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DIAMINE OXIDASE ACTIVITY IN HUMAN MELANOMA CELL LINES WITH DIFFERENT TUMORIGENICITY IN NUDE MICE

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Summary.—The activity of diamine oxidase (DO, EC 1.4.3.6.) which converts putrescine into γ-aminobutyraldehyde in the degradative pathway of polyamine, was studied in 4 human melanoma cell lines, 2 of which produce tumours in >80% of nude mice (M_3Dau, M_4Beu), whereas the other 2 induce tumours in <25% (M_1Dor, M_2GeB). The activity of DO in these cells varies with the growth rate: 24 h after seeding there is an initial increase in DO activity, followed by a steep decline during exponential growth. At 96 h, when cells reach saturation density, the activity of DO is significantly greater in the highly tumorigenic cell lines than in the poorly tumorigenic cell lines. Kinetic studies show that for the highly tumorigenic lines apparent K_m values are $10{\cdot}6 \times 10^{-6}$M $\pm 0{\cdot}2$ (M_3Dau) and $14{\cdot}2 \times 10^{-6}$M $\pm 0{\cdot}6$ (M_4Beu), whereas for the poorly tumorigenic lines the values are $4{\cdot}5 \times 10^{-6}$M $\pm 0{\cdot}3$. After transplantation into nude mice, the M_1Dor cell line, which exhibits a low K_m (app.) for DO yielded tumour cells the DO of which had high K_m (app.) value. K_m (app.) determination of DO could be an approach for characterizing human melanoma cells differing in their tumorigenic potential in nude mice.

THE LEVELS OF POLYAMINES and the activities of the enzymes involved in their biosynthesis increase during the early growth phase of normal cells cultivated *in vitro* (Russell & Snyder, 1968; Jänne *et al.*, 1978). Similarly, increases in the level of putrescine after the stimulation of ornithine decarboxylase have also been seen in cells either treated by carcinogens such as dimethylaminoazobenzene (Scalabrino *et al.*, 1978) and tumour-promoting agents (O'Brien, 1976) or infected by oncogenic viruses (Don & Bachrach, 1975). Moreover, other enzymic activities such as those of S-adenosyl methionine decarboxylase, spermidine or spermine synthetase are enhanced in neoplastic cells (Kallio *et al.*, 1977). However, the level of putrescine in cells also depends on the activity of enzymes belonging to the catabolic pathway, such as polyamine oxidase and diamine oxidase (DO). Quash *et al.* (1979) have shown that the activity of DO (EC 1.4.3.6.), the enzyme which oxidatively deaminates putrescine to γ-aminobutyraldehyde, varies with the growth phase of rat kidney cells in tissue culture. This variation has been found for both the parent cell line and its virally transformed counterpart. At 24 h after seeding, the increase in DO activity is greater in transformed than in normal cells. However, 96 h after seeding, the activity is greater in normal than in virally transformed cells.

This fluctuation in DO with the growth phase may provide an explanation for apparently conflicting results: an increase in DO activity was found in endocrine tumour biopsy specimens, such as medullary thyroid carcinoma (Baylin *et al.*, 1972) and ovarian cancer (Lin *et al.*, 1975),

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when compared to corresponding normal tissue; conversely, in tumours of the stomach or intestinal tract, the reverse findings have been made by other authors (Quash *et al.*, 1979; Kusche *et al.*, 1980).

From these observations it seemed that there might well be a relationship between DO activity and malignancy, but that its nature was unclear. To investigate further the relationship between DO activity and the expression of malignancy, we have studied variations in DO activity in 4 human malignant-melanoma cell lines differing in several characteristics, including heterotransplantability into athymic nude mice. The ability of human tumours or human tumour cell lines to grow in nude mice has been shown to be one of the criteria of malignancy, and, at least for heterotransplantable tumours such as melanomas, to reflect the invasive potentiabilities of the tumour cells (Stiles *et al.*, 1976; Giovanella *et al.*, 1976).

In these cell lines, DO activity was studied as a function of *in vitro* cell growth and the kinetic properties of the enzyme from the different lines were compared.

