Modification of Diamine Oxidase Activity *in vitro* by Metabolites of Asparagine and Differences in Asparagine Decarboxylation in Normal and Virus-Transformed Baby Hamster Kidney Cells

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1. The oxidation of putrescine *in vitro* by pig kidney diamine oxidase (EC 1.4.3.6) was increased in the presence of 2-oxosuccinamic acid and malonic acid. 2. It was inhibited by 3-aminopropionamide, oxaloacetate and pyruvate. 3. 2-Oxosuccinamate was derived from asparagine in virus-transformed baby hamster kidney (BHK) cells growing in tissue culture. 4. Asparagine was decarboxylated more efficiently by transformed than by normal BHK cells. 5. In BHK cells transformed by polyoma virus (Py BHK), 2-oxosuccinamate is the most likely immediate precursor of the $^{14}\text{CO}_2$ arising from $[\text{U-}^{14}\text{C}]$ asparagine, and there was some evidence for its formation in an asparagine-dependent clone of BHK cells before and after their transformation by hamster sarcoma virus (respectively Asn⁻ and HSV Asn⁻). 6. The relationship between 2-oxosuccinamate and pyruvate and the possible roles of these two substances in controlling cellular diamine oxidase activity are discussed.

The increase in putrescine and spermidine concentrations that accompanies increased growth of animal cells is well documented (Raina *et al.*, 1966; Moruzzi *et al.*, 1968; Dion & Herbst, 1970). Since putrescine itself is an activator of mammalian S-adenosyl-methionine decarboxylase, a key enzyme in spermidine and spermine biosynthesis, it can be said that the concentrations of spermidine and spermine in mammalian cells are dependent on the intracellular concentration of putrescine (Pegg & Williams-Ashman, 1969).

One explanation for the elevated putrescine concentrations observed in growth is the increased activity of ornithine decarboxylase (EC 4.1.1.17) (Russell & Snyder, 1966), the enzyme which decarboxylates ornithine to putrescine with pyridoxal phosphate as cofactor. But another enzyme which must play a role in determining its concentration is diamine oxidase (EC 1.4.3.6). This enzyme converts putrescine into γ -aminobutyraldehyde, which can then either cyclize to Δ^1 -pyrroline or be oxidized enzymically to γ -aminobutyric acid. However, putrescine is not the only substrate for diamine oxidase; this enzyme exhibits a wide specificity, including the aliphatic diamines having the general formula $\text{NH}_2[\text{CH}_2]_n\text{NH}_2$ where $n = 3-6$, the mono-amino substituted derivatives of the above-mentioned

amines, and aromatic amines such as histamine (Bardsley & Ashford, 1972).

Thus the intracellular putrescine concentration depends on the relative concentrations of the different substrates of diamine oxidase, the K_m values of the enzyme for these different substrates and the presence of inhibitors or activators of the enzyme.

The inhibition of this enzyme by hydrazine derivatives is well-documented (Kapeller-Adler, 1970) and a comprehensive study (Seiler & Eichenkopf, 1975) using amino guanidine and hydrazine sulphate and carboxymethoxylamine has permitted the intra- and extra-mitochondrial pathways of putrescine metabolism to be elucidated.

We were primarily concerned with the endogenous activators and inhibitors of the enzyme, especially those that could be derived from substances known to be implicated in controlling growth. For these reasons and the fact that the immediate product of diamine oxidase action on any substrate is the corresponding aldehyde, we chose, in this first study, the amide analogue of diaminopropane ($\text{NH}_2[\text{CH}_2]_3\text{NH}_2$), which is itself a substrate of diamine oxidase (Quash & Taylor, 1970) and has also been identified in human urine (Walle, 1973). Further, this amide could be obtained theoretically by the direct decarboxylation of asparagine, which is essential for the growth of certain lymphomas (Capizzi *et al.*, 1971). Thus for comparative purposes, and in an attempt to elucidate the role

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of asparagine in growth, other amide derivatives of asparagine were also included in this study.

The effect of these amide derivatives, namely 3-aminopropionamide ($\text{NH}_2\text{COCH}_2\text{CH}_2\text{NH}_2$), 2-oxosuccinamic acid ($\text{NH}_2\text{COCH}_2\text{COCO}_2\text{H}$) and malonamic acid ($\text{NH}_2\text{COCH}_2\text{CO}_2\text{H}$), on diamine oxidase activity *in vitro* will be reported here. Further, evidence will be presented for the formation of 2-oxosuccinamate from asparagine by baby hamster kidney (BHK) cells and the differential decarboxylation of asparagine by normal as compared with viral-transformed BHK cells in tissue culture.

