CHANGE IN THE STRUCTURE OF SHOPE PAPILLOMA VIRUS-INDUCED ARGINASE ASSOCIATED WITH MUTATION OF THE VIRUS*

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Much inferential evidence has been reported suggesting that the arginase induced by the Shope virus is synthesized according to virus rather than rabbit genetic information (1–4). A direct approach to the question is to study the arginase induced by a mutant line of the virus, to find out whether the mutation influences the structure of the enzyme. The wild-type papilloma virus, though readily recoverable from "spontaneous" field-infected or laboratory-inoculated Kansas cottontail rabbit papillomas, is seldom recoverable from tumors induced in the domestic rabbit and then only in very small quantity. However, Shope et al. (5) have isolated a mutant line of the virus which can be passed readily in domestic rabbits. In this report the arginase induced by the two virus lines in domestic rabbits is compared.

Materials and Methods

Virus.—The wild-type virus was harvested in the laboratory from papillomas of trapped cottontail rabbits obtained from Mr. Earl Johnson of Rago, Kansas. The mutant line of papilloma virus, recoverable in domestic rabbits, was obtained originally from Dr. Richard Shope and since passed in domestic rabbits in this laboratory. It has been found to be free of rabbit kidney vacuolating virus (RKV),[1] both by Dr. Shope[2] and in recent tests in this laboratory carried out by Dr. Raymond Tennant. RKV has been found occasionally to be a passenger in extracts of wild rabbit papillomas (6). Domestic rabbits are obtained from the Southern Rabbitry, Birmingham, Ala.; only brown agoutis are used. Rabbits are inoculated in the standard way after turpentine-acetone pretreatment of the skin (7). Only the living squamous cell epithelium of the tumors is used, either for enzyme purification or for studies of the free amino acid pool of the tumors. The keratin and underlying fibrous tissue and hair follicles are cut away.

Preparations.—Photomicrographs of the material used have been reported (8). The detailed method of preparing this material is as follows. Concentrated or cesium-banded virus preparations are inoculated, yielding confluent papillomas, which at the end of about 2 wk have only a

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[1] *Abbreviations used in this paper:* ORD, optical rotary dispersion; RKV, rabbit kidney vacuolating virus.

[2] Shope, R. E. Personal communication.

thin film of keratin and form a flat, elevated surface about 1 mm high. This surface is washed with a brush and soap and water, followed by rinsing with dilute alcohol and water. The superficial keratin is shaved off, leaving only the living tissue beneath. This is separated from the underlying connective tissue and hair follicles by dissection. A dissecting microscope is used to check the material. The usually sterile tissue is then extracted for enzymes or extracted with 5% trichloroacetic acid for studies of the free amino acid pool. Essentially the same procedure is carried out when similar studies of the normal or hyperplastic squamous epithelium of the rabbit are made. All amino acid analyses are made using the Moore and Stein system (9) and a 120B or a 120C Beckman-Spinco amino acid analyzer (Spinco Div. of Beckman Instruments, Inc., Palo Alto, Calif.).

The separation, purification, and physical and chemical characterization of rabbit liver and papilloma arginase have been described previously (3). However, due to changes in the characteristics of the resins over the years it is worthwhile to include in detail the methods currently used.

Chemicals and Resins.—Diethylaminoethyl (DEAE) (Bio-Rad Laboratories, Richmond, Calif., anion exchange cellulose, Cellex-D, exchange capacity 0.70 meg/g, coarse porosity), Sephadex G-75 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden, particle size 40–120 μ), Sephadex G-200 (Pharmacia Fine Chemicals, particle size 40–120 μ), and carboxymethyl cellulose (Whatman CM23, fibrous, mean small ion capacity 0.6 meg/g) are used.