MATERIAL AND METHODS

Reagents.—[1,4-^{14}C] Putrescine dihydrochloride (sp. act. 122 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks.

Putrescine dihydrochloride and diamine oxidase were obtained from Sigma Chemical Co., St Louis, Mo, U.S.A.

Cells.—Four cell strains were initiated from metastatic human malignant melanoma. The characteristic of these strains have already been described (Jacubovich & Doré, 1979). Cells were maintained as monolayers in RPMI 1629 medium supplemented with 10% fetal calf serum (FCS) from Gibco Laboratories, Detroit, Mi, U.S.A., 100 u/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamicin and 2mM glutamine from Gibco Laboratories. For growing cells for enzymic assays, $2{\cdot}5 \times 10^6$ cells were seeded in 100mm plastic dishes in the presence of 10 ml of medium without antibiotics, and incubated at 37°C in a humid atmosphere of 5% CO_2 in air. All the human melanoma cell lines used in these studies were between the 30th and 50th passages.

The normal rat kidney cells and normal rat kidney cells transformed by avian sarcoma virus (B77 strains) were grown as previously described (Quash *et al.*, 1979) in Eagle's minimum essential medium (MEM) containing glutamine (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% tryptose phosphate broth (from Difco Laboratories, Detroit, Mi, U.S.A.), and 10% FCS.

Growth of melanoma lines in the nude mouse.—Athymic, 4–6-week-old, male nude mice (*nu/nu*) from Iffa-Credo (Les Oncins 69210 L'Arbresle, France) were inoculated s.c. into the anterior lateral thoracic wall, with viable cells (2×10^6) suspended, after trypsination, in 0·1 ml of phosphate-buffered saline. Four melanoma cell lines were used: 2 of them (M_1Dor and M_2GeB) are poorly tumorigenic (*i.e.*, $<25\%$ of the athymic nude mice develop tumours within 40 days of inoculation); the other 2 (M_3Dau and M_4Beu) are highly tumorigenic, 90–100% of the mice developing tumours within 40 days.

Preparation of homogenates from cells in tissue culture.—The cell layer was washed twice with cold PBS containing 0·14M NaCl and 0·014M sodium phosphate buffer (pH 7·2), after which the cells were scraped off with a rubber-covered rod, washed once and centrifuged at 600 *g* for 5 min. The cell pellet was stored at −70°C until use. Cells were disrupted by sonication in a Branson Sonifier for 2 sec at output 2 with a microprobe, and finally suspended in 0·10M sodium phosphate buffer (pH 7·0) for the assay of DO activity.

Assay of DO activity.—To 0·3 ml of cell homogenate containing various amounts of protein in screw-cap culture tubes was added 0·1 μCi of [1,4-^{14}C] putrescine ($0{\cdot}75–50 \times 10^{-6}$M). Chloral hydrate at 10^{-2}M was used to inhibit aldehyde dehydrogenase (Andersson *et al.*, 1979). The volume was brought to 2·0 ml with 0·1M sodium phosphate buffer (pH 7·0).

After incubation at 37°C for 2 h the reaction was stopped by the addition of 0·2 ml of aminoguanidine bicarbonate (10 mM) in 2% Na_2CO_3, and the reaction product extracted with 10 ml of toluene scintillant (5 g of 2,5-diphenyloxazole plus 0·1 g of 1,4 bis (4-methyl-5-phenyloxazol-2-yl) benzene in 1 l of toluene, as described Kobayashi, 1963). Control tubes contained the same consti-

tuents, except the cell homogenate, which was replaced by the phosphate buffer. Control values were substracted for each determination.

Samples were counted on an Intertechnique liquid scintillation spectrometer SL30, at an efficiency of 80%. Enzyme activity, expressed as units, refers to pmol of Δ^1-pyrroline formed/h/0·1 mg of protein. All determinations were in duplicate, and all duplicates agreed within 6%.

All figures for enzyme kinetics expressed as 1/V *vs* 1/S were drawn by computer, using a linear-regression programme.

