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Identification and quantitation of 1,4-butanediol in mammalian tissues: an alternative biosynthetic pathway for gamma-hydroxybutyric acid

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Gamma-hydroxybutyric acid (GHB) occurs naturally in mammalian brain [1] and has been shown to produce marked central nervous system depression [2, 3] and a trance-like behavioral state, associated with paroxysmal electrical activity, quite similar to that observed in petit mal epilepsy [4–7].

The parent compound for GHB in brain is considered to be gamma-aminobutyric acid (GABA) [8–13]. However, several lines of evidence have been developed that suggest the presence of precursors for GHB other than GABA: (1) GHB in extraneural sites (liver, heart, kidney, muscle) notable for their absence or small concentrations of GABA [14–17], (2) liver and kidney concentrations of $NADP^+$-dependent oxidoreductase (D-glucuronate reductase; EC 1.1.1.10), the primary catabolic enzyme for GHB, ten to twenty times that in brain [15] and (3) the GHB ontogeny significantly different from that of GABA and the enzymes involved in its conversion to GHB [18]. These data suggest that in the periphery, and perhaps in the brain, there is an alternative source for GHB.

In this regard, it has been shown that the compound 1,4-butanediol (BD), administered either i.v. or i.p., is metabolized to GHB, producing increases in both the blood and brain levels of this compound [19, 20]. Thus, BD would appear on the surface to be a likely candidate for an alternative source of GHB in brain and liver.

A method for the extraction, derivatization and gas chromatographic/mass spectrometric (GC/MS) identification and quantification of BD by selected ion monitoring/isotope dilution techniques is presented. The presence of BD as an *in vivo* component of human and rat brain and of rat liver is demonstrated and the level of BD in these tissues is reported.

Deuterated 1,4-butanediol (DBD: 1,1,2,2,3,3,4,4-$^2H_8$-BD, 99.99 atoms% with respect to BD by GC/MS) was obtained from Merck Isotopes, Montreal, Canada. The BD, heptafluorobutyric anhydride (HFBA) organic solvents and other materials were obtained from commercial sources and were of the highest available purity.

Rat brains and livers were obtained from adult male Sprague–Dawley rats (CrL: CS(SD)BR, Charles River; 200–350 g, fed and watered *ad lib.* and maintained on a 12 hr light–dark cycle) that were killed by decapitation; the brain and liver were rapidly removed, dissected, and subjected to the assay procedure outlined below.

Post mortem human brain tissue (frontal cortex and cerebellum) was obtained from patients, ranging in age from 32 to 68 years (mean = 43.7), dying of non-neurologic disease who were autopsied within 12 hr of death (mean = 4.23 hr). The brain tissue samples were then frozen at −76° until the assay procedure was conducted.

The extraction of BD from liver and brain aqueous and lipid fractions was accomplished via modification of the method reported by Bergelson *et al.* [21], who reported the presence of trace amounts of BD in rat liver lipid fractions in 1966 using thin-layer chromatography.

Lipid fraction. To a 15.0-ml conical glass tube in an ice-water bath were added 2.0 ml of water, 0.5 ml of 70% perchloric acid and 0.5 μg of DBD. Tissue was weighed (0.2 to 0.5 g of liver or brain), added to the tube, and thoroughly homogenized. The sample was centrifuged and the supernatant fraction was transferred to a clean 15-ml conical glass-stoppered tube. The supernatant fraction was extracted with 2 × 1.0 ml of isooctane:hexane (15:85, v/v) by shaking for 15 min. The combined organic phases, containing lipids and other neutral compounds, were evaporated to dryness under nitrogen in a 50-ml RB flask; 0.5 μg of DBD and 20.0 ml of 0.4 M methanolic potassium hydroxide were added and the mixture was refluxed for 2 hr. On cooling, the pH was adjusted to 7.0 with 0.25 M sulfuric acid and lyophilized to dryness. The residue was extracted with 6 × 5.0 ml of chloroform:methanol (95:5, v/v), and the combined extracts were evaporated to dryness under nitrogen in 5.0-ml portions in a 15-ml conical glass-stoppered tube. HFBA (250 μl) was added, vortexed, and heated at 50° for 30 min. On cooling, 1.0 ml of methylene chloride was added and then extracted with 3 × 1.0 ml of deionized water. The methylene chloride was transferred to an ampoule, 0.25 g anhydrous sodium sulfate was added and the ampoule was sealed.