Enzyme Purification Procedure.—The living papilloma epithelium is ground with sand in the cold, 3 vol of 30% alcohol in 0.03 M sodium acetate, pH 5.8, is added, and the tissue is extracted at −8°C for 30 min. After centrifugation at 6000 rpm for 15 min, the residue after discarding the supernatant is extracted at −5°C for 1 hr with 19% alcohol in 0.02 M sodium acetate, pH 5.8. After centrifugation the supernate is dialyzed overnight against 0.001 M manganese maleate, then lyophilized. The lyophilisate is brought up in distilled water and loaded on an 18 × 1 cm DEAE column, having been equilibrated with 0.01 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 8.0, and eluted with the same buffer, using a 1 cc/min flow rate. The tubes showing arginase activity are then dialyzed overnight against 0.001 M manganese maleate, lyophilized, and loaded onto a Sephadex G-75 column (18 × 1 cm) previously equilibrated with 0.001 M manganese maleate, pH 6.9, and eluted with the same buffer. Tubes containing activity are dialyzed overnight and lyophilized. The lyophilisate is brought up in water and loaded onto a carboxymethyl cellulose column, 18 × 1 cm, after equilibration at pH 5 with 0.01 M sodium acetate. The column is eluted sequentially with 0.01 M sodium acetate in 0.001 M manganese maleate, pH 5, then the same buffer at pH 5.8, then 0.025 M buffer at pH 5.8, then 0.01 M Tris buffer at pH 7.6. The flaw rate used is 0.8 cc/min. All columns are eluted at 10°C.

Arginase activity is measured in the standard way (10), except that the paper chromatography with butanol:acetic acid:water (40:10:50) to detect qualitatively the presence of ornithine rather than urea formation, is used. This method facilitates the testing for the presence or absence of arginase activity in large numbers of tubes used in purification. For specific activity of the enzyme the method of Greenberg (10) is used.

Purification and Characterization of Viruses.—The wild-type papilloma virus is purified in the standard way (11), using sequential pelleting in the Spinco Model L centrifuge followed by banding in cesium chloride, 5-25% glycerin gradients, or D_2O.

The recoverable virus line is also pelleted three to five times, being brought up in 0.05 M phosphate buffer, pH 6.5. The protein coat of this virus, however, is much less stable than wild-type and is lost in cesium chloride, leaving only DNA and protein coat fragments. Therefore, rate banding in glycerin gradients or in D_2O is used. The absolute density of this virus was not determined but it moves in gradients much more slowly than the wild-type virus. Preliminary electron micrographs (kindly made by Dr. W. W. Harris of the Molecular Anatomy Program of the Oak Ridge National Laboratory) revealed particles of about the same size as wild-type

virus but without capsomers. The virus is thus far "contaminated" with lipid-like spherules. These are probably due to breakdown of the virus structure, as they would not be expected to appear in banded material. In working with this virus it is essential not to freeze the preparation after the original extraction from glycerinated papillomas, as it loses its infectivity. This loss is associated with a proteinaceous precipitate appearing in the previously opalescent supernate.

For studies using optical rotatory dispersion (ORD), the virus-induced enzyme is dissolved in 0.001 M phosphate buffer, pH 7. Liver arginase is dissolved in 0.001 M phosphate buffer, pH 7, or in 0.05 M manganese sulfate, pH 7, for activation studies. Manganese has no influence on the ORD pattern of virus-induced enzyme. The concentration of the enzymes is determined using the Lowry test for protein. The ORD curves are made using a Jasco Model ORD/UV-5 recording spectropolarimeter (Durrum Instrument Corp., Palo Alto, Calif.) in cells of 1 mm path length at room temperature (24°–26°C).

The data are expressed in terms of specific rotation $[\alpha] = \alpha/dc$, where α is the observed rotation in degrees at a given wavelength, d is the path length in decimeters, and c is the concentration in grams per milliliter (12).

TABLE I

Comparison of Lysine, Arginine, and Ornithine Levels in Free Amino Acid Pools of Papillomas Induced by Wild-Type and Recoverable Virus Lines

	Skin	Wild-type	Recoverable
		mμmole	
Lysine	0.5	3.0	0.6
Arginine	20.1	0.4	0.16
Ornithine	0	2.0	0.4

RESULTS

Table I shows the relative proportions of basic amino acids in domestic rabbit tumors induced by the two virus lines as compared to normal skin. It is clear that arginine is relatively depleted in the virus-induced papillomas and that ornithine is present in considerable amounts in both tumor types although not in normal skin. This result has been reported previously (1). The proportion of basic amino acids in tumors induced by the mutant virus is between that in skin and that in papillomas induced by wild-type virus. This finding raises the question as to whether there is less arginase induced by the mutant line virus or whether the enzyme is less active.