Protein determination.—The protein content of the homogenates was determined by the method of Lowry, with bovine serum albumin as standard.

RESULTS

Before undertaking a systematic examination of DO activity in the different melanoma cell lines, we first verified the linearity of the enzymic reaction as a function of time and of cellular protein added. It was found that activity was linear up to 2·5 h (Fig. 1a) and for increasing quantities of cell homogenate up to 400 μg (Fig. 1b). In all further assays incubations were carried out with 300 μg protein for 2 h. However, it has been shown by Andersson *et al.* (1979) and Sessa *et al.* (1981) that γ-aminobutyraldehyde, the immediate product of putrescine oxidation by DO, can be oxidized to γ-aminobutyric acid by the action of aldehyde dehydrogenase, and that this oxidation can be inhibited by chloral hydrate. As a precautionary measure, chloral hydrate at 10^{-2}M was therefore added routinely to all determinations, to ensure that any γ-aminobutyraldehyde produced would not be lost *via* this oxidative pathway. In addition we verified that at the concentration used there was no inhibition of DO (Fig. 1b).

DO as a function of human melanoma cell growth

Cells were seeded and harvested at 24 h intervals as described in the Material and

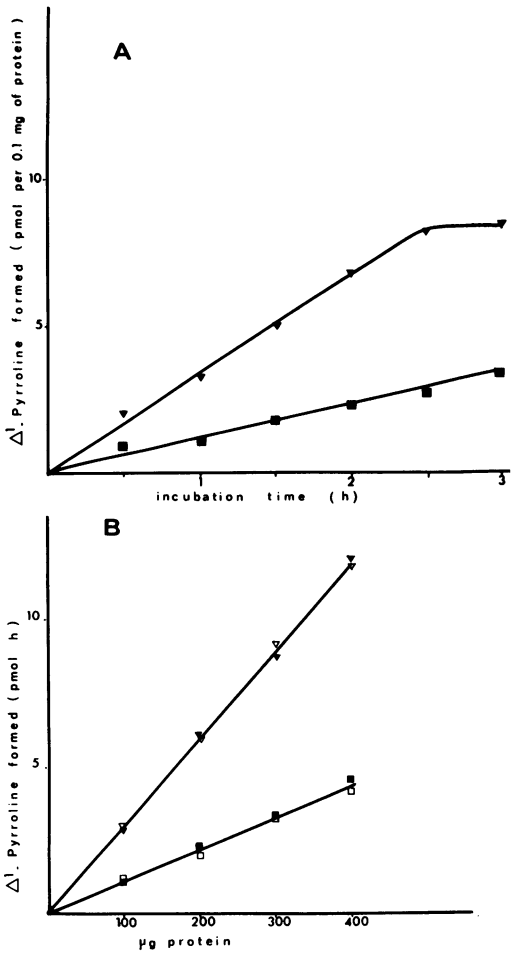

FIG. 1—(A) Diamine oxidase (DO) activity in homogenate from melanoma cell lines as a function of time. (B) Effect of chloral hydrate on Δ^1-[^{14}C] pyrroline formation from [1·4-^{14}C] ($2{\cdot}5 \times 10^{-6}$M) putrescine in homogenates from melanoma cell lines. Symbols: ▽, M_3 Dau + 10^{-2}M chloral hydrate; ▲, M_3 Dau − chloral hydrate; □ M_1 Dor + 10^{-2}M chloral hydrate; ■, M_1Dor − chloral hydrate.

