STUDIES OF THE MECHANISM OF ACTION OF THE SHOPE RABBIT PAPILLOMA VIRUS*

I. Concerning the Nature of the Induction of Arginase in the Infected Cells

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Plates 24 and 25

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The Shope papilloma virus induces an arginase in the squamous epithelium of wild Kansas cottontail and domestic rabbits (1). Animals carrying the virus-induced papillomas develop antibodies (precipitins) against the purified enzyme (2) as well as against the virus (3). These sera from either wild or domestic rabbits cross-react with the purified enzyme derived from papillomas of both rabbit sorts. The antibodies do not cross-react with liver arginase derived from either wild or domestic rabbits. The virus itself is immunologically distinct from the purified arginase and has no arginase activity (2). These findings strongly suggest that the information for the synthesis of the enzyme in rabbit cells is derived from the virus rather than induced in some way by the virus from rabbit chromosomal information (2).

In contrast to normal or hyperplastic squamous epithelium, the Shope virus papillomas have an extreme paucity of arginine-rich nuclear histones (2). This observation is remarkably similar to that of Allfrey and Mirsky who reported an inverse relationship between the amount of nuclear histones and nuclear synthetic activity (4). It seems, therefore, that the virus brings in information for the synthesis of an arginase for which the cells have no control mechanism. The arginase, in turn, depletes cellular arginine which inhibits nuclear histone (2), thus possibly freeing the cells for abnormally rapid growth, and leading to the formation of the papilloma. The following report is concerned with an extension of these findings.

The antigenic difference between papilloma and liver arginase necessitated comparison of the physiochemical characteristics of the available arginases of

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the wild and domestic rabbit. These studies have included sedimentation velocity, movement in sucrose gradients, partial specific volume, molecular weight, amino acid analysis, peptide pattern, requirements of enzyme activation; content of manganese, nucleic acid, and polysaccharide; and enzyme activity per milligram nitrogen. The following purified, or in some instances partially purified, arginases were used: papilloma arginase from wild and domestic rabbits, liver arginase from wild and domestic rabbits, domestic rabbit kidney arginase, an arginase derived from a tar-induced tumor of the rabbit, and horse liver arginase. Papilloma arginase derived from wild rabbits (WR) is identical with that derived from domestic rabbits (DR) in respect to each of these determinations. The papilloma arginase, on the other hand, differs strikingly in all qualities tested from the other arginases studied.

One would expect, as concerns the relation of the enzyme to the neoplastic state of the cells, that if the depletion of cellular arginine and nuclear histone is related to the rapid growth of the cells, the growth rate of the tumors would be slowed by supplying sufficient arginine to by-pass the enzyme. The studies reported below demonstrate that the growth rate of the papillomas can be greatly reduced by giving large supplements of arginine parenterally in the presence of a small amount of an arginase inhibitor, canavanine.

Materials and Methods

Materials.—

Virus.—The virus was extracted as a 10 per cent solution using papillomas from field-infected wild cottontail rabbits obtained from Mr. Earl Johnson of Rago, Kansas. The papillomas were plucked and stored in glycerine in this laboratory. The extracting solvent was phosphate buffer (0.05 M, pH 6.5).

Rabbits.—Wild cottontail rabbits with and without papillomas were obtained from Earl Johnson of Rago, Kansas. Domestic rabbits were obtained principally from Riley's Bunny Haven, Durham, North Carolina. All rabbits were brown agouti, kept one to a cage, fed Purina rabbit chow and given water at liberty. Canavanine and arginine were given subcutaneously, and 6-mercaptopurine riboside, intraperitoneally. All injections were given as sterile isotonic solutions and at pH 7. Weights of the animals were recorded daily.

Tar Tumors.—Skin tumors were induced by applying coal tar on the ears of the rabbits 2 times weekly for varying lengths of time. Benign tumors of two sorts were obtained, papillomas and carcinomatoids (5, 6), the latter being papillomas so stimulated temporarily by the tar as to look like squamous cell carcinomas. The carcinomatoid tissue used was not of a single cell type but included an almost equal amount of a reactive, mucoid-appearing, epithelioid stroma from which the squamous epithelium could not be manually separated. The tar papillomas resembled in general the Shope virus-induced papillomas (6), but were histologically distinguishable.