Aqueous fraction. The pH of the aqueous phase was adjusted to 12.0 with 45% potassium hydroxide, the mixture was centrifuged, and the supernatant fraction was transferred to a 15-ml conical glass-stoppered tube. The supernatant fraction was extracted with 2 × 1.0 ml of chloroform, and the aqueous phase was adjusted to a pH of 7.0 with perchloric acid. The sample was centrifuged and the supernatant fraction was lyophilized to dryness. The remainder of the procedure was conducted as described above for extraction and derivatization.

GC/MS analyses were conducted using a Hewlett Packard 5985A GC/MS equipped with a RTE-6-VM data system. Gas chromatography was conducted in the splitless mode on a 25 m × 0.25 mm i.d., 0.12 mm coating of BP5 (J & W Scientific Inc., Rancho Cardova, CA) fused silica glass capillary column using helium as the carrier gas. A temperature program was used to effect separation of DBD and BD: 50° for 1.0 min, increasing 10°/min to 300°. The purge function was activated at 0.5 min into the run. The injection port and source temperatures were maintained at 200° while the transfer zone temperature was held at 300°. Analyses were conducted in the positive ion/electron

impact mode, collecting either total ion or selected ion data, at an ionizing voltage of 70 eV.

Pure standards of BD and DBD were derivatized as described above and analyzed in the total ion/electron impact mode to determine fragmentation patterns. Characteristic ions from these data were used to monitor samples in the selected ion monitoring mode. Spiked (0.0 to 0.5 μg BD, 0.5 μg DBD) water and homogenized liver and brain samples (0.5 g tissue) were treated as described above and used to determine percent recovery, to determine linearity of recovery to response, and to provide standard curves for quantitation.

GC characteristics, mass spectra and proposed fragmentation patterns for the diheptafluorobutyrl (di-HFB) derivatives of BD and DBD are shown in Fig. 1A, B and C respectively. The recovery of BD from spiked brain and liver aqueous and lipid fractions was 74 ± 10% (N = 5) and 88 ± 9% (N = 5) respectively. A linear recovery and