Materials and Methods

Reagents

[1,4- ^{14}C]Putrescine (21 mCi/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. *N*-Methylbenzo-2-thiazolonehydrazine hydrochloride was purchased from Koch-Light, Colnbrook, Bucks., U.K. L-Asparagine, L-amino acid oxidase and catalase were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Succinamide, succinamic acid, succinic acid and propionamide were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. [U- ^{14}C]Asparagine (100 mCi/mmol) was obtained from the C.E.A., Saclay, France. It was purified by chromatography on Dowex 1 (Merck-France S.A., Paris, France) before use. Strong acid SA-2 and strong base SB-2 ion-exchange papers were purchased from Reeve Angel, Clifton, NJ, U.S.A.

Cells

The cells used were baby hamster kidney cells-C₁₃ (strain BHK-21/C13) and baby hamster kidney cells transformed by polyoma virus (Py BHK). An asparagine-dependent clone of this C13 strain (Asn⁻) and this clone transformed by hamster sarcoma virus (HSV Asn⁻) were generous gifts from Dr. L. Montagnier, Institut Pasteur, Paris, France. Eagle's medium containing glutamine (as supplied by Eurobio, Paris, France), supplemented with 3% sodium bicarbonate, 10% Tryptose phosphate broth and 10% calf serum was used for all cultures. Roux bottles were incubated at 37°C in a humid atmosphere of air+CO₂ (95:5).

Preparation and partial purification of diamine oxidase

Diamine oxidase was partially purified by a modified method of Bardsley *et al.* (1970). Fresh pig kidney cortex (234 g) was homogenized in 0.1 M-potassium phosphate buffer (pH 7.0) and centrifuged at 12000g for 30 min. The supernatant, which contained most of the diamine oxidase, was heated

at 60°C for 10 min, cooled and re-centrifuged at 12000g. The supernatant was fractionated with (NH₄)₂SO₄. The precipitate formed between 1.2 M- and 2.5 M-(NH₄)₂SO₄ was collected and dialysed against 0.1 M-potassium phosphate buffer (pH 7.0). After concentration, this fraction was applied to a DEAE-cellulose (Whatman, Maidstone, Kent, U.K.) column (50 cm × 1.5 cm) equilibrated with the same buffer. Gradient elution with 0.2–0.5 M-potassium phosphate buffer (pH 7.2) was carried out and the active fractions were pooled. The fractions contained 33% of the original activity and had been purified approx. 400-fold.

Assay of diamine oxidase activity

The reaction mixture contained 0.5 ml of enzyme (2.5 mg of protein), 0.5 ml of putrescine at various concentrations, 0.5 ml of 0.14 M-NaCl and 4 mM-Tris/HCl buffer (pH 7.7). After incubation at 37°C for 1 h the tubes were cooled and the aldehydes determined as described by Bachrach & Reches (1966) by using *N*-methylbenzo-2-thiazolonehydrazine hydrochloride.

The radioactive assay method of Kobayashi (1963) was also used with [1,4- ^{14}C]putrescine as substrate.

Protein was determined by the method of Lowry *et al.* (1951).

Preparation of 2-oxosuccinamic acid

2-Oxosuccinamic acid was prepared from L-asparagine essentially by the method of Meister (1953) by using L-amino acid oxidase and catalase. After removal of the protein with 0.5 M-HClO₄, the supernatant was made alkaline with KOH and the precipitated KClO₄ removed by centrifugation. The supernatant was applied to a column of Dowex 50 (H⁺ form), to remove any unchanged asparagine, and eluted with water. The acid eluate was brought to 0.2 M with respect to NaOH and stored at -20°C to avoid dimerization (Stephani & Meister, 1971). The pH was adjusted to pH 8 with 2 M-HCl just before use.

Preparation of 3-aminopropionamide

3-Aminopropionamide hydrogen sulphate was prepared by hydrolysis of 2-aminopropionitrile as described by Beutel (1964). The free amine was released, when required, by Dowex 50 resin (H⁺ form).

Decarboxylation of [^{14}C]asparagine

Cells were treated with trypsin, washed and suspended in 0.9% NaCl buffered with 0.014 M-sodium phosphate buffer, pH 7.2 (phosphate-buffered saline),

at the concentrations shown. Cells (1 ml) were introduced into Warburg flasks fitted with a side arm containing 1 ml of 2M-citric acid for stopping the reaction. Erlenmeyer flasks (25 ml), fitted with rubber stoppers from which were suspended plastic wells, were also used. In addition to the cells the incubation medium contained 0.25 μ mol of pyridoxal. Flasks were preincubated at 37°C for 15 min before the addition of [U-¹⁴C]asparagine at the concentrations shown. Incubation was terminated either by tipping in the citric acid from the side arm or placing the flasks on ice. Hyamine in the centre wells was transferred to counting vials, the wells were rinsed twice with 0.5 ml of toluene scintillant (4 g of 2,5-diphenyloxazole plus 0.1 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of toluene) and the washings added to the vials. Samples were counted in a Beckman liquid-scintillation counter at an efficiency of 80%. A blank consisting of all the constituents except cells was always run so as to check non-enzymic decarboxylation of the asparagine under the conditions used, and this value was subtracted. All determinations were done in duplicate.