Cellulose Columns.—Diethylaminoethyl cellulose,[1] Bio Rad Laboratories, Richmond, California; sephadex G-75, Pharmacia, Uppsala, Sweden; carboxymethyl cellulose, Bio Rad Laboratories.

[1] DEAE, diethylaminoethyl cellulose; EDTA, ethylenediaminetetraacetate; 6-MPR, 6-mercaptopurine riboside; TNBA, tetra-*n*-butyl ammonium.

Chemicals.—Arginine, Nutritional Biochemicals Corporation, Cleveland; ethylenediaminetetraacetate, Matheson Coleman and Bell, East Rutherford, New Jersey; tetra-*n*-butyl ammonium iodide, Eastman Kodak Co.; fluoro-chemical oil, kind gift of Dr. J. S. Johnson, Oak Ridge National Laboratories, Oak Ridge; canavanine, California Corporation for Biochemical Research, Los Angeles; trypsin (2 times crystallized), Worthington Biochemical Corporation, Freehold New Jersey; sheep anti-rabbit globulin, Dr. David Novelli, Oak Ridge National Laboratories, Oak Ridge, Tennessee; TC-199, Difco Laboratories, Inc., Detroit; 6-mercaptopurine riboside, National Chemotherapy Service Center, National Institute of Health, Bethesda; sucrose, Nutritional Biochemicals Corporation; tar, commercial coal tar sources; horse liver arginase, generous gift of Dr. David Greenberg, University of California, San Francisco, California.

Methods.—

Virus Inoculation.—Scarification method following three pretreatments with 50:50 turpentine and acetone (7).

Arginase Purification.—The papilloma tissue was freed of keratin and other extraneous tissues by cutting them away. It was then ground with sand and extracted at −8°C with 3 volumes of 30 per cent ethyl alcohol in sodium acetate buffer at an ionic strength of 0.03 and a pH of 5.8. The supernate was discarded and the sediment extracted with 2 volumes of 19 per cent ethyl alcohol in sodium acetate buffer at an ionic strength of 0.02 and pH 5.8 at −5°C. The supernate was centrifuged at 15,000 RPM for 30 minutes, dialyzed overnight against 0.001 M maleate and lyophilized. The dried material was dissolved in a small volume of water and passed through a DEAE column that had been equilibrated and eluted with tris chloride buffer at pH 8. The fractions containing enzyme activity were pooled, dialyzed, lyophilized, and passed through a sephadex G-75 column previously equilibrated and eluted with 0.001 M Mn maleate (pH 6.5). The active fractions from this run were dialyzed, lyophilized, dissolved in water, and put on a carboxymethyl cellulose column, equilibrated with 0.01 M NaOAc buffer containing 0.001 M Mn maleate (pH 5.0), and eluted with the same buffer at pH 5.8. The active fractions were dialyzed and lyophilized. Arginase from liver was purified in an identical manner. This method, previously described in detail (2), is a modification of one devised by Anderson (8) for purification of proteins.

The preparations of "purified" arginase were then checked for homogeneity. Fractions used for subsequent work showed the following: a single peak in the analytical centrifuge with schlieren optics and with monochromatic ultraviolet at 280 mμ, a single band in sucrose gradients, a single peak in free electrophoresis, and a straight line upon plotting the log of the concentration against the square of the radial distance at sedimentation equilibrium (9).

Molecular Weight.—The interferometric system of Richards and Schachman (9) at sedimentation equilibrium in the Spinco model E centrifuge was used except with the wild rabbit papilloma arginase. In the latter instance, not enough material was available for use in the synthetic boundary cell for the determination of the concentration equivalent of the fringe numbers. Therefore, the method was modified in the following way: with a short column (about 0.08 cc) of arginase in solution on top of a column of fluoro-chemical oil, the rotor was spun at 8000 RPM until sedimentation equilibrium was reached (as determined by following the position of the middle fringe using a Gaertner microcomparator from day to day until no further change occurred, reference 9). Since the number of fringes crossed in measuring the lengths of the column (the method used by Richards and Schachman to measure the concentration throughout the column of fluid at sedimentation equilibrium) is directly proportional to the curvature of the fringes as one approaches the bottom of the cell, plotting the log of the increments in slope of the middle fringe against the square of the radial distance yields a straight line with the same slope as that obtained with the Richards and Schachman method.

