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Short-Chain Carboxylic Acids, A New Class of Teratogens: Studies of Potential Biochemical Mechanisms

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Certain short-chain carboxylic acids (SCCA) appear to share a common teratogenic potential, although the structural requirements for activity remain obscure. By using a whole rat embryo culture model system, several biochemical processes have been examined, either as potential initial sites of teratogenic action or as early steps in the pathway to malformation. Valproate, methoxyacetate, and butyrate were the prototype SCCA examined. Measurement of [^{14}C]glucose utilization and lactate production confirmed that energy production by the early organogenesis embryo is predominantly from glycolysis. While the positive control agent, iodoacetate, caused a significant inhibition of lactate production, none of the SCCA affected this process or glucose utilization at teratogenic concentrations. Valproate did not influence embryonic acetyl CoA levels, in marked contrast to the reported response of adult liver, the other major target of valproate toxicity. Pinocytosis by the visceral yolk sac (VYS) was measured by the uptake of [^{125}I]polyvinylpyrrolidone. This process ultimately supplies the embryo with amino-acids and is essential for normal development. SCCA induce morphological abnormalities of the VYS in embryo culture. Pinocytosis was slightly reduced by valproate, but not the other SCCA. However, comparison with the action of an antiserum, for which inhibition of pinocytosis is the initial teratogenic insult, suggests that this is not the mechanism for valproate. Incorporation of [^{3}H]thymidine into embryo or yolk sac was not affected after 3 hr of SCCA exposure, but there was a marked effect of the positive control, hydroxyurea. This suggests that DNA synthesis is not directly influenced by SCCA. It can be concluded that SCCA do not exert their teratogenic effects by actions on glycolysis; maintenance of cellular acetyl CoA; pinocytosis or DNA synthesis. These observations contrast with preliminary results which suggest significant effects of SCCA on embryonic and yolk sac lipid metabolic pathways.

Introduction

A series of observations from experiments *in vivo* and *in vitro* suggest that short-chain carboxylic acids (SCCA) share a common property of teratogenic potential and may represent a new class of teratogens of human significance. The anticonvulsant drug valproic acid (Depakene, Epilim) is a simple SCCA of systematic name 2-propylpentanoic acid and is teratogenic in several laboratory species (*1–3*) and probably also in the human (*4,5*). Methoxyacetic acid is the major metabolite of the glycol ether, 2-methoxyethanol (ethylene glycol monomethyl ether) (*6*). We have shown that the teratogenic effect of 2-methoxyethanol in the rat is probably mediated by this alkoxy-SCCA metabolite (*7*). Metabolism appears to occur in the maternal, but not the embryonic, compartment since direct exposure of isolated rat embryos to the glycol ether is without effect, while the acid is dysmorphogenic (*8*).

Using a whole embryo culture model system, we have studied the potential teratogenicity of about 30 SCCA and related compounds (*2,8–10* and unpublished observations). Many SCCA induce a similar spectrum of structural abnormalities in embryo culture, although potency varies over two orders of magnitude. Of all SCCA tested to date, butyrate is the most potent, although this compound would not be expected to pose a major hazard *in vivo* because of rapid clearance. Noteworthy aspects of the structure-activity relationships (SAR) *in vitro* include: an absolute requirement for a free carboxyl group; a reduction in potency with any substitution of C in the alkyl chain (e.g., N, S, or O); maximum activity at overall alkyl chain lengths of 4 or 5. So far, this SAR has resisted any mechanistic explanation, and there are no simple relationships with lipid solubility, pK_a or any known pharmacological or biochemical activities.

There have been no direct comparisons of the tera-

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togenic activities of several SCCA *in vivo*. However, reported malformation profiles are broadly similar, which is in general support of the proposal that SCCA share common activities and, perhaps, common mechanisms of action. In the mouse, treatment in early organogenesis with valproate (*2*), valproate analogs (Nau, personal communication) or 2-methoxyethanol (*11*) induces exencephaly and abnormalities of the axial skeleton, while later treatment can cause limb defects. In the rat, defects of the axial skeleton predominate (*1,3,7*). It is interesting that most of these malformations are "general," that is to say, commonly induced by many treatments, including maternal toxicity (*12,13*). There is little doubt, however, that at least some SCCA have effects on the embryo *in vivo* at significantly lower doses than those which induce overt maternal toxicity. In other words, these compounds are "teratogenic hazards" (*2,11,14*).