Fig. 1 shows the elution characteristics from carboxymethyl cellulose of rabbit liver arginase, arginase induced with the wild-type virus, and arginase induced with the mutant, recoverable line. They are shown both singly and when all three enzymes are mixed together and separated in a single run. The three enzymes may be readily separated from one another. The molecular weight and homogeneity of the enzyme preparations were determined using sedimentation equilibrium. It has been reported previously that rabbit liver and

wild-type papilloma arginase may be separated by this method, yielding homogeneous molecular populations with molecular weights of 37,000 and 43,000, respectively. Fig. 2 shows the log c/dx data (13) for the recoverable virus-induced enzyme at the end of carboxymethyl cellulose purification. Two

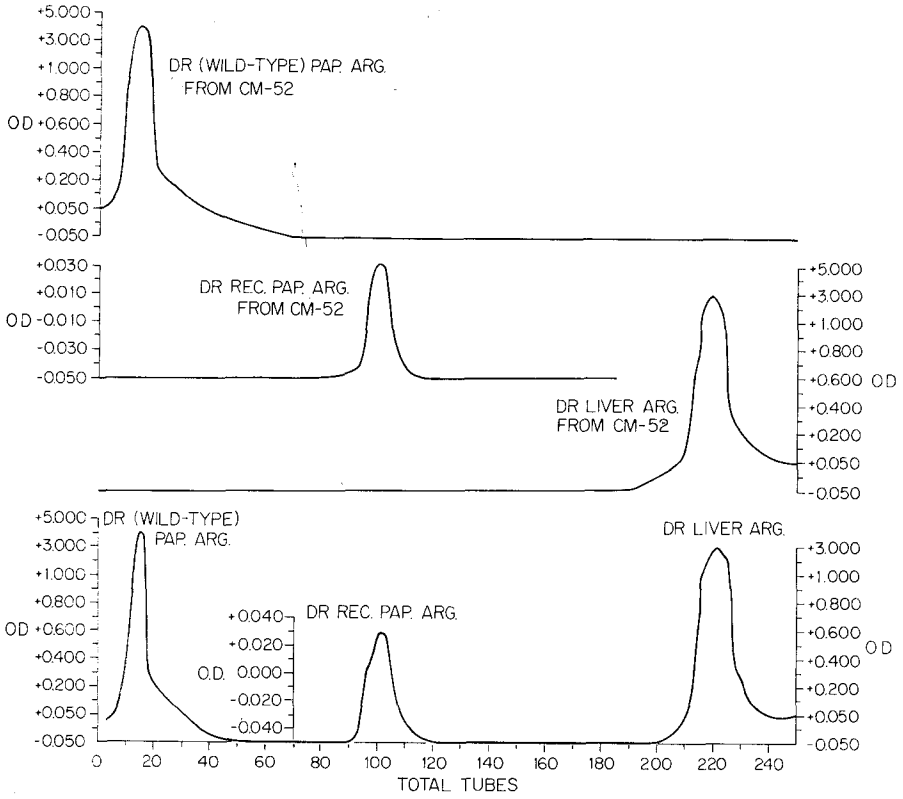

FIG. 1. Elution characteristics of arginase from wild-type virus-induced papilloma, mutant virus-induced papilloma, and domestic rabbit liver. Curves are labeled domestic (wild-type) papilloma arginase from CM-52, domestic rabbit recoverable papilloma arginase from CM-52, domestic liver arginase from CM-52, domestic rabbit (wild-type) papilloma arginase, domestic rabbit recoverable papilloma arginase, and domestic rabbit liver arginase.

components were found, one small component with a molecular weight of ~43,000 and a larger component with a molecular weight of ~400,000. This mixture showed arginase activity without manganese activation after extensive (48 hr) dialysis against 0.001 M phosphate buffer, pH 6.5. The activity was very small in amount considering the concentration of protein. It was therefore decided to put this mixture through Sephadex G-200 to eliminate the smaller molecular weight component. Fig. 3 shows the result. Only the large molecular

weight component remained after the elution time used. When the enzyme was tested without manganese activation after dialysis, no activity was found. When manganese was added, however, large amounts of enzyme activity appeared, though less than that found with similar amounts of wild-type virus–induced enzyme. The relative amounts were 800 and 2500 arginase units/mg of protein nitrogen from mutant and for wild-type virus–induced arginase, respectively. This activity, together with the centrifugal studies, indicates that the peaks shown in Fig. 1 consist mainly of single-protein molecular popula-