The slope of this line gives an estimate of molecular weights with reproductability of about ±2 per cent. An estimate can be obtained when as little as 0.25 mg of pure protein is available.

Partial Specific Volume.—The partial specific volumes were estimated from the density (10) of the sucrose solution required to float the enzyme (as measured by activity and by banding) in a centrifugal field of 125,000 *g* for 60 hours at 20°C. The Spinco model L centrifuge with the swinging cup (SW-39) rotor was used.

Peptide Patterns.—The enzymes were freed of potential bacteria by centrifugation, hydrolyzed with 1 per cent trypsin, chromatographed ascendingly in butanol:acetic acid:water (40:10:50) in one dimension and then electrophorized in the other direction using a pyridine:glacial acetic acid:water (10:100:2390) buffer at 2000 volts for 1 hour (11). The sheets were sprayed with ninhydrin to locate the material.

Amino Acid Analysis.—The enzymes were solubilized, freed of any potential bacteria by centrifugation for 1 hour at 15,000 RPM, and hydrolyzed with 6 N HCl (12) at 100°C in sealed evacuated tubes. The hydrolysate was then dried, washed, freed of HCl, and put on a Spinco amino acid analyzer (Moore and Stein) for determination of amino acid composition (12).

Manganese, Sulfur, and Nitrogen Content.—Elemental analysis was carried out by Galbraith Laboratories, Knoxville, Tennessee.

Unit Enzyme Activity.—The number of arginase units was calculated from the amount of urea nitrogen released per minute upon incubation of the activated arginase with a 3.7 M arginine (13) at pH 8.6. The nitrogen content of the purified enzyme was determined, and the enzymatic activity was expressed as arginine units per milligram of nitrogen. Activities were assayed with arginine at pH 8 (incubation for 2 to 4 hours). The production of ornithine was detected chromatographically.

Preparation of Sucrose Gradients.—Plastic or quartz tubes for the Spinco SW-39 rotor were layered with the solution of higher density in the bottom half of the tubes and the lower density above. The tubes were stored at 3°C for 24 hours; the protein solution (0.1 or 0.2 cc) was then layered on top.

Precipitin Reaction.—Sera were heated at 65°C for 30 minutes to eliminate possible nonspecific antitissue antibodies (14), and diluted with saline as indicated. The antigens were dissolved in saline and layered over the serum. After incubation at 37°C for 2 hours, the tubes were examined and the presence of a precipitin band at the antigen-serum interface recorded.

PHYSIOCHEMICAL CHARACTERIZATION

Separation of Arginases on Sucrose Gradients.—1 mg amounts of the individual arginases were dissolved in 0.25 cc of water, and 0.2 cc of this solution was layered on top of the sucrose gradients as indicated in Table I. The tubes were spun at 39,000 RPM for 20 hours using the Spinco SW-39 rotor. The bottoms of the plastic tubes were then punctured with a No. 20 gauge short bevel needle and the solution allowed to drip out. Three drops were collected per tube and all tubes analyzed for enzyme activity. In Table I, the tube where the first enzyme activity was detected is recorded.

It is clear that wild and domestic rabbit papilloma (pap.) arginase acted alike in these gradients and could be readily separated from liver or carcinomatoid arginase. The difference in the position of the arginase has only significance within the individual gradient sort. The conditions of prior dialysis varied from experiment to experiment and possibly modified the density of carcinomatoid and liver arginase because of manganese binding.