Methods section. They were pelleted and stored at −70°C until use. Fig. 2 shows that in all 4 cell lines there are increases in DO activity 24 h after seeding, followed by a progressive decrease up to 96 h, when the cells approach confluence. As there was no further increase in protein content per plate at 120 h, cells were harvested at 96 h. At this time, the residual activity of DO in the highly tumorigenic lines M_3Dau

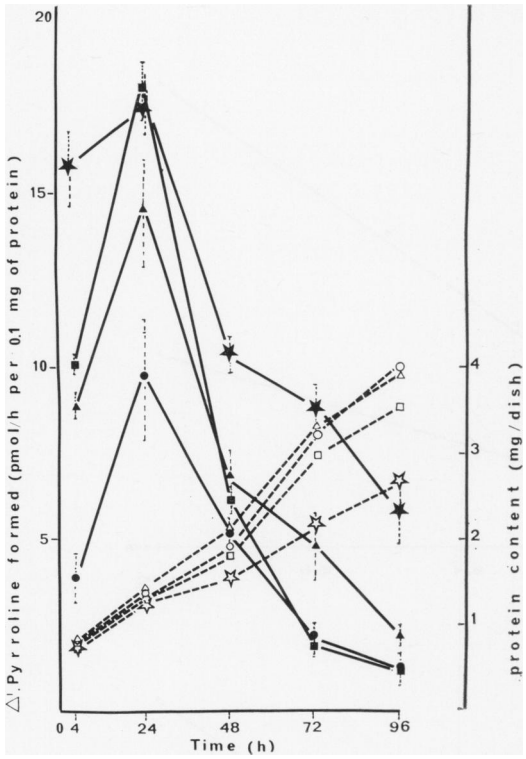

FIG. 2.—DO activity as a function of cell growth. Δ^1-[^{14}C] Pyrroline formation from [1·4-^{14}C] putrescine ($2{\cdot}5 \times 10^{-6}$M) by 0·1 mg of homogenate from 4 human melanoma cell lines at different times after seeding. Four separate cultures for each cell line were investigated, and each point represents mean ±s.e. Symbols: ●, ○, M_2GeB; ■, □, M_1Dor; ▲, △, M_4Beu; ★, ☆, M_3 Dau. ———, Δ^1-Pyrroline formed; – – –, protein content/dish.

and M_4Beu is significantly greater than in the less tumorigenic ones M_1Dor and M_2GeB (Table). The variation in enzymic activity with growth confirms our previous results with both normal and transformed rat kidney cells. However, the increase in DO activity seen in the highly tumorigenic melanoma cell lines contradicts the decrease previously reported for the transformed compared to the normal rat kidney cells. Though the differences in DO activity between the highly tumorigenic and less tumorigenic cell lines are statistically significant ($P \leqslant 0{\cdot}01$), it was clear from the results shown in Fig. 2 that minor modifications in the growth rate could influence the enzyme activity at confluence. We therefore attempted to determine whether kinetic properties such as the K_m and the V_{max} of DO from confluent cells of the same cell line, but with different growth rates, were constant.

Kinetic studies

Fig. 3 shows that the V_{max} of DO from cells harvested at confluence from different subcultures of the same cell line is different, but the K_m remains constant. It must be stated straight away that the values for the kinetic parameters refer to the apparent (app.) K_m and V_{max} since measurements were not made on the pure enzyme on account of difficulties in obtaining pure enzyme in sufficient quantity from cells in culture. Nevertheless free intracellular putrescine does not alter the K_m (app.) values, since no differences in K_m of DO were found when dialysed or undialysed homogenates were used. These results indicate that the value of K_m (app.) could be a more reliable criterion for comparing DO from cells with different tumorigenic potential in nude mice.

Accordingly, the K_m (app.) was determined for the 4 melanoma lines, with putrescine concentrations ranging from 1·25 to 50×10^{-6}M in the presence of

TABLE.—*Determination of diamine oxidase activity of 4 melanoma cell lines at confluence*

Athymic nude mice developing tumours (%)	Cell lines	Δ^1-Pyrroline formed (pmol/h/0·1 mg protein)
100	M_3Dau	3·56 ± 0·60 (17) + +
88	M_4Beu	2·36 ± 0·23 (23) +
26	M_1Dor	1·43 ± 0·27 (11)
25	M_2GeB	1·53 ± 0·20 (21)

Significance of excess over M_1Dor and M_2GeB by t test + + $P < 0{\cdot}001$; + $P < 0{\cdot}01$.
The numbers of cultures are shown in parentheses. Mean values ±s.e. are given.

