases in the ^{14}C label contained in succinate plus fumarate plus malate. Thus, even the low oxygen environment, in which there is no inhibition of alpha-ketoglutarate dehydrogenase activity to facilitate the GABA transaminase reaction—as in HPO exposure—resulted in a marked increase in the ^{14}C level of succinate, fumarate, and malate. Since GABA-^{14}C was injected, we concluded that under the condition of HPO stress or hypoxia (4% O_2)—both of which have been shown to decrease tissue ATP concentration (12, 13, 17–19)—there is a rapid shunting of GABA to succinate. This supports the concept of the GSH–glutamate–GABA–succinate pathway functioning as a secondary system for the maintenance of brain ATP levels.

The hypothesis that the GSH–glutamate–GABA–succinate shunt is a reserve system for maintenance of normal brain energetics gives a logical explanation to (i) oxidized glutathione protection against HPO toxicity [Gershman *et al.* (21)], and (ii) marked decreases in GSH and/or GABA in tissues from animals subjected to various stress conditions (Table I). The observations from the literature as outlined above, and the experimental data included in this report, support the hypothesis that a GSH–glutamate–GABA–succinate shunt functions as a protectant to brain under stress conditions.

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