Sedimentation Velocities.—0.5 per cent solutions of wild or domestic rabbit papilloma arginase were centrifuged at 59,600 RPM following 48 hours' dialysis against water, and the

sedimentation velocity at 10°C determined using the Spinco model E centrifuge. Liver arginase from the wild or domestic rabbit was handled similarly but it had to be dialyzed against 0.001 M manganese maleate or against 0.001 M phosphate buffer because of its relative insolubility in water. The comparative sedimentation velocities corrected for temperature to 20°C are recorded in Table II.

TABLE I

Sucrose Gradient Studies of DR Shope Papilloma, Rabbit Liver Arginase, and Arginase from Tar-Induced Carcinomatoids

	Tube 1st positive
Gradient: 10 to 85 per cent	
Tar pap. I	Neg.
Tar pap. II	Neg.
Carcinomatoid	45
Shope pap	41
DR liver	45
DR pap	41
DR pap. + liver	41-fade-46
	Tube*
Gradient: 25 to 35 per cent	
Carcinomatoid	12
Shope pap	7
DR liver	13
Gradient: 25 to 50 per cent	
WR pap	53
WR liver	59
DR pap	53

4°C, 20 hours' SW-39, 39,000 RPM.

* 1st tube 1.2 cc; 3 drops/tube thereafter.

TABLE II

*S^{w}_{20} of Arginases**

DR Liver	4.4
WR Liver	4.6
DR Pap	3.7
WR Pap	3.8

* Determined in 0.001 M Mn maleate at 59,780 RPM in the Spinco model E centrifuge.

It is evident that again the papilloma arginases resembled each other, and differed from liver arginase of either the wild or domestic rabbit.

Molecular Weights.—The molecular weights were determined using the system described by Richards and Schachman (9). The results are given in Table III (domestic rabbit liver arginase) and Table IV (domestic rabbit papilloma arginase), both the number average and weight average molecular

TABLE III A

Analysis of Plate Depicting DR Liver I at Sedimentation Equilibrium

	x	x^2	Δm	$(\Delta m)\,x$
Centripetal reference edge	5.706			
Meniscus	6.677	44.582		
Crossover 1	6.688	44.729	0.211	1.41
Crossover 2	6.745	45.495	1.211	8.168
Crossover 3	6.795	46.172	2.211	15.02
Crossover 4	6.837	46.745	3.211	21.95
Crossover 5	6.872	47.224	4.211	28.94
Meniscus	6.908	47.720	5.234	36.16

x signifies distance from axis of rotation and Δm signifies differential fringe numbers between meniscus and point x.

TABLE III B

Molecular Weight Determination for DR Liver

Components of equation	Value	Equation	Molecular weight
R	8.313×10^7 ergs/degree mole	$\text{MW} = \dfrac{2RT\,(c_b - c_m)}{(1 - \bar{V}\rho)w^2(x_b^2 - x_m)^2 c_o}$	38,100
T	283 degrees Kelvin		
w^2	8440×10^2 radians/sec.		
$(1 - \bar{V}\rho)$	0.208		
$c_b - c_m$	5.234		
c_o	11.824		
$x_b^2 - x_m^2$	3.138 cm^2		
$\bar{V}$	0.79	$\text{MW} = \dfrac{2RT\,\dfrac{d \ln c}{dx^2}}{(1 - \bar{V}\rho)w^2}$	36,600
ρ	1.0026		
$\dfrac{d \ln c}{dx^2}$	0.136		
$c_b - c_m)/c_o(x_b^2 - x_m^2)$	0.141		

The value c_o was determined using a synthetic boundary cell and the concentration value in terms of fringes.

w = angular velocity in radians per second. R = gas constant. T = absolute temperature. $\bar{V}$ = partial specific volumn. ρ = density of a solution. x_b = radial distance from center of rotor to the bottom of column. x_m = radial distance from center of rotor to menicus. c_b = concentration at the bottom of the column. c_m = concentration at the menicus. c_o = original concentration. ln = natural log

TABLE IV A

Analyses of Plate Depicting DR Pap. I at Sedimentation Equilibrium

	x	x^2	Δm	$(\Delta m)x$
Centripetal reference edge	5.705			
Meniscus	6.696	44.836		
Crossover 1	6.704	44.944	0.202	1.35
Crossover 2	6.742	45.455	1.202	8.104
Crossover 3	6.779	49.955	2.202	14.93
Crossover 4	6.807	46.335	3.202	21.80
Crossover 5	6.832	46.676	4.202	28.71
Crossover 6	6.852	46.950	5.202	35.64
Crossover 7	6.873	47.238	6.202	42.63
Meniscus	6.985	47.541	7.202	49.66

x signifies distance from axis of rotation in centimeters and Δm signifies differential fringe number between meniscus and point x.