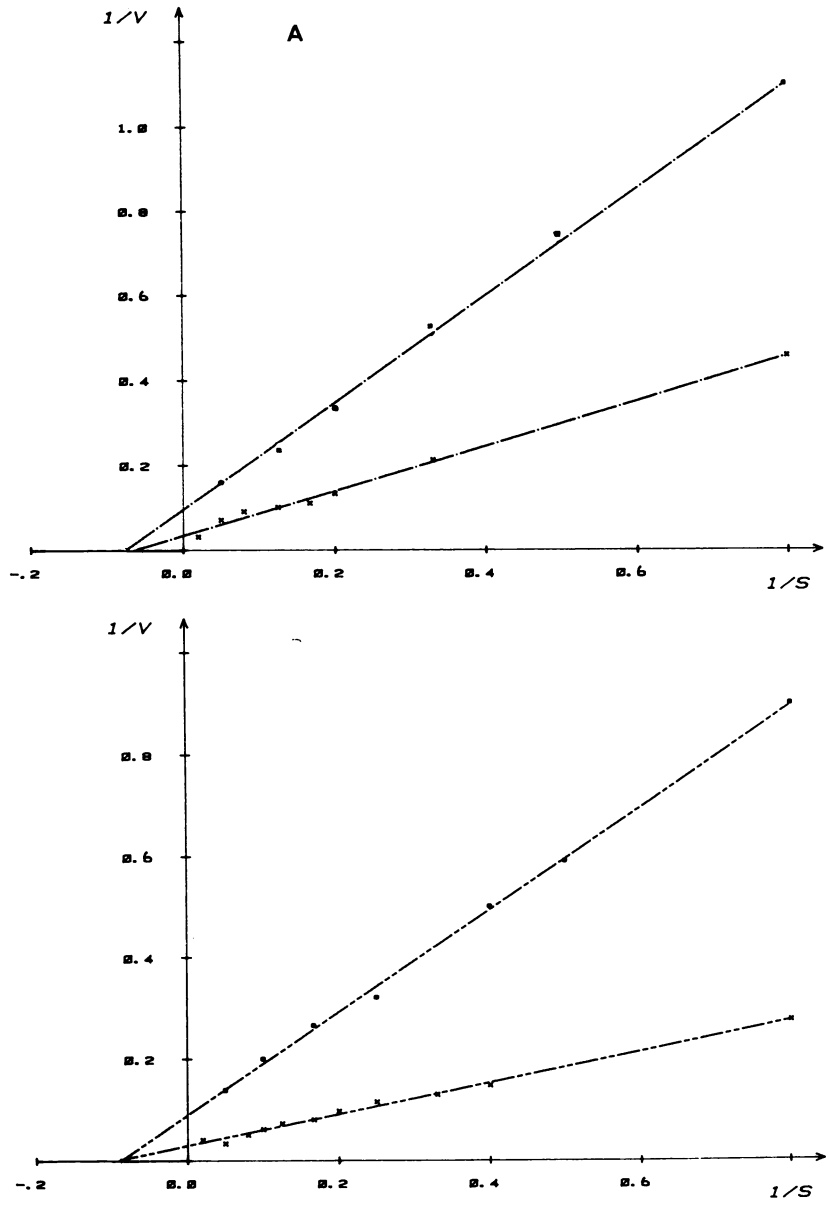

FIG. 3.—Lineweaver–Burk plots of DO. Two differents subcultures (x, o) of the same melanoma cell line were incubated in the reaction mixture described in Material and Methods. Concentrations of [1·4-^{14}C] putrescine were varied from 1·25 to 50×10^{-6}M. $1/S = 10^{-6}/$[putrescine] $1/V = 1/$pmol Δ^1-pyrroline formed/h/0·1 mg protein. A, M_3Dau cell line; B, M_4Beu cell line.