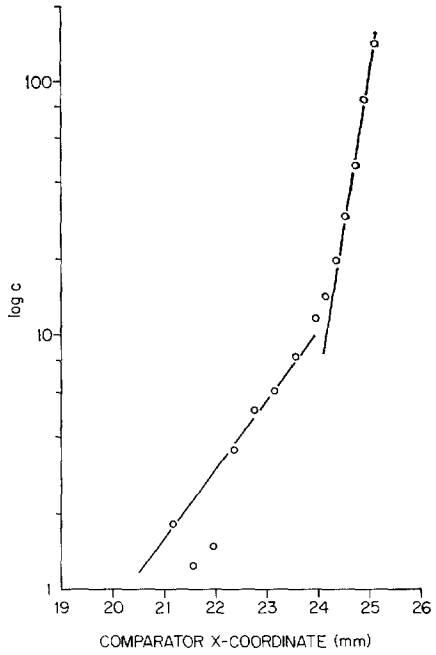

FIG. 2. Plot of mutant virus–induced arginase concentration in increments of curvature of middle fringes against comparator x-coordinate.

tions. The smaller molecular weight material described above appears to be a degradation product of the large polymer, and with a molecular weight of 43,000 it resembles the wild-type virus–induced enzyme purified on carboxymethyl cellulose. In contrast to the larger polymer, and like the wild-type virus–induced enzyme, it has no requirement for manganese (3).

Using Sephadex G-200, the crude wild-type virus–induced enzyme comes off the column as does the mutant virus–induced one, with the void volume indicating that the polymer is of high molecular weight. The wild-type arginase polymer is more unstable and breaks down into 43,000 molecular weight units as it is being eluted from the cellulose column at pH 5. It has also been reported

elsewhere that when arginase is purified at low pH the molecule breaks down into smaller units (14).

Amino Acid Analysis of the Purified Enzymes.—Table II shows the amino acid composition of the wild-type and recoverable virus-induced enzymes as compared with values reported elsewhere in the literature. The prime difference between the virus-induced enzymes and liver arginase is the relative number of basic amino acids. The smaller number of basic amino acids in the virus-

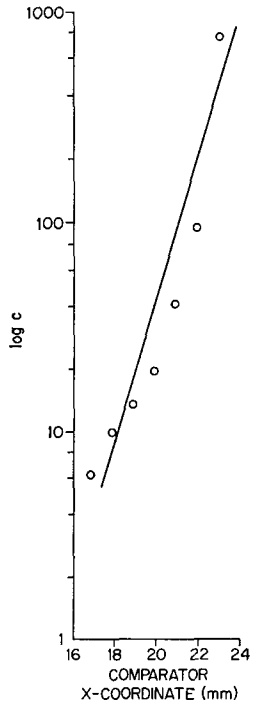

FIG. 3. Plot of mutant virus-induced arginase concentration after elution on Sephadex G-200 to eliminate the smaller molecular weight component.

induced enzymes are also reflected in their relative movement on carboxymethyl cellulose, the virus-induced enzymes being more acidic.

Structural Comparisons of the Enzymes.—The differences in the elution characteristics of the two papilloma arginases and liver arginase from carboxymethyl cellulose, as well as their amino acid constitutions, show clearly that the three differ from each other. In addition, previous results concerning density, molecular weight, and sedimentation velocity suggested different molecular shapes (3), so it was determined to compare the ORD characteristics of the enzymes both with and without manganese activation.

Fig. 4 shows the ORD's of papilloma virus–induced arginase in phosphate buffer, pH 7, and liver arginase in 0.05 M $MnSO_4$, pH 7, both at zero time and after 3 hr of activation at 37°C. As there was no observable difference in the characteristics of arginase in phosphate buffer and in $MnSO_4$ before incubation, the curve for liver arginase in phosphate is not included in the diagram. Both wild-type and recoverable papilloma arginase showed a negative Cotton effect

TABLE II

Amino Acid Composition of Purified Arginase

Amino acid	Horse liver*	Domestic rabbit liver‡		Wild-type virus papilloma‡		Recoverable virus papilloma‡	
	Amino acid residues/100 g protein	*μmole*	%	*μmole*	%	*μmole*	%
Aspartic acid	6.46	0.6930	9.4	0.2919	7.1	0.0285	10.84
Glutamic acid	8.98	0.7940	10.8	0.3200	10.3	0.0360	11.63
Glycine	4.61	0.5950	8.1	0.3890	9.3	0.0193	7.33
Alanine	1.85	0.5430	7.4	0.3800	9.2	0.0221	8.40
Serine	4.49	0.2321	3.1	0.2620	6.3	0.0151	5.74
Threonine	4.53	0.3958	5.4	0.2650	6.4	0.0177	6.73
Valine	7.36	0.4270	5.8	0.2530	6.1	0.0179	6.80
Isoleucine	11.81	0.5020	6.8	0.2185	5.3	0.0113	4.29
Leucine		0.7030	9.5	0.3630	8.7	0.0335	12.73
Phenylalanine	3.66	0.3175	4.3	0.1429	3.3	0.0140	5.32
Histidine	NR§	0.1948	2.6	0.1309	3.1	0.0073	2.77
Tyrosine	0.87	0.2351	3.2	0.1001	2.4	0.0127	4.82
Lysine	6.91	0.7840	10.6	0.2910	6.9	0.0182	6.91
Arginine	4.30	0.3750	5.1	0.1252	3.0	0.0070	2.66
Proline	5.65	0.5610	7.6	0.2775	6.7	0.0078	2.96
Methionine	1.43	0.0105	0.14	NR§	—	—	—
Cystine	NR§	0.0135	0.18	NR§	—	—	—
Ornithine	—	—	—	—	—	—	—