The aims of current studies are to identify the embryonic site(s) of biochemical action of SCCA and to characterize the initial biochemical response(s). The embryo culture model system has been used for two main reasons. The exposure conditions required for SCCA to induce structural defects consistently are well established in this laboratory. In addition, the use of radiotracers and other biochemical manipulations are facilitated, compared to the whole animal. We report here the potential actions of three prototype SCCA—valproate, methoxyacetate, and butyrate—on four processes: glycolysis, maintenance of cellular acetyl CoA levels, pinocytosis, and DNA synthesis.

Glycolysis is the major source of energy in the rodent organogenesis embryo (*15*) and disruption of this process can lead to so-called "fuel-mediated" teratogenesis (*16*). In addition, it has been suggested that methoxyacetate exerts its testicular toxicity by interference with lactate production and utilization in Sertoli cells (*17,18*). In adult liver, valproate profoundly affects cellular acetyl CoA levels leading to various biochemical derangements and, ultimately, hepatotoxicity (*19,20*). [Some of our studies on embryonic acetyl CoA and valproate have been published (*21*)]. Pinocytosis by the visceral yolk sac was examined because it is essential for normal development and the yolk sac is morphologically affected by SCCA (*2,8,10*). Finally, potential actions on DNA synthesis were examined because SCCA are growth retarding and appear to be capable of affecting most organs of the conceptus. Normal DNA synthesis is required for the development of all embryonic primordia.

Materials and Methods

Culture Techniques

All the studies reported here used explanted whole conceptuses (i.e., embryo, amnion, visceral yolk sac, and placenta) maintained *in vitro*, as follows. LAC-P rats were mated overnight; those females with plugs the following morning were considered to be in the first day of gestation. Embryonic ages used throughout this report were calculated from zero time as the mid-point of the mating period. The whole embryo culture techniques used have been described in detail elsewhere (*22*). In brief, standard 9.5-day 48-hr cultures were performed as follows. Conceptuses were explanted on day 10 of gestation. Two or three conceptuses were cultured in 50-mL Soveril bottles containing 4 mL of standard medium, comprising 75% immediately centrifuged, heat-inactivated rat serum and 25% Eagle's Minimal Essential Medium with Earle's salts (MEM). Bottles were rotated at 30 rpm and incubated at 37°C for 48 hr. The initial gas phase was 5% O_2, 5% CO_2, 90% N_2. The oxygen content was increased to 20% at 16 hr and 40% at 26 hr (5% CO_2 at all times). Where SCCA were added, the acid was neutralized by dissolving in an equimolar volume of 1 N NaOH, followed by dilution to volume with MEM to form a neutral stock solution in MEM. Throughout the studies, measurement of total protein content was by the method of Lowry.

Glycolysis

After 16 hr of standard culture, three or four conceptuses were transferred into 53 × 15 mm plastic tubes (minivials, Gordon-Keeble) containing 1 mL standard medium including 1.1 μCi [U-^{14}C]glucose (270 mCi/mmole, Amersham). The mean glucose concentration of batches of rat serum was 1.5 mg/mL, as determined by a glucose oxidase colorimetric assay (Sigma). MEM contains 1 mg/mL glucose, giving a final medium specific activity for glucose of 0.144 mCi/mmole. Conceptuses were incubated for 8 hr, with or without treatment, at 37°C with 20% O_2 and rolling at 60 rpm. Conceptuses grew well under these conditions, with active heart beat and yolk sac circulation throughout. At the end of incubation, 10 μL samples of medium were taken for descending paper chromatography with butanol/water/acetic acid (4:5:1) as solvent, in order to separate glucose from metabolites. Paper was cut into strips that were counted for radioactivity. The R_f values for glucose and lactate were determined from standards.