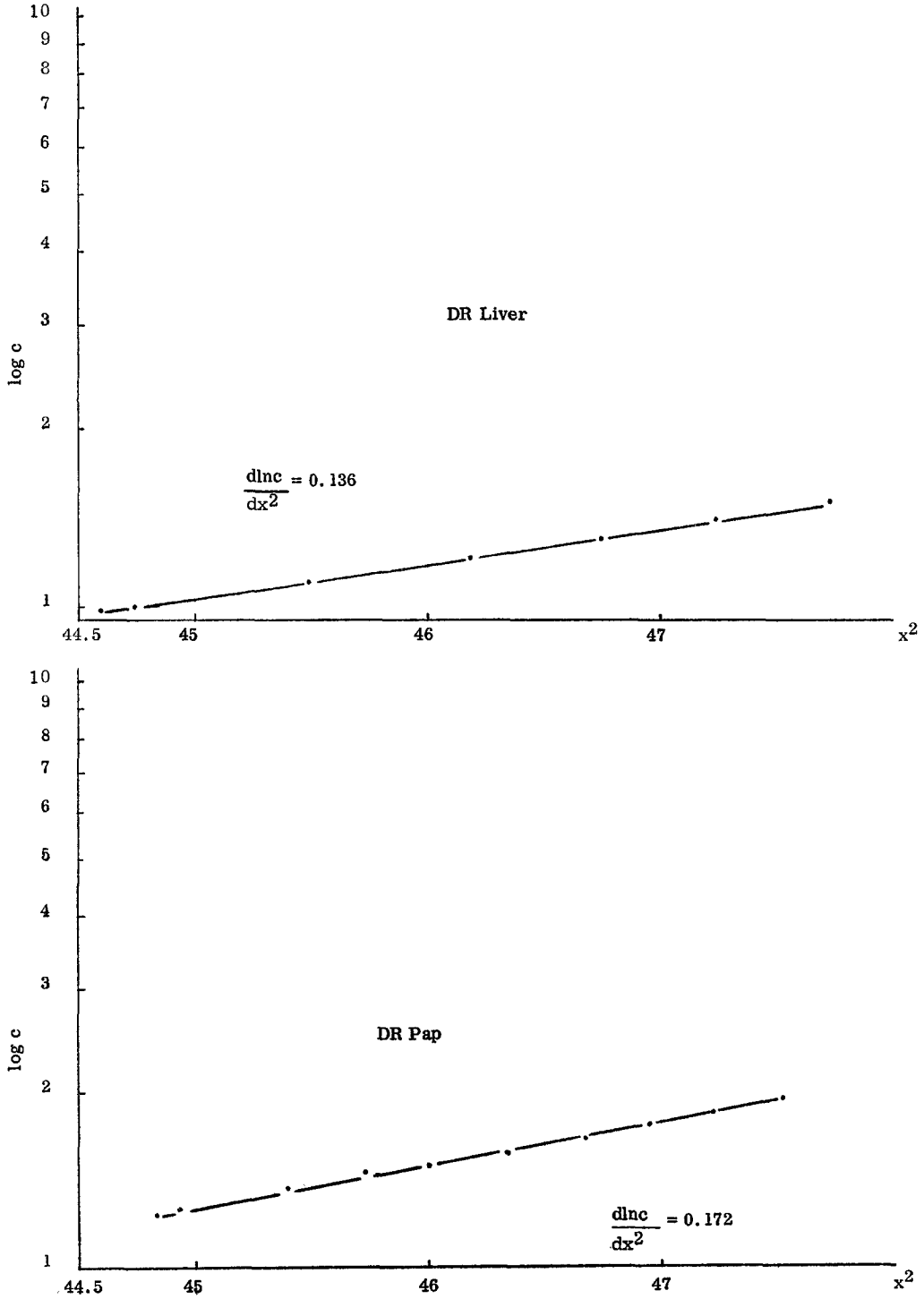
TABLE IV B

Molecular Weight Determination of DR Pap.