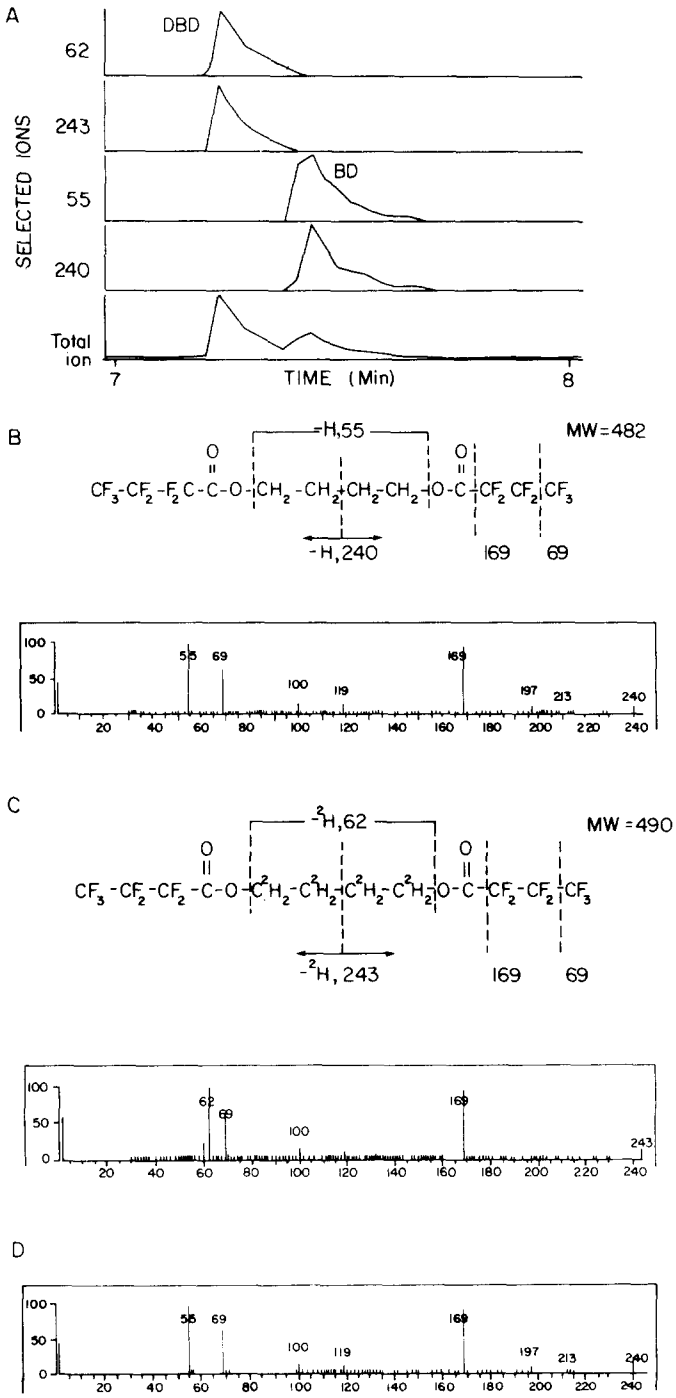

Fig. 1. GC characteristics (A) and mass spectra and proposed fragmentation patterns for the diheptafluorobutyrl derivatives of 1,4-butanediol (B) and $^2H_8$-1,4-butanediol (C). Figure 1D is the total ion mass spectrum of 1,4-butanediol obtained from a rat liver lipid fraction.

Table 1. Concentration (ng/g) and/or presence (+) of BD in the various tissue lipid and aqueous fractions examined*

Tissue	N	BD (ng/g wet weight of original tissue) Lipid fraction	Aqueous fraction
Rat brain			
(whole)	7	+	65 ± 29
Cortex	2	+	17
Sub-cortex	2	32	21
Cerebellum	2	+	36
Liver	7	167 ± 62	29 ± 8
Human brain			
Cortex	2	15	+
Cerebellum	2	26	+

* For N = 7, values are expressed as mean ± S.D. For N = 2, values are expressed as the average of the two samples.

response for BD were observed in all cases (0.0 to 1.0 μg BD/g wet weight of tissue, $r = 0.99$, $N = 5$) examined. The limit of detection for BD was 0.01 μg/g wet weight of tissue.

The criteria for identification of BD in the tissue fractions examined were: (1) correct retention time, as determined from standards, and (2) presence of, and correct ratio for, diagnostic ions (m/z 55/240) as determined from standards. Sufficient quantities of endogenous BD were obtained from rat liver lipid fraction to obtain a total ion mass spectrum (Fig. 1D).

Quantitation of BD in the various tissues was accomplished using the criteria above for identification and the ratio of the ions (m/z) 55/62 for BD/DBD. The ratio obtained was compared to those from the standard curves to give total ng of BD/g wet weight of tissue (Table 1).

The results of the present study indicate that BD is a naturally occurring endogenous compound, existing in both the free (aqueous phase) and combined (lipid phase) forms, in rodent brain and liver and in human cortex and cerebellum. The presence of BD *in vivo*, coupled with the data regarding the conversion of BD to GHB following its i.p. or i.v. administration [19, 20], provides support for the hypothesis that BD could serve as an alternative source for GHB in peripheral and neural tissues. This is particularly true in liver where GABA concentrations are significantly lower than in brain [14–17] yet the activity of GHB dehydrogenase is high [15]. The metabolic source of BD is not known at this time. Conceivably this compound could be formed from dietary precursors or be synthesized in liver and brain.

The demonstration of the presence of BD as a naturally occurring compound in brain and liver and the knowledge that this substance is converted to GHB by a pathway utilizing alcohol dehydrogenase [22–25] add another complex dimension to thinking about GHB metabolism in neural and extraneural tissues. This is especially true in view of the protean neuropharmacologic and neurophysiologic properties of GHB [26] and a proposed role for this compound as a neurotransmitter or neuromodulator in its own right [27].

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Anti-inflammatory drug inhibition of transport of cystine and glutamate in cultured human fibroblasts

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In mammalian cells, several transport systems for amino acids with different specificity have been characterized [1]. Recent studies have shown that nonsteroidal anti-inflammatory drugs (NSAID) inhibit the transport of amino acids through system A in a variety of cell lines [2, 3]. System A is a Na^+-dependent, neutral amino acid transport system characterized by the transport of methylaminoisobutyric acid. Transport of amino acids through system N or system L is unaffected or slightly stimulated by NSAID [2, 3]. Inhibition by NSAID of system A amino acid transport develops slowly and probably is not the initial action of NSAID [2]. Maximum inhibition by indomethacin was observed after a 2-hr incubation with the drug. In previous papers we have shown that there exists an anionic amino acid transport system responsible for the uptake of cystine and glutamate in cultured human diploid fibroblasts [4, 5]. Similar transport systems have been found in rat hepatoma cells [6] and in isolated rat hepatocytes [7]. This communication describes our observations on the immediate and potent inhibition by NSAID of cystine and glutamate transport in human fibroblasts.