Acetyl CoA

Two experimental designs were used. (1) After 16 hr of standard culture, valproate was added to the medium to a concentration of 1.5 mM and cultures were continued for 8 hr. Conceptuses were then harvested, washed, snap-frozen in liquid N_2 and stored overnight at −70°C. (2) After 40 hr of standard culture, valproate was added, as above, and cultures continued for 4 hr. During this period, conceptuses were taken each hour for immediate analysis. Acetyl CoA was determined by the citrate synthase method of Pande and Caramancion (*23*). The assay method was as described, but tissue preparation was scaled down so that single 11.3-day (40–44 hr) embryos or yolk sacs, or three 10.5-day (24 hour) conceptuses could be assayed.

Pinocytosis

The method for measuring fluid-phase pinocytosis by visceral yolk sacs was essentially that of Williams et al. (*24*), as we have described in detail elsewhere (*25*). Briefly, 11.3-day conceptuses were cultured individually in minivials containing 1 mL of 25% rat serum, 75% MEM including 1.0 μCi [^{125}I]polyvinyl-pyrollidone (PVP). After 4 hr incubation, with or without treatment, at 37°C, 40% O_2, and rolling at 60 rpm, yolk sacs were isolated, washed, homogenized, contained radioactivity counted and protein content measured. Pinocytosis is expressed as a clearance, in units of microliters of medium whose contained radioactivity was taken up per milligram yolk-sac protein per hour (*25*).

DNA Synthesis

Thymidine incorporation was measured over two exposure periods. Following 16 hr standard culture, SCCA was added to the medium. After 2 hr of exposure, 3 μCi/mL [^{3}H-methyl]thymidine (25 Ci/mmole) was added, and incorporation allowed to proceed for an additional 1 or 2 hr. Alternatively, 11.3-day conceptuses were cultured in minivials in 1 mL standard medium. Following a 1-hr pre-equilibration, SCCA was added, and experiments proceeded as above. Preliminary observations showed that incorporation of activity into acid-insoluble fractions of embryo and yolk sac was linear over 2 hr. At the end of incubation, embryos and yolk sacs were isolated, washed, homogenized, and a sample taken for DNA determination (*26*). Protein carrier (serum) was added and samples were TCA precipitated, centrifuged, the soluble fraction taken for counting and the pellet was washed twice by resuspension before counting.

Results

Glycolysis

The amount of [^{14}C]glucose utilized and lactate produced by 10.5-day conceptuses is shown in Table 1. Overall, conceptuses utilized about 900 pmole glucose/μg protein/hr and produced from it about 1500 pmole lactate/μg protein/hr, which is greater than 80% conversion. This conversion is consistent with previous data concerning mouse and rat embryos (*15,27*) and the rate of lactate production is very similar to published data on rat embryos at this stage (*27*). Exposure of conceptuses to the positive control compound, iodoacetate, which inhibits glycolysis at the glyceraldehyde phosphate dehydrogenase step, resulted in a 90% inhibition of lactate production (Table 1). Concentrations of iodoacetate as low as 3 μM significantly reduced lactate production (data not shown). Valproate, butyrate, and methoxyacetate had no significant effect on glucose utilization or lactate production, nor on total protein content (Table 1).

Table 1. Glucose utilization and lactate production by 10.5-day rat conceptuses *in vitro* during an 8-hr exposure to carboxylic acids.

Treatment	Glucose utilized, pmole/μg/hr[a]	Lactate produced, pmole/μg/hr[a]	Protein, μ/conceptus[a]	Number of samples
Control	928 ± 47	1508 ± 46	119 ± 4	12
Valproate (1.5 mM)	797 ± 56	1417 ± 62	103 ± 2	6
Butyrate (0.75 mM)	1049 ± 149	1656 ± 69	114 ± 2	6
Control	863 ± 87	1365 ± 52	119 ± 6	6
Methoxyacetate (5.0 mM)	827 ± 111	1255 ± 31	116 ± 6	6
Iodoacetate (0.1 mM)	867 ± 63	183 ± 14**	64 ± 7**	3

[a] Data as $\bar{X}$ ± SE. Each sample was three or four conceptuses.
** Significantly different from control, $p < 0.01$, ANOVA.