Identification of reaction products

After incubation, mixtures from duplicate flasks were pooled, and to 1 ml samples was added 1 ml of 1M-HClO₄. After 30 min in an ice bath, the tubes were centrifuged at 3000 rev./min for 15 min in a refrigerated International PR₂ centrifuge. The supernatant was neutralized with KOH, the precipitated KClO₄ removed by centrifugation and a 1 ml sample placed on a column (5 cm $\times$ 1 cm) of Dowex 50 (H⁺ form). The columns were eluted with water until no longer acid; the eluate was brought to pH 10 with aq. NH₃ and concentrated by vacuum distillation at room temperature (20°C). The columns were then eluted with 2M-HCl to remove adherent material; the eluate was neutralized and concentrated by vacuum distillation at room temperature.

The separation of material eluted from Dowex 50 (H⁺ form) by 2M-HCl was achieved by descending chromatography on strips of SA-2 paper (Na⁺ form) with 0.01M- or 0.5M-sodium acetate, pH 5.2. The presence of amine-containing substances was revealed by ninhydrin. Radioactivity was detected on duplicate dried strips cut into 1.5 cm segments, which were then placed in counting vials containing toluene scintillant. Samples were counted for radioactivity in a Beckman liquid-scintillation counter.

The neutralized water eluate obtained as described above was subjected to t.l.c. on alkaline cellulose in 98% (v/v) ethanol/25% (w/w) NH₃/water (8:2:1, by vol.). Carbonyl-containing compounds were located by spraying the plates with 2,4-dinitrophenylhydrazine in methanol/HCl followed, after drying,

by methanolic KOH. Carboxylic acids were located by spraying the plates with 0.04% Bromocresol Green in propan-2-ol, adjusted to pH 7 with 1M-NaOH. Radioactivity was determined on 1 cm bands of the dried cellulose support, which were scraped off and introduced into counting vials containing toluene scintillant.

Identification of 2-oxosuccinamic acid was achieved by comparison with authentic 2-oxo[U-¹⁴C]succinamic acid prepared from L-[U-¹⁴C]asparagine by Meister's (1953) procedure and chromatographed on the same plate.

Further identification was carried out by descending chromatography on strips of SB-2 paper (OH⁻ form) in 0.1M-NH₃ as solvent.

Results

3-Aminopropionamide tested as a substrate for diamine oxidase

3-Aminopropionamide is oxidatively deaminated by diamine oxidase to yield an aldehyde as assessed by the formation of a coloured derivative with *N*-methylbenzo-2-thiazolonehydrazine hydrochloride in the presence of FeCl₃. From the double-reciprocal plot in Fig. 1, the *K_m* for 3-aminopropionamide was determined to be 0.9 mM. Since the enzyme did show affinity for 3-aminopropionamide, we investigated whether its presence affects the oxidation of putrescine by diamine oxidase.

Effect of 3-aminopropionamide on putrescine oxidation

With [1,4-¹⁴C]putrescine as substrate, it was found (Fig. 2) that 3-aminopropionamide behaved like a competitive inhibitor with an apparent *K_i* of 437×10^{-3} mM. The *K_m* for putrescine is 5.7×10^{-5} mM. It is thus possible that 3-aminopropionamide, if present in cells, could affect putrescine oxidation *in vivo*. However, as we were unable to detect its presence in Py BHK cells in which decarboxylation of [¹⁴C]asparagine did take place (see below), the derivative 2-oxosuccinamate, for whose presence there was evidence in Py BHK cells as well as in rat liver (Meister, 1965), was investigated for its effect on diamine oxidase.

Effect of 2-oxosuccinamic acid and its decarboxylated derivatives on diamine oxidase activity

Experiments were carried out to investigate the effect of various concentrations of 2-oxosuccinamate on putrescine oxidation. Maximum activation of 55.7% occurred with 37.0 mM-2-oxosuccinamate (Table 1). The oxidative decarboxylation of 2-oxo acids to the corresponding carboxylic acids by H₂O₂ has been reported (Bunton, 1949; Rowsell *et al.*, 1972) and for 2-oxosuccinamic acid, in particular,

Fig. 1. Double-reciprocal plot of $1/[S]$ against $1/v$ for diamine oxidase of pig kidney, with 3-aminopropionamide as the substrate

Incubations (2.0ml) contained 0.5ml of a suitably diluted enzyme in 4mM-Tris/HCl buffer (pH 7.7)/35mM-NaCl. After incubation at 37°C for 1h, the aldehyde formed was determined by the method of Bachrach & Reches (1966).