10^{-2}M chloral hydrate. The results in Fig. 4 show that for the highly tumorigenic lines the K_m (app.) values are $10{\cdot}6 \times 10^{-6}\text{M} \pm 0{\cdot}2$ (5) and $14{\cdot}2 \times 10^{-6}\text{M} \pm 0{\cdot}6$ (5) respectively, whereas for the poorly tumorigenic lines they are $4{\cdot}5 \times 10^{-6}\text{M} \pm 0{\cdot}3$ (8). Thus DO from highly tumorigenic lines has a greater apparent K_m than that from the poorly tumorigenic lines. These findings prompted us to examine the kinetic parameters of DO from normal and transformed rat kidney

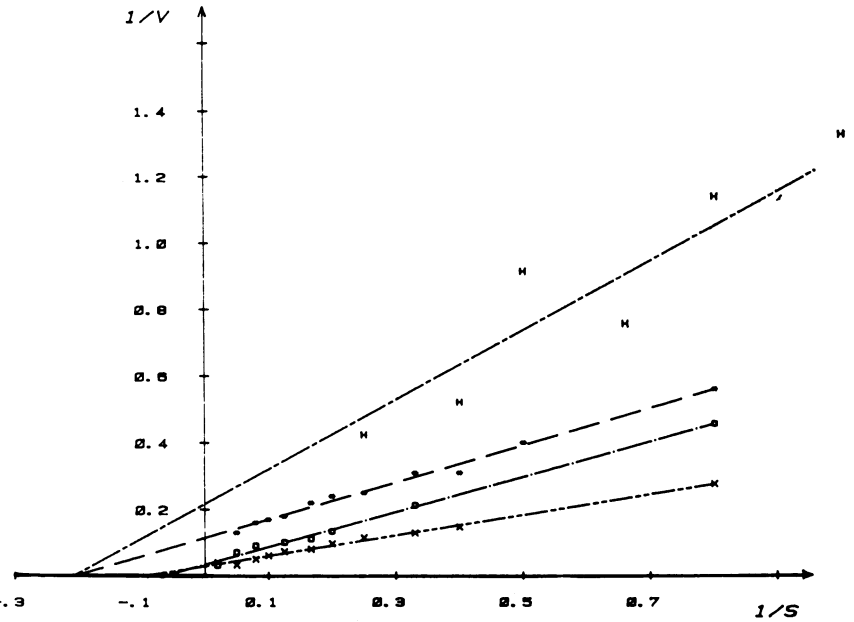

FIG. 4.—Lineweaver–Burk plots of DO in different melanoma cell lines. Concentrations of [1·4-^{14}C] putrescine were varied from 1·25 to 50×10^{-6}M. $1/S = 10^{-6}/$[putrescine]; $1/V = 1/$pmol Δ^1-pyrroline formed/h 0·1 mg protein. H, M_2GeB line; M_1Dor; □, M_4Beu; ×, M_3Dau.