Materials and methods

Chemicals. L-[3,3'-^{3}H]Cystine was obtained from Amersham. Indomethacin and phenylbutazone were purchased from Sigma. 5-Methoxy-2-methylindole-3-acetic acid was from Aldrich. Mefenamic acid and piroxicam were gifts from the Sankyo Pharmaceutical Co. and the Pfizer Taito Co. respectively.

Cells. Experiments were performed with a strain of human diploid fibroblasts derived from fetal lung (IMR-90). The cells were cultured in Eagle's basal medium supplemented with 10% fetal calf serum.

Uptake method. L-Cystine uptake was measured as described previously [4]. Cells grown in a 35-mm diameter dish were rinsed three times in warmed phosphate-buffered saline, pH 7.4, and then were incubated in 0.5 ml of the uptake medium at 37°. The uptake medium consisted of the same buffer used to rinse the cells plus labeled amino acid (1 μCi/0.5 ml). NSAID were dissolved in dimethyl sulfoxide and added to the uptake medium (less than 0.2% dimethyl sulfoxide at final concentration). pH was adjusted when necessary. The incubation was terminated by rinsing the cells three times in ice-cold phosphate-buffered saline, and the radioactivity taken up by the cells was determined as described before.

Results and discussion

Indomethacin inhibited the uptake of cystine in human diploid fibroblasts (Fig. 1). Inhibition was immediate and reversible; uptake was inhibited within 10 sec and upon removal of the drug the rate of uptake was restored without a detectable delay (Fig. 1A). Since cystine is rapidly metabolized (i.e. reduced to cysteine) in the cells, it is possible that an inhibition of the metabolism was responsible for the decreased net rate of the cystine uptake. It has been shown that *N*-ethylmaleimide almost completely inhibits the intracellular metabolism of cystine with only a slight effect on uptake of cystine [4]. Therefore, uptake of [^{3}H]-cystine was measured in the presence of 0.1 mM *N*-ethylmaleimide, where about 95% of ^{3}H-labeled compounds recovered from the cells was identified as cystine. Figure 1B demonstrates apparent uphill transport of cystine and the direct inhibition of transport by indomethacin. Indomethacin decreased the influx V_{max} with little change in the influx K_m for cystine (Fig. 2). In the presence of 0.5 mM indomethacin, the influx V_{max} decreased to about one-fifth that of the control cells. The effects of indomethacin on the uptake of other amino acids were examined. The uptake of alanine, cysteine and leucine was inhibited only weakly by indomethacin at 0.5 mM, whereas the uptake of glutamate, which is largely mediated by the same transport system as cystine, was inhibited to the same extent as that of cystine (data not shown).

Figure 3 shows the effects of various NSAID on cystine uptake. All of these agents inhibited the uptake in a concentration-dependent manner. Mefenamic acid appears to be the most potent inhibitor. Several compounds, which are structurally related to indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] but do not possess anti-inflammatory activity, were also tested. Indole-3-acetic acid and 5-methoxy-2-methylindole-3-acetic acid at 1 mM were ineffective, and *p*-chlorobenzoic acid was weakly inhibitory at 1 mM (about 30% inhibition). Correlation between the inhibition of cystine uptake and the anti-inflammatory action is not clear. However, the order of potency in the inhibition of cystine uptake was roughly similar to that reported for their anti-inflammatory activity.

The amino acid transport system for cystine and glutamate in human diploid fibroblasts is Na^+-independent and shared by anionic amino acids such as homocysteate and α-aminoadipate [4, 5]. Indomethacin has been shown to affect Na^+ influx in human fibroblasts [9]. It is possible that the inhibition of A system amino acid transport may be linked with the inhibition of Na^+ influx, because the inhibition of Na^+ influx by indomethacin occurs more rapidly than the inhibition of A system amino acid transport [9] which is Na^+-dependent. However, it is unlikely that the inhibition of cystine transport by NSAID is related to the inhibition of Na^+ influx. Cystine transport is Na^+-independent and the inhibition of cystine uptake occurs more rapidly than the inhibition of Na^+ influx. In fact, indomethacin inhibited cystine uptake in Na^+-free medium to the same extent as that in the normal, Na^+-containing medium (data not shown). Correlates with other biochemical variables, e.g. cellular ATP levels, might be considered. However, it has been shown that ATP levels do