Acetyl CoA

The acetyl CoA content of 10.5-day conceptuses is shown in Table 2. These values were measured after samples had been frozen and stored overnight. We have found that recovery of acetyl CoA is about 40% efficient by this procedure, compared to 95% for unfrozen, unstored samples. Freezing of these samples was necessary because conceptuses were removed from culture in the late afternoon, and the acetyl CoA assay requires a full working day. There was no significant effect of an 8-hr exposure to 1.5 mM valproate on acetyl CoA levels (Table 2). Total protein content of conceptuses was also not affected by valproate. Protein values in these experiments are lower than those shown in Table 1 because conceptuses were TCA precipitated, and the pellet washed and redissolved prior to protein determination.

As described for the younger conceptuses, there was also no effect of 1.5 mM valproate on acetyl CoA levels in 11.3-day embryos or yolk sacs exposed for 4 hr (Fig. 1). The acetyl CoA level was about 100 pmole/mg protein in these freshly analyzed tissues, which is comparable to adult liver (*23*). The level in 10.5-day conceptuses is also of this order, the lower values shown in Table 2 being due to loss during freezing and storage, as described.

Pinocytosis

Uptake of [^{125}I]PVP was measured as an index of fluid-phase pinocytosis by the visceral yolk sac of 11.3-

Table 2. Acetyl CoA content of 10.5-day rat conceptuses after 8 hr exposure to valproate *in vitro*.

Treatment	Acetyl CoA, pmole/mg protein[a]	Protein, μg/conceptus[a]
Control	41.5 ± 6.0	84.5 ± 5.0
Valproate (1.5 mM)	46.3 ± 6.0	92.4 ± 3.1

[a] Data as $\bar{X}$ ± SE, four samples, three conceptuses each. No significant differences between treatment and control, Student's *t* test, $p > 0.2$.

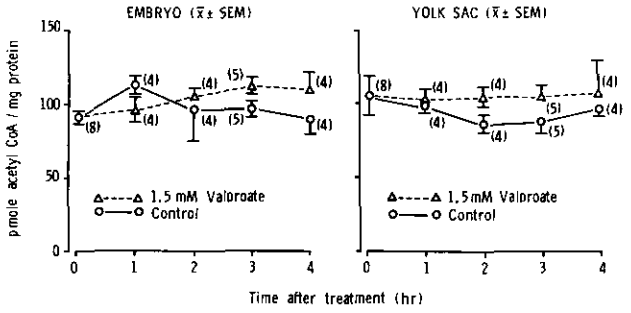

FIGURE 1. Acetyl CoA content of 11.3-day rat conceptuses *in vitro* during a 4-hr exposure to 1.5 mM valproate. There are no significant time or treatment-related effects (analysis of variance).

day conceptuses. Four series of experiments are summarized in Table 3. Uptake was slightly, but significantly, reduced by 5 mM valproate exposure, and there were small, insignificant reductions with 5 mM butyrate and 20 mM methoxyacetate. Lower concentrations of each acid were without effect. The positive control treatment, an antivisceral yolk-sac endoderm antiserum, induced significant reductions of uptake at both doses tested (Table 3). In each series, total yolk-sac protein content did not vary between treatment and control groups (data not shown).

DNA Synthesis

The incorporation of [^{3}H]thymidine into acid-soluble and -insoluble fractions of 11.3-day conceptuses is shown in Table 4. Although incorporation (on the basis of DPM/μg DNA) into both fractions was greater for yolk sac than embryo, fractional incorporation (insoluble/total) was identical for the two tissues over 1- and 2-hr thymidine exposures (data not shown). Overall, SCCA exposure had little influence on incorporation. Table 4 shows data obtained after 1 hr thymidine, but similar results were collected at 2 hr. From the analysis of variance, three significant treatment-related differences were detected. Incorporation into the acid-insoluble fraction of yolk sacs was slightly increased by both butyrate (2 mM) and valproate (5 mM), whereas soluble counts were increased in yolk sacs exposed to 20 mM methoxyacetate (Table 4).