Fig. 2. Double-reciprocal plot illustrating inhibition of the oxidation of putrescine by 3-aminopropionamide

Enzyme was incubated with $[1,4-^{14}\text{C}]$ putrescine with (○) and without (●) 1.25mM-3-aminopropionamide.

by Meister (1953). Since H_2O_2 is formed during the oxidation of putrescine by diamine oxidase, the activation observed in these experiments could have been due to 2-oxosuccinamate itself and/or malonamic acid formed by the action on 2-oxosuccinamate of this H_2O_2 .

The effect of H_2O_2 on the 2-oxosuccinamate-mediated activation of diamine oxidase was investigated by adding it to the assay mixture at twice the concentration of 2-oxosuccinamate. As shown (Table 1), H_2O_2 alone had no measurable effect, but in the presence of 2-oxosuccinamate brought about a further stimulation of diamine oxidase activity. Verification of the products formed by the action of H_2O_2 on 2-oxosuccinamate showed that malonamate was the other product formed (Fig. 6c). We therefore investigated the effect of malonamic acid on diamine oxidase activity and to determine the specificity of activation included other amides and carbonyl compounds structurally related to 2-oxosuccinamate.

Effect of amides and 2-oxo acids on diamine oxidase activity

Table 2 shows that apart from 2-oxosuccinamic acid and its decarboxylated derivative malonamic

acid, none of the amides listed has any effect on diamine oxidase. Thus the activation observed is specific and of the same order of magnitude (30%) as both malonamate and 2-oxosuccinamate. The increased activation that takes place with 2-oxosuccinamate in the presence of H_2O_2 cannot be attributed to malonamate and for the moment remains unexplained.

2-Oxosuccinamate can also be deamidated by ω -amidase (EC 3.5.1.3) to oxaloacetate (Meister, 1965). When the effect of this compound on diamine oxidase activity was investigated, 78% inhibition was observed (Table 2). However, as oxaloacetate is unstable and is rapidly decarboxylated both enzymically (Dean & Bartley, 1973) and spontaneously (Bessman & Layne, 1950) to pyruvate, the effect of a similar concentration of pyruvate on diamine oxidase activity was determined. With this compound an even greater inhibition (94%) was obtained (Table 2). Thus of the possible intermediates of asparagine metabolism tested here, the amidated derivatives 2-oxosuccinamate and malonamate are activators, whereas 3-aminopropionamide and the deamidated metabolites are inhibitors of

Table 1. *Effect of 2-oxosuccinamate on diamine oxidase activity in the presence and absence of H₂O₂*

To 0.5 ml of enzyme (150 μ g of protein) in screw-cap culture tubes was added 0.3 μ Ci of [1,4-¹⁴C]putrescine in 0.5 ml, and the volume brought to 2 ml with 0.1 M-potassium phosphate buffer (pH 7.0). This series served as the control. Then 0.2 ml each of 2-oxosuccinamate and/or H₂O₂ (final concentrations as stated), were added to the same amount of enzyme and substrate as in the control tubes above, and the final volume was adjusted to 2 ml with buffer. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 200 mg of NaHCO₃, and the reaction product extracted with toluene scintillant as described by Kobayashi (1963). For the blanks, enzyme was added after the reaction was stopped with NaHCO₃. All assays were done in triplicate.

2-Oxosuccinamate (final concentration, mM)	2.9	5.8	37.0
% activation	31.7	46.6	55.0
% activation in the presence of H ₂ O ₂ at twice 2-oxosuccinamate concentration	57.0	63.3	78.0
% activation with H ₂ O ₂ alone in incubation at twice 2-oxosuccinamate concentration	0	0	0

Table 2. *Effect of various amines, amides and oxo acids on diamine oxidase activity*

Diamine oxidase activity was measured as described for Table 1. —, No activation or inhibition.

Substances added at final concentration of 2.9 mM	Inhibition (%)	Activation (%)
2-Oxosuccinamate	—	31.7
Malonamate	—	32
Oxaloacetate	78.5	—
Pyruvate	94.4	—
Propionamide	—	—
Succinamate	—	—
Succinamide	—	—
Spermine	—	—
Spermidine	—	—

diamine oxidase activity *in vitro*. No structure-activity relationship is immediately apparent.