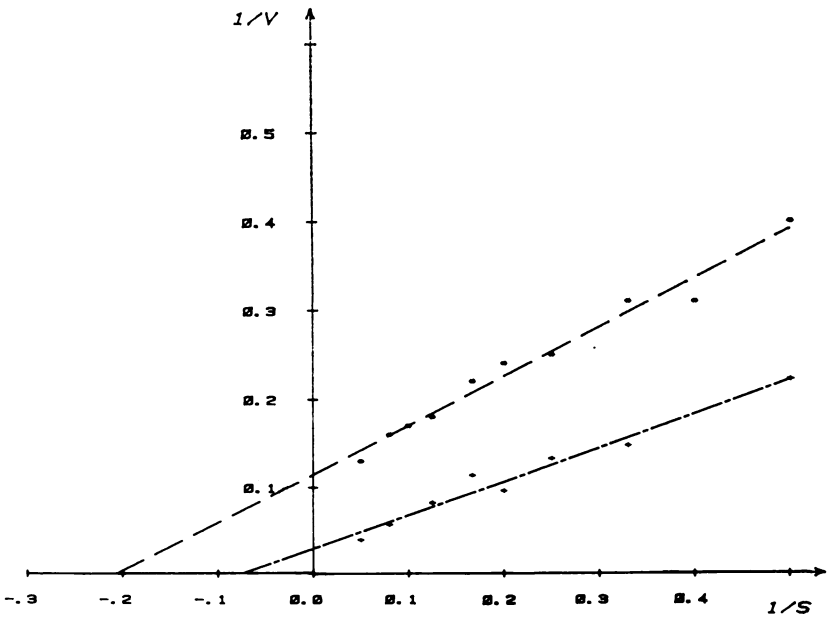

FIG. 5.—Lineweaver–Burk plots of DO from M_1Dor melanoma cells (——) and cells established from tumours appearing in nude mice after injection of M_1Dor cells (—·—·). Concentration of [1·4-^{14}C] putrescine was varied from 2 to 20×10^{-6}M. $1/S = 10^{-6}/$[putrescine]. $1/V = 1/$pmol Δ^1-pyrroline formed/h/0·1 mg protein.

cells, in which residual DO activity at confluence was lower in the cells transformed by Rous sarcoma virus than in their normal counterparts. It was found here too that the K_m (app.) of DO from the transformed rat kidney cells is $2{\cdot}5 \times 10^{-6}$M, compared to $5{\cdot}5 \times 10^{-7}$M in the normal cells. It thus seems reasonable to conclude that an increased K_m (app.) is characteristic of both highly tumorigenic and transformed cells.

In view of these results, plus the findings of Kanzaki *et al.* (1979) and Brüggen *et al.* (1981) that a population of melanoma cells is heterogeneous, the question could be asked whether the low K_m (app.) in the poorly tumorigenic lines was characteristic of the enzyme from all the melanoma cells in the population, or whether it represented the overall value of a heterogeneous population containing a mixture of highly tumorigenic and poorly tumorigenic cells. To assess this, cell cultures were established from tumours appearing in nude mice after injection of the poorly tumorigenic line M_1Dor.

Determination of K_m (app.) of DO from these tumour cells revealed that it was 14×10^{-6}M, whereas that of the original poorly tumorigenic line was $4{\cdot}5 \times 10^{-6}$M (Fig. 5). Thus this melanoma line does contain a mixture of highly tumorigenic cells (responsible for tumour formation) and of poorly tumorigenic ones. The results provide further evidence for the correlation established so far between DO and tumorigenicity. It must be noted that the K_m (app.) of DO shows no variation in M_3Dau (which exhibits 100% tumorigenicity in nude mice) whether the cultures were established from human or mouse tumours. This suggests that the cell population in the poorly tumorigenic line is heterogeneous, and that the more tumorigenic cells of this population have a K_m (app.) typical of the 100% tumorigenic lines.

DISCUSSION

It is apparent from the results presented that modifications in DO activity take place with the growth of human malignant melanoma cells in culture, as has been shown for rat kidney cells. Contrary to observations made in studies comparing DO activity in homogenates of confluent cultures from normal rat kidney cells and from these same cells transformed by the wild-type strain of Rous sarcoma virus B77, the DO activity, expressed as pmol Δ^1-pyrroline formed/h/0·1 mg protein, in confluent cells from the highly malignant lines is greater than that of the less malignant lines. Though these results were statistically significant (Table) they must be interpreted with caution, since V_{max} (app.) values obtained on kinetic analysis showed that this parameter varied with the number of subcultures undergone by the cell line (Fig. 3) and also with the presence of dialysable modulators of the enzyme (unpublished results). Evidence for such natural modulators of DO has been previously presented (Quash *et al.*, 1976) and whereas 2-oxosuccinamate derived from asparagine transamination was an activator, the deaminated derivative of this ketoacid oxaloacetate, and its decarboxylated product pyruvate, were inhibitors. Variations in the intracellular level of such modulators with passage number and culture conditions could provide one explanation for the differences in V_{max} described here. They could also explain the apparently conflicting findings, showing on the one hand increased DO activity in human malignant cells from lung (Baylin *et al.*, 1980) and thyroid (Andersson *et al.*, 1980), and on the other decreased DO activity in malignant cells from oesophagus (Quash *et al.*, 1979) and intestine (Kusche *et al.*, 1980).