[^{3}H]Thymidine incorporation was also measured using 10.5-day conceptuses in an identical protocol (data not shown). Again, SCCA exposure had little influence, excepting a significant increase in acid-insoluble counts in methoxyacetate (20 mM)-treated yolk sacs at 2 hr (data not shown). None of the data, at either embryonic age, give any indication of an inhibition of [^{3}H]thymidine incorporation caused by SCCA.

In contrast to the lack of effect shown by SCCA, the positive control compound, hydroxyurea (2.5 mM), induced marked changes. Counts in the soluble fractions of 11.3-day conceptuses were increased to 209% and 170% of control for embryos and yolk sacs, respectively (Table 4). Acid-insoluble counts were reduced to about 2% of control for both embryos and yolk sacs (Table 4).

Table 3. Pinocytosis of ^{125}I-PVP by the visceral yolk sac of 11.5-day rat conceptuses *in vitro* during a 4-hr exposure to carboxylic acids.

Treatment	Concn	Fluid pinocytosed μL/mg yolk sac protein/hr[a]	
Control		2.25 ± 0.05	(n = 6)
Valproate	2 mM	2.00 ± 0.20	(n = 4)
	5 mM	1.85 ± 0.09*	(n = 6)
Control		2.08 ± 0.10	(n = 6)
Butyrate	2 mM	2.31 ± 0.40	(n = 3)
	5 mM	1.76 ± 0.18	(n = 7)
Control		1.51 ± 0.20	(n = 5)
Methoxyacetate	10 mM	1.59 ± 0.07	(n = 6)
	20 mM	1.48 ± 0.14	(n = 5)
Control		2.01 ± 0.09	(n = 12)
Antiserum	10 μL/mL	1.61 ± 0.08†	(n = 9)
	50 μL/mL	0.88 ± 0.10†	(n = 4)

[a] Values as $\bar{X} \pm SE$ (n = number of conceptuses).
*,† Statistics by ANOVA; *$p < 0.05$; † $p < 0.01$ compared to control.

Discussion

Experimental Design

The rationale for performing these experiments using the *in vitro* whole embryo culture model was that both exposure and response are more reproducible in this system, compared to *in utero*, and that radiotracer studies are greatly facilitated. It is important, however, to ensure that the model accurately reflects teratogenesis *in vivo*. In other studies, we have shown that manipulation of concentration and timing of exposure to valproate *in vitro* enables the production of embryos with abnormalities of widely differing severity and morphology (*2,28*). Almost all embryos exposed to 1.5 mM valproate from 16 to 24 hr of standard culture and examined at 48 hr show abnormalities in mesoderm segmentation manifest as somite defects. These defects are apparently identical to those of 11.5-day embryos *in vivo* following valproate treatment on gestation day 11 (*28*). These embryos, both *in vivo* and *in vitro*, are relatively subtly affected, and we have observed that embryos that are more grossly abnormal at this stage *in vivo* do not survive to term. In designing biochemical studies of teratogenesis, it is important to avoid measurements on grossly defective embryos, which may have artifactual biochemical abnormalities.

Another major problem in biochemical studies of teratogenesis is the timing of measurements in relation to exposure so that observed effects are restricted to initial insults, or early events in the sequence leading to malformation. The majority of the studies described here utilized the experimental design described above, with exposure to SCCA from 16 to 24 hr of culture. Measurements were made during, or at the end of, this period, since it is clear that the teratogenic insult has been delivered by 24 hr and is not reversed by returning embryos to control conditions. Any biochemical change

Table 4. Incorporation of [^{3}H]-thymidine by 11.3 day rat conceptuses *in vitro* during the final hour of a 3-hr exposure to carboxylic acids.