This list of possible amidated and deamidated derivatives of asparagine is by no means exhaustive. However, before examining the effect of others and those which could be derived from, e.g. glutamine, we investigated whether any of the metabolites tested are produced *in vivo*. Further, as the effective concentration of activators is relatively high (2.9 mM), the question was also raised whether the activation observed is of physiological significance.

Accordingly we incubated BHK cells grown in tissue culture with [U-¹⁴C]asparagine and measured ¹⁴CO₂ evolution, as well as the production of 2-oxo[¹⁴C]succinamate in the presence or absence of unlabelled intermediates.

Metabolism of [U-¹⁴C]asparagine by Py BHK cells

Py BHK cells suspended in phosphate-buffered saline (2 $\times$ 10⁷ cells/ml), were incubated in Warburg flasks with 0.9 μ Ci of [U-¹⁴C]asparagine (3 μ Ci/ μ mol). The flasks contained in addition 0.25 μ mol of pyridoxal in a total volume of 1.25 ml. The reaction

Fig. 3. ¹⁴CO₂ production from [U-¹⁴C]asparagine by Py BHK cells as a function of incubation time

Some 0.9 μ Ci (3 μ Ci/ μ mol) was added per flask. Cells (2 $\times$ 10⁷) were suspended in 1.25 ml of phosphate-buffered saline containing 0.25 μ mol of pyridoxal. The reaction was stopped at the times indicated by placing the flasks in an ice bath. Other conditions were as in the text.

was stopped at intervals of 15, 30, 60 and 120 min. The results obtained are shown in Fig. 3. There appears to be a slight lag in ¹⁴CO₂ production for the first 15 min, and at 120 min the rate of ¹⁴CO₂ production appears to have diminished from the maximum. In the light of these results, cells were first preincubated at 37°C for 15 min before the addition of [U-¹⁴C]asparagine, and the reaction was allowed to proceed for 30 or 60 min.

Fig. 4. ${}^{14}\text{CO}_2$ production from $[\text{U}-{}^{14}\text{C}]$ asparagine by (●) Py BHK cells and (○) BHK cells as a function of cell concentration

Some $0.1 \mu\text{Ci}$ ($0.1 \mu\text{Ci}/\mu\text{mol}$) was added per flask. Cells were suspended in 1.25 ml containing $0.25 \mu\text{mol}$ of pyridoxal. The reaction was stopped by tipping in 1.0 ml of 2 M -citric acid from the side arm.

The variation in ${}^{14}\text{CO}_2$ production with cell number was investigated for both normal BHK and Py BHK cells at concentrations varying from 8×10^5 to 4×10^7 cells/ml. Incubation was carried out for 30 min after adding $0.1 \mu\text{Ci}$ of $[\text{U}-{}^{14}\text{C}]$ -asparagine. The results obtained are shown in Fig. 4. It is clear that, whereas there is a linear relationship between ${}^{14}\text{CO}_2$ production and cell number for Py BHK cells, no such relationship holds for normal BHK cells. In fact at cell concentrations below 5×10^6 , ${}^{14}\text{CO}_2$ production by normal BHK cells was negligible. Further, even at cell concentrations (10^7) at which ${}^{14}\text{CO}_2$ production did take place, the decarboxylation of $[\text{U}-{}^{14}\text{C}]$ asparagine by Py BHK cells was fourfold that observed with normal BHK cells.

To determine the immediate precursor of this ${}^{14}\text{CO}_2$ with Py BHK cells, we undertook the identification of labelled metabolites in the total incubation mixture after disrupting the cells by two successive cycles of freezing and thawing. After elimination of high-molecular-weight constituents by HClO_4 , the supernatant was treated as described in the Materials and Methods section.

Fig. 5. Chromatography of products of $[\text{U}-{}^{14}\text{C}]$ asparagine metabolism after elution from Dowex 50 (H^+ form) by HCl

Py BHK cells were incubated for 1 h at 37°C with $0.9 \mu\text{Ci}$ of $[\text{U}-{}^{14}\text{C}]$ asparagine ($3 \mu\text{Ci}/\mu\text{mol}$). The fraction obtained by elution in 2 M - HCl after applying an extract to Dowex 50 (H^+ form) was chromatographed on SA-2 paper (Na^+ form) with 0.01 M -sodium acetate, $\text{pH} 5.2$. Dried strips were cut into 1.5 cm segments for radioactivity determination. Unlabelled marker compounds were chromatographed on the same paper to locate running positions. See the Materials and Methods section for further detail.