* Greenberg et al. (15).

‡ Rogers and Moore (3).

§ NR = not recorded.

at 233 nm without manganese activation, with α values of 6900 and 4900, respectively. On the other hand, liver arginase requires manganese activation before a significant Cotton effect develops. Incubation of liver arginase in 0.05 M $MnSO_4$ at room temperature produced no change in the ORD spectrum, whereas incubation at 37°C produces the changes shown in Fig. 4. These results correlate with the temperature dependence previously observed for enzymatic activation of liver arginase (15). Manganese had no influence on the virus-induced enzyme. Using the Moffitt equation (16) the b_o for wild-type papilloma arginase is -250; for inactive and activated liver arginase the b_o values are

+20 and −130, respectively. In all three instances the wavelengths used in the calculations were 240–254 nm. These are equivalent to an estimated 40% helicity for papilloma arginase and less than 20% for activated liver arginase. Inactive liver arginase has no detectable negative deviation using this method. The Cotton effect and enzyme activity of the activated liver arginase are destroyed by 4 M urea. 10 M urea is required to destroy the Cotton effect and enzyme activity of the virus-induced enzymes. The Cotton effect of the activated liver arginase is lost following dialysis for 18 hr against 0.001 M manganese sulfate.

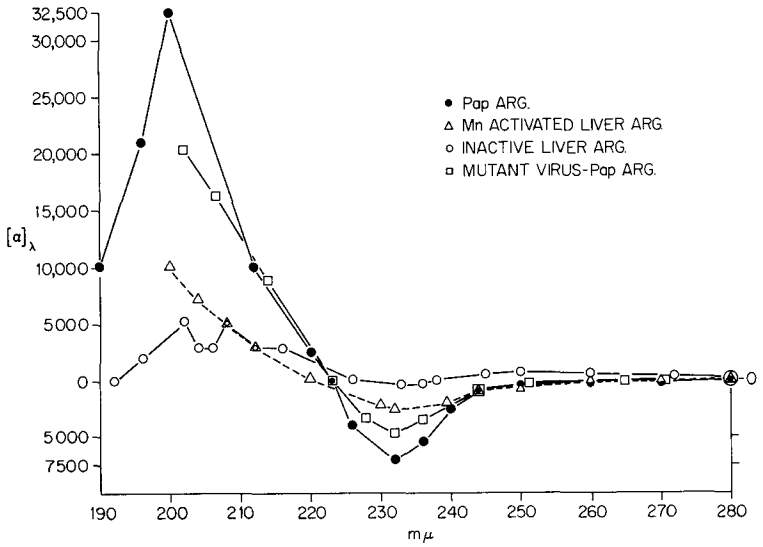

FIG. 4. Optical rotary dispersion characteristics of wild-type virus-induced, mutant virus-induced, and liver arginase. Papilloma arginase, manganese-activated liver arginase, inactive liver arginase, and mutant virus-papilloma arginase are shown.