		DPM × 10^{-3}/μg DNA					
		Soluble		Insoluble		DNA (μg)	
		E	YS	E	YS	E	YS
Control	(n = 11)	1.1 ± 0.3	2.2 ± 0.7	3.1 ± 0.6	6.5 ± 1.2	24 ± 5	5.2 ± 1.0
Butyrate, 2 mM		1.1 ± 0.3	2.4 ± 0.5	3.0 ± 0.7	7.3 ± 2.1*	21 ± 7	4.6 ± 1.2
Control	(n = 4)	0.9 ± 0.2	1.4 ± 0.1	3.0 ± 0.9	5.2 ± 0.3	24 ± 4	6.2 ± 1.0
Valproate, 5 mM		0.8 ± 0.2	1.5 ± 0.3	3.2 ± 0.6	6.0 ± 0.6*	22 ± 7	5.8 ± 1.3
Control	(n = 4)	0.8 ± 0.1	1.9 ± 0.3	2.3 ± 0.4	5.6 ± 0.4	29 ± 5	6.4 ± 0.9
Methoxyacetate, 20 mM		1.0 ± 0.1	2.4 ± 0.2*	2.5 ± 0.1	5.3 ± 0.1	37 ± 6	8.3 ± 1.2
Control	(n = 4)	1.1 ± 0.1	2.0 ± 0.1	3.6 ± 0.3	6.7 ± 0.4	28 ± 5	7.0 ± 1.1
Hydroxyurea, 2.5 mM		2.3 ± 0.2†	3.4 ± 0.4†	0.07 ± 0.006‡	0.16 ± 0.02‡	23 ± 4	6.7 ± 0.6

[a] Data as $\bar{X}$ ± SD, *,†, ‡ Significance by ANOVA: *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$.

that cannot be detected at 24 hr cannot be an early, "initiating" event.

From studies of different SCCA exposure levels and times, it is clear that virtually all tissues of the conceptus can be rendered morphologically abnormal by SCCA treatment *in vitro*. When exposure is manipulated to result in subtle defects of specific organs, we would propose that a similar biochemical insult (e.g., enzyme inhibition) is delivered to most, or all, cells of the conceptus. However, primordia respond differently, depending upon the developmental processes underway at the time of insult. (The induction of somite defects suggests that some aspect of mesoderm segmentation is especially sensitive to the changes induced by SCCA exposure.) Following this argument leads to the conclusion that the whole embryo can be taken for analysis of biochemical changes, rather than just those tissues which give rise to defective structures.

Glycolysis

The current data on glucose utilization by early organogenesis embryos are in agreement with previous studies of carbohydrate metabolism and energy production at this stage, which show that glycolysis is the major (~90%) pathway (*15,27*). The observed rate of lactate production (1300–1500 pmole/mg protein/hr, Table 1) is very similar to that in an earlier study of rat conceptuses *in vitro* (*27*) and also to that in a study of mouse conceptuses, when reported inaccurate calculations are corrected (*29*). A second study of the mouse reports much higher rates, but this is probably due to differences in protein determinations (*15*).

None of the three SCCA tested had any effect on glucose utilization or lactate production (Table 1), in contrast to the positive control, iodoacetate. It has been suggested that methoxyacetate exerts its testicular toxicity by interfering with lactate production and/or utilization by Sertoli cells (*17,18*). The structure-activity relationships for SCCA and teratogenic potency in whole embryo culture are remarkably similar to those for SCCA in a Sertoli/germ cell culture model of testicular toxicity (Gray et al., unpublished). Whether or not the testicular toxicity of SCCA involves effects on glycolysis, it seems clear that their teratogenic action does not.

Acetyl CoA

The major adverse effect of valproate in the adult, both clinically and in laboratory animals, is hepatotoxicity (*30*). The exact mechanism of this action is not known, but it is clear that there are many hepatic biochemical disruptions which can be explained by reduced concentrations of cellular CoA and derivatives (*19,20*). Hepatic valproate treatment *in vivo* or *in vitro* results in a dramatic fall in cellular acetyl CoA levels within 30 min (*19,20*). The results presented in Table 2 confirm our previous observations that valproate has no action on embryonic acetyl CoA (*21*). This is thought to be due to the lack of enzymatic conversion of valproate to valproyl CoA (*21*).

In other studies (Rawlings et al., unpublished), we have found no evidence for embryonic conversion of methoxyacetate to methoxyacetylglycine—the major metabolite in the adult (*31*)—which also proceeds through an acyl-CoA intermediate. In contrast, butyrate is extensively incorporated into acid-insoluble material by the yolk sac and embryo (*32*), suggesting competence at forming the CoA derivative of this natural SCCA. Since these three SCCA exert similar actions on the isolated embryo, it seems unlikely that embryonic metabolism of the compounds has a major role in the teratogenic action.