Identification of reaction products

Metabolites were eluted from Dowex 50 (H^+ form) with 2 M - HCl and separated by chromatography on SA-2 paper (Na^+ form). The major radioactive peak was associated with compounds moving in the region of asparagine and aspartic acid. There was no radioactivity in the region of the chromatogram corresponding to 3-aminopropionamide. In the event that the amide group may have been hydrolysed by the acid conditions used during the isolation procedure, we looked for the presence of labelled β -alanine: this was again by chromatography on SA-2 paper (Na^+ form), but using 0.01 M -sodium acetate as solvent (Fig. 5). The absence of any radioactivity from β -alanine and 3-aminopropionamide led us to conclude that no direct decarboxylation of asparagine was taking place.

Fig. 6. T.l.c. of products of $[U-^{14}C]$ asparagine metabolism not retained on Dowex 50 (H^+ form)

(a) The fraction of the extract not retained on the Dowex column, (b) 2- $[U-^{14}C]$ oxosuccinamate and (c) 2- $[U-^{14}C]$ oxosuccinamate pretreated with H_2O_2 were subjected to t.l.c. on alkaline cellulose in 98% (v/v) ethanol/aq. 25% (w/w) NH_3 /water (8:2:1, by vol.). Dried plates were cut into 1 cm bands, and cellulose was scraped off for radioactivity determination. See the legend to Fig. 5 and the Materials and Methods section for further detail.

The separation of the products not retained on Dowex 50 (H^+ form) was therefore undertaken by t.l.c. on alkaline cellulose in 98% ethanol/25% (w/w) NH_3 /water (8:2:1, by vol). The position of authentic 2-oxosuccinamate was revealed by spraying the dried plates with 2,4-dinitrophenylhydrazine followed, after drying, by methanolic KOH. 2- $[U-^{14}C]$ Oxosuccinamate was also prepared and used as a marker. Figs. 6(a) and 6(b) show respectively the migration of the eluate from Dowex 50 and that of authentic 2-oxosuccinamate under the conditions described. Radioactivity was associated with 2-

oxosuccinamate and with another unidentified compound which was not malonamate (Fig. 6c). Additional evidence for the presence of 2-oxosuccinamate in the water eluate of Dowex 50 (H^+ form) was obtained by descending chromatography on SB-2 paper (OH^- form) with 0.1M- NH_3 as solvent. Radioactivity was again present in a spot having the same migratory characteristics as authentic 2-oxosuccinamate.

Inhibition studies on $[U-^{14}C]$ asparagine decarboxylation

With the identification of 2-oxosuccinamate in the reaction mixture of Py BHK cells after asparagine decarboxylation, we tried to assess the importance of this deamination pathway relative to that well defined for the deamidation of asparagine to aspartic acid. We therefore undertook a series of experiments to measure the decarboxylation of $[U-^{14}C]$ asparagine in the presence of an excess of unlabelled 2-oxosuccinamate and unlabelled aspartate.

These experiments were performed on an asparagine-requiring mutant of BHK-21/C13 (Asn^-) and this same mutant transformed by hamster sarcoma virus (HSV Asn^-). The use of Asn^- and HSV Asn^- cells, whose characteristics have been previously described (Montagnier *et al.*, 1971), had three advantages.

As the cells were transformed by hamster sarcoma virus instead of polyoma virus, if an increase in $^{14}CO_2$ were again observed in the transformed as compared with the normal cells, it would be additional evidence for linking this observation to viral infection and/or transformation, rather than to the virus used. On the other hand, if there was no difference in $^{14}CO_2$ production between HSV Asn^- and Asn^- cells, it would indicate that this metabolic pathway is characteristic of asparagine metabolism in general. Moreover, in these cells no conversion of aspartate into asparagine can occur (Montagnier *et al.*, 1971), and by comparing the dilution of the radioactivity of the $^{14}CO_2$ evolved in the presence of 2-oxosuccinamate and aspartate, it should be possible to obtain evidence bearing on the identity of the immediate precursor(s) of the $^{14}CO_2$ in the normal and transformed cells.

The results presented in Table 3 show that in the presence of a 500-fold excess of 2-oxosuccinamate (1.2 μ mol), there is about a 50% decrease in the amount of $^{14}CO_2$ produced by both Asn^- and HSV Asn^- cells. At a concentration of aspartate (1.2 μ mol) similar to that of 2-oxosuccinamate, there is almost total dilution of the $^{14}CO_2$ evolved by both Asn^- (86%) and HSV Asn^- (99%) cells. HSV Asn^- cells also decarboxylate $[^{14}C]$ asparagine more efficiently than do Asn^- cells (twofold).