The investigation of the other kinetic parameter, K_m (app.), gave results which did not fluctuate either with the number of subcultures (Fig. 3) or with the dialysis or not of cell homogenates. Using this criterion, the 4 melanoma cell lines were classified into 2 groups of high and low K_m (app.) and this classification corresponded to the highly tumorigenic and low

tumorigenic lines respectively. Using this same parameter, it was found that the K_m (app.) of DO from rat kidney cells transformed by Rous sarcoma virus showed a 20-fold increase over that of the enzyme from normal rat kidney cells. Thus even if increases or decreases in enzyme activity and V_{max} (app.) take place in tumour cells according to their growth phase and/or anatomical origin, it would appear that an increased K_m (app.) of DO is characteristic of all tumour cells.

This example of an altered K_m of an enzyme in tumour cells is not an isolated observation. White *et al.* (1981) have very recently provided evidence for an altered K_m in the sugar-transport system for deoxyglucose in tumour cells. Moreover the character of an altered K_m for hexose transport segregates as a character of tumorigenicity in hybrids made between malignant and normal cells. In fact using heterotransplantability in syngeneic hosts as the assay for tumorigenicity, they were further able to show a definite correlation between tumorigenicity and an altered K_m in the enzyme system responsible for deoxyglucose transport. Other examples of enzymes with altered kinetic properties in human neoplastic tissues have been documented for 7 melanoma cultures xenografted into athymic nude mice. In such cultures Liau *et al.* (1980) demonstrated the presence of S-adenosyl-methionine synthetase isoenzyme with an altered intermediate K_m which was attributable to the malignant evolution of the cells. Such an alteration is analogous to that found for the leucine aminotransferase isoenzymes reported by Roth & Kaji (1979). The transition of normal isoenzyme I into tumour isoenzyme III for leucine aminotransferase is caused by the transforming-gene product of Rous sarcoma virus. The alteration of isoenzymes in malignant tissues appears to be a general phenomenon.

In the results of all the studies cited above, as well as in those presented here, there was one inherent difficulty: the validity of the correlation between enzyme kinetics and tumorigenicity depended on the homogeneity of the cell population used. A cell population may be heterogeneous and be composed of cells with different tumorigenic potential in nude mice. With cell lines exhibiting 100% take or no take at all in nude mice the cell population is most probably homogeneous, and the interpretation is straightforward. For those showing intermediate percentages, differences may reflect the heterogeneity of the cell population rather than an altered characteristic of the enzyme from each cell. This possibility was investigated directly by examining the K_m (app.) of DO in cells of the poorly tumorigenic line M_1Dor before and after transplantation into nude mice. Since cells growing in these animals are by definition tumorigenic, a population of tumorigenic cells can then be selected from a poorly tumorigenic cell line. The fact that tumours arising under these conditions result from population selection in nude mice was documented by their karyotype, which contained multiples of chromosomes 3, 7, 16 and 22, relatively few copies of chromosomes 9 and 21, and was similar to that of highly tumorigenic melanoma cell lines (Bertrand *et al.*, in preparation). When kinetic studies were performed on DO in cells subcultured from xenografts, it was found that the K_m (app.) of DO was typical of a highly tumorigenic line (K_m (app.) 14×10^{-6}M), whereas the K_m of the enzyme in the cell population from which the cells were selected was $4{\cdot}5 \times 10^{-6}$M. This result provides evidence not only for the heterogeneity of the cell population, but gives additional support to the observations made with M_3Dau and M_4Beu, that increased tumorigenesis is accompanied by an increase in the K_m of DO.

The basis of this altered K_m of DO will not be fully understood until the enzyme is rigorously purified and appropriate antibodies have been made. Nevertheless the pursuit of investigations along the lines outlined here with other xenografted human tumours should allow conclusions

to be reached as to whether increases in K_m (app.) of DO are a general characteristic of all tumorigenic cells.

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