Pinocytosis

Treatment of rat or mouse conceptuses *in vitro* with SCCA induces morphological abnormalities of the visceral yolk sac at media concentrations only slightly in excess of the minimum concentrations that cause embryonic defects (*2,10*). These VYS abnormalities are apparent on gross observation and it is likely that more subtle changes induced by lower concentrations would be revealed by histological examination. In other studies we have shown that an anti-VYS endoderm anti-

serum induces teratogenesis by interfering with VYS pinocyotic function (*25*). In whole embryo culture, this antiserum can induce abnormality of 67% of embryos at a concentration where only 20% of VYS have any observable defect (*25*). Thus, embryonic defects can be induced by impaired VYS function even when there is no apparent action on VYS morphology and it was thought that the SCCA may mediate their effects by a similar mechanism.

The data in Table 3 show that the SCCA have no effect on VYS pinocytosis at high teratogenic concentrations. There was a significant reduction induced by valproate at 5 mM, but this is not thought to be significant, from the following comparison. The lower concentration of positive control antiserum used (10 μL/mL) significantly reduced pinocytosis (Table 3). This concentration induces 67% embryonic abnormality in 9.5-day rat conceptuses exposed for 48 hr, but is not grossly toxic to the VYS, inducing only 20% defects (*25*). In cultures of the same design, 2 mM valproate, 2 mM butyrate, or 10 mM methoxyacetate would induce grossly abnormal VYS and embryos, but these concentrations did not affect VYS pinocytosis (Table 3).

DNA Synthesis

It is widely accepted that agents which interfere with DNA synthesis or function in the embryo are likely to be teratogenic (*33*). At the early organogenesis phase all embryonic primordia are actively dividing, although the exact rate varies from organ to organ at any one time. Because the SCCA are growth-retarding and seem capable of affecting virtually all tissues of the embryo, under appropriate exposure conditions, DNA synthesis was considered as a potential target because it is one of the processes common and essential to all tissues.

The design of the experiments was to attempt to measure an initial action on DNA synthesis (thymidine incorporation). Since SCCA, at appropriate concentrations, can induce significant growth retardation in embryo culture over 24 or 48 hr (*2,10*), it would undoubtedly be possible to measure a reduction in DNA synthesis over such a time period. However, this is likely to be a secondary, rather than an initial, response. Thus, thymidine incorporation was measured during the third or fourth hours of exposure to teratogenic concentrations of SCCA. It is clear from the data shown in Table 4 that there is no initial inhibition of thymidine incorporation into acid-insoluble pools by SCCA. There is an apparent small stimulation of incorporation into the insoluble fraction of yolk sacs, but this is variable and of unknown significance. These experiments do not rule out the possibility of some effect on *de novo* purine or pyrimidine synthesis, as has been suggested for the related compound 3-hydroxybutyric acid (*34*). It is not clear whether such an effect would influence thymidine incorporation *via* the salvage pathway over the time scale examined. The response to the positive control compound, hydroxyurea, does not help to resolve this issue, as it inhibits DNA synthesis at the ribonucleoside diphosphate reductase step.

Conclusions

It is thought that the experimental designs used in these studies minimize the chances of measuring secondary biochemical changes after teratogen exposure. In addition, they ensure that embryonic tissues are examined during realistic exposures to SCCA, which are known to result in malformation. Therefore, the lack of effect of SCCA on glycolysis; acetyl CoA; pinocytosis and thymidine incorporation is not due to insensitive procedures, but strongly suggests that these processes are not involved in the early steps of the teratogenic mechanisms of these compounds. Confidence in this conclusion is bolstered by our recent experiments, of similar design, in which the effects of SCCA on incorporation of [^{3}H]acetate into lipid fractions has been measured (*35*). Both valproate and butyrate appear to induce highly significant changes. These changes affect specific classes of lipids and the responses of the embryo and the yolk sac appear to differ (*35*). The significance of the changes is not yet established, but they do illustrate that early biochemical changes can be detected in this model system. This strengthens the conclusion that the processes measured in this study are not involved in the teratogenic mechanism of carboxylic acids.

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