Thus increased $^{14}CO_2$ production from asparagine

Table 3. Decarboxylation of [$U\text{-}^{14}\text{C}$]asparagine by BHK cells in the presence of 2-oxosuccinamate or aspartate

Cells suspended in phosphate-buffered saline (2.0 ml) at the concentrations shown were incubated for 60 min at 37°C with [$U\text{-}^{14}\text{C}$]asparagine. For Asn⁻ and HSV Asn⁻ cells, 0.25 μCi (100 $\mu\text{Ci}/\mu\text{mol}$) was added. For BHK and Py BHK cells, 0.10 μCi (10 $\mu\text{Ci}/\mu\text{mol}$) was used. In addition the flasks contained 0.1 μmol of pyridoxal and 1.2 μmol of 2-oxosuccinamate or aspartate as shown. Controls consisted of all the constituents except cells and these values were subtracted.

Cell type	No. of cells	Presence of		$^{14}\text{CO}_2$ (c.p.m.)	% inhibition
		2-Oxo-succinamate	Aspartate		
Asn ⁻	10 ⁷	—	—	10447	
Asn ⁻	10 ⁷	+	—	4680	55
HSV Asn ⁻	10 ⁷	—	—	20726	
HSV Asn ⁻	10 ⁷	+	—	11558	44
Asn ⁻	10 ⁷	—	—	10447	
Asn ⁻	10 ⁷	—	+	1482	86
HSV Asn ⁻	10 ⁷	—	—	20726	
HSV Asn ⁻	10 ⁷	—	+	39	99
BHK	10 ⁷	—	—	807	
BHK	10 ⁷	—	+	95	88
Py BHK	2 × 10 ⁶	—	—	1187	
Py BHK	2 × 10 ⁶	—	+	1452	0

Table 4. Decarboxylation of 2-oxo[$U\text{-}^{14}\text{C}$]succinamate by liver cells

$^{14}\text{CO}_2$ production from 2-oxo[$U\text{-}^{14}\text{C}$]succinamate (40000 c.p.m.; 3.4 μmol) by rat liver cells incubated for 1 h at the temperature indicated is shown. Incubation buffer: 0.14 M-NaCl, 0.1 M-sucrose, 0.016 M-sodium succinate, 0.02 M-Tris/HCl, pH 7.6. Total volume was 2 ml.

	No. of cells	Temperature of incubation (°C)	$^{14}\text{CO}_2$ (c.p.m.)
Experimental	10 ⁶	37	1043
Control	10 ⁶	4	206
Control	—	37	285

does appear to be linked to viral infection and/or transformation, but in view of the results obtained with aspartate, the immediate precursor remains unidentified.

However, this total dilution of radioactivity by aspartate in Asn⁻ and HSV Asn⁻ cells was not observed in Py BHK cells. As shown in Table 3, aspartate almost completely diluted the $^{14}\text{CO}_2$ arising from [^{14}C]asparagine in normal BHK cells, whereas in Py BHK cells, not only was there no dilution, but a further increase (22%) in $^{14}\text{CO}_2$ production took place. Asparagine decarboxylation in Py BHK cells is thus qualitatively different from that in normal BHK cells and 2-oxosuccinamate is the most likely direct precursor of this $^{14}\text{CO}_2$ in Py BHK cells.

As the results reported so far were obtained with cells growing in tissue culture, we tried to determine whether this pathway, involving 2-oxosuccinamate

production and decarboxylation, also took place *in vivo*. To do this we measured the decarboxylation of 2-oxo[$U\text{-}^{14}\text{C}$]succinamate in rat liver cells isolated by perfusion with Ca^{2+} -free Locke's solution at 37°C followed by gentle mechanical dispersion. Table 4 shows that uptake and decarboxylation has taken place.

Discussion

The results presented here show that diamine oxidase activity can be affected *in vitro* by metabolites of naturally occurring amino acids: 2-oxosuccinamate derived from asparagine by transamination was an activator; oxaloacetate, which can be formed from aspartate also by transamination or from 2-oxosuccinamate by enzymic deamination (Meister, 1965), was an inhibitor; pyruvate, the decarboxylated product of oxaloacetate, was also inhibitory.

Oxaloacetate and pyruvate production from glucose and amino acid metabolism is well documented in eukaryotic cells *in vivo*, but only rarely has mention been made of 2-oxosuccinamate. We have presented evidence from t.l.c. and inhibition studies that 2-oxosuccinamate is an intermediate of asparagine catabolism and is decarboxylated in BHK cells growing in tissue culture as well as in rat liver cells. Further, we have observed an increase in $^{14}\text{CO}_2$ production from [$U\text{-}^{14}\text{C}$]asparagine in BHK cells transformed by both hamster sarcoma virus and polyoma virus, as compared with their normal counterparts.