DISCUSSION

A structural change in the arginase induced by the Shope papilloma virus is associated with the mutation of the wild-type virus line to the domestic rabbit recoverable line, providing additional evidence that the synthesis of the enzyme is mediated by virus rather than rabbit information. This is revealed by the difference in elution characteristics from carboxymethyl cellulose, the degree of polymerization of the enzymes, their specific activity, and their ORD characteristics. The previously reported physical and chemical data showing consistent differences between the wild-type virus-induced enzyme and liver arginase provided only suggestive evidence. The finding that rabbits carrying

their own tumors develop antibodies not only against the virus but also against purified induced enzyme, and that there is no cross-reaction against purified rabbit liver arginase, also was of interest, as was the finding that sheep anti-rabbit globulin reacts strongly against purified liver arginase but not against papilloma arginase. The previously reported differences (3) between the manganese requirements of papilloma arginase and liver arginase may be related to the physical states of the enzymes at the time of testing. This is suggested by the manganese requirement of the purified polymerized form of the recoverable virus-induced enzyme after cellulose purification and passage through the Sephadex G-200 column and by the manganese requirement of wild-type virus–induced arginase after passage through Sephadex G-200 before elution on carboxymethyl cellulose. There the wild-type enzyme breaks down into smaller units of about 43,000 molecular weight that do not require manganese for activity. This breakdown is in contrast to the mutant virus–induced enzyme, most of which has a molecular weight of 400,000, although there is some breakdown into units with a molecular weight of 43,000. It is significant that these units elute at the same position as the heavier polymer, indicating that the difference in elution characteristics is due to other changes than just the state of polymerization. The influence of the state of the enzyme before purification with carboxymethyl cellulose played a role in the report of Orth et al. (17), who found little arginase activity in crude extracts of papillomas without prior treatment with manganese.

Satoh et al. (18) reported that they were unable to separate liver and papilloma arginase using carboxymethyl cellulose. This is related to the fact that they used a column washed at pH 7.4 and equilibrated in the sodium form before loading, and then began their elution. In this report, as in previous ones from this laboratory relating to arginase purification, carboxymethyl cellulose in the acid form has been used, with equilibration at pH 5 and the elution begun at pH 5. As shown in Fig. 1, the wild-type virus–induced enzyme is eluted at pH 5 at low ionic strength. The mutant virus–induced enzyme elutes at pH 5.8 with a higher ionic strength, and liver arginase at pH 7.6 at a much higher ionic strength. The virus-induced enzyme, either crude or purified, is precipitable with 25% ammonium sulfate. Liver arginase requires 37% at 4°C (10). The relative solubility in ammonium sulfate is consistent with the elution characteristics of these enzymes from carboxymethyl cellulose as concerns charge differences. This is further substantiated from the amino acid analyses of liver and papilloma arginase (Table II), which shows the relative lack of basic amino acids in the virus-induced enzymes.

The finding of small amounts of arginase activity in some normal rabbit skin preparations (17, 18) seems most likely related to the preparation of the material and contaminating microorganisms. Such contamination also makes difficult the evaluation of enzyme activity in chemically induced papillomas. Such

activity has been reported by us (3) and others (17, 18). Due to the convoluted state of the thin line of living epithelium in chemically induced tumors it has not been possible to eliminate keratin, bacteria, fungi, etc. In partial purifications of arginase derived from coal tar–induced papillomas, the enzyme, irrespective of its source (microbial or otherwise), was separable from papilloma virus–induced arginase in sucrose density gradients (3). The absence of ornithine in the free amino acid pool of normal squamous epithelium (8) substantiates the lack of arginase in such epithelium (Table I).

The decrease in relative arginase activity in tumors induced by the recoverable virus line, as compared to the wild-type (Table I), is of interest since their growth rate is slower than that of papillomas induced with wild-type virus (5). This is consistent with the finding that the amount of available arginine in the infected cells is a determining factor in controlling the synthesis of an arginine-rich histone associated with growth of the virus-infected cells (2, 8).

ORD characteristics of papilloma virus–induced arginase are very different from those of rabbit liver arginase, as might be expected from the previously reported differences in physical and chemical characteristics of these enzymes. The most striking difference reported is that the virus-induced enzyme has a slower sedimentation velocity, even though it is more dense and has a higher molecular weight (3). These characteristics suggest that the enzyme is more linear in structure than liver arginase.

SUMMARY

The change in the state of the virus-induced enzyme associated with a mutation in the virus provides additional evidence that the enzyme is synthesized from virus rather than rabbit genetic information. This change in structure results in differences in stability of polymerization, degree of optical rotary dispersion (ORD) specific rotation, change in elution characteristics from carboxymethyl cellulose, and a reduction in specific activity of the arginase.

Liver arginase differs markedly in ORD characteristics from the virus-induced enzyme. In contrast to the virus-induced enzyme, it showed no negative Cotton effect at 233 nm until it was activated with manganese. Manganese had no influence on the ORD spectrum of virus-induced arginase. In addition, liver arginase is denatured by 4 M urea, while the virus-induced enzyme requires 10 M urea for denaturation.

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