The immediate precursor of the $^{14}\text{CO}_2$ in transformed BHK cells is different, depending on whether the cells are asparagine-dependent or not. In Py

BHK cells there is no dilution of the radioactivity of $^{14}\text{CO}_2$ in the presence of aspartate. This observation plus the identification of 2-oxo[^{14}C]succinamate in the incubation mixture makes 2-oxosuccinamate the most likely immediate precursor. However, confirmation must await the identification of the decarboxylated product. Nevertheless, for Py BHK cells the small increase in the $^{14}\text{CO}_2$ evolved from [U- ^{14}C]asparagine in the presence of aspartate also raises the possibility that aspartate itself or a metabolite thereof is involved in controlling the activity of the enzyme(s) responsible for 2-oxosuccinamate decarboxylation. This possibility will only be evaluated when the enzymes involved have been isolated.

In Asn⁻ and HSV Asn⁻ cells approx. 50% dilution occurs with 2-oxosuccinamate and total dilution with aspartate. Since the requirement for asparagine by these mutants cannot be met by aspartic acid (Montagnier *et al.*, 1971), we can eliminate the possibility of conversion of the unlabelled aspartic acid added into asparagine. There are two possible reasons for the dilution observed: (a) dilution of precursor pools and (b) action of the unlabelled precursor on the enzymes involved. Considering (a), the only intermediate common to 2-oxosuccinamate and aspartate is oxaloacetate, which can be produced from aspartate by transamination and from 2-oxosuccinamate by deamidation. But oxaloacetate is itself unstable and can be decarboxylated both spontaneously (Bessman & Layne, 1950) and enzymically (Dean & Bartley, 1973) to pyruvate; the increase in $^{14}\text{CO}_2$ observed after viral transformation of Asn⁻ cells could therefore be due to increased activity of ω -amidase, yielding oxaloacetate which is then decarboxylated. Attempts to obtain evidence for this pathway by using unlabelled oxaloacetate did not give meaningful results owing to its inherent instability and the problem of its transport across cell membranes. The use of more stable precursors of oxaloacetate should resolve this problem. Possibility (b) cannot be ignored; its relevance here, as in Py BHK cells, will only be determined when the enzymic system(s) involved in this decarboxylation have been assayed *in vitro*.

However, if the detailed mechanism(s) of asparagine decarboxylation remains to be elucidated, its increased decarboxylation in cells infected with transforming viruses as compared with normal cells is clearly established, and in Py BHK cells, at least, our evidence favours decarboxylation via 2-oxosuccinamate rather than via aspartate. Is this difference in [U- ^{14}C]asparagine decarboxylation a further feature of transformed cells or is it linked to viral infection itself? A systematic study of [U- ^{14}C]asparagine decarboxylation by cells infected with transforming and non-transforming viruses should provide an answer.

Regardless of this relationship, the question has to

be asked whether the observed activation of diamine oxidase by 2-oxosuccinamate is of importance *in vivo*? From the present results it would seem that this effect is of limited physiological significance: a relatively high concentration (2.90 mM) is needed to produce a comparatively small activation (30–50%) of putrescine oxidation. Its real physiological role may be as an allosteric effector of the enzyme for substrates other than putrescine. It is well documented that diamine oxidase is capable of oxidatively deaminating a wide variety of substrates (see the introduction). Further, spermine and spermidine, which do not affect the activity of partially purified diamine oxidase from pig kidney (see Table 2, and Bardsley & Ashford, 1972), are themselves oxidized to iminoaldehydes by the pure human seminal plasma enzyme (Hölttä *et al.*, 1975). Though this difference in reactivity towards spermine and spermidine could be due to a species difference, it could also be due to differences in the degree of purification of the enzyme and the presence or absence of allosteric effectors. This hypothesis remains to be verified.

On the other hand, the diamine oxidase-inhibition studies reported here could have physiological significance. Almost total inhibition (94%) occurs with pyruvate, which can be derived from 2-oxosuccinamate via oxaloacetate, and increased glycolysis resulting in elevated pyruvate and lactate are characteristic of some types of malignant cells (Weinhouse, 1972). Moreover, increases in putrescine and spermidine have been observed in all dividing cells (Raina *et al.*, 1966), but increases in putrescine alone are characteristic of transformation by temperature-sensitive mutants of Rous sarcoma virus at the permissive temperature (Don & Bachrach, 1975). It thus seems reasonable to suggest that the modulation of intracellular diamine oxidase activity by some of the inhibitors (e.g. pyruvate) and activators (e.g. 2-oxosuccinamate) here described may be a link between these observations.

The identification of the labelled decarboxylated product(s) in HSV Asn⁻ cells would provide direct evidence for the deamidation and decarboxylation of 2-oxosuccinamate to pyruvate and/or lactate.

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