Components of equations	Value	Equation	Molecular weight
R	8.313×10^7 ergs/degree mole		
T	283 degrees Kelvin		
w^2	8440×10^2 radians/sec.	$\mathrm{MW} = \dfrac{2RT\,(c_b - c_m)}{(1 - \bar{V}\rho)w^2(x_b^2 - x_m^2)c_o}$	42,900
$\bar{V}$	0.77		
ρ	Assumed 1.003		
$(1 - \bar{V}\rho)$	0.228		
$c_b - c_m$	7.202		
c_o	15.192		
$x_m^2 - x_b^2$	2.705	$\mathrm{MW} = \dfrac{2RT\,\dfrac{d\ln c}{dx^2}}{(1 - \bar{V}\rho)w^2}$	42,200
$\dfrac{d\ln c}{dx^2}$	0.172		
$(c_b - c_m)/c_o(x_b^2 - x_m^2)$	0.175		

The value c_o was determined using a synthetic boundary cell and the concentration value in terms of fringes.

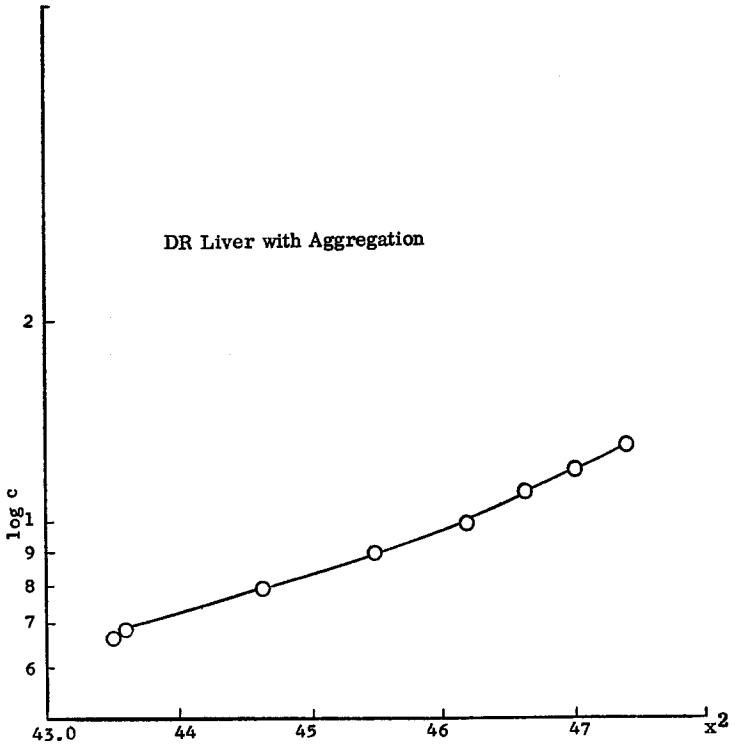
weights being shown. The number average molecular weight, computed graphically, is less accurate but is not influenced by aggregation, while the weight average value can be misleading if aggregation has occurred. The test for nonidealty and polydispersity (Text-fig. 1) revealed no detectable aggregation with



TEXT-FIG. 1. Plots of log of concentration in fringes against square of the radial distance. The straight lines are indicative of the degree of homogeneity and their slope a function of individual molecular weight. The apparent slope is distorted by the semi-log plot.

these preparations, although this sometimes occurs with liver arginase (Text-fig. 2).

The amounts of wild rabbit papilloma arginase were not sufficient for measurement in the synthetic boundary cell. For this reason, the modification described under Methods was used. The results are shown in Table V and



TEXT-FIG. 2. Plot of another preparation of liver arginase using log of concentration fringes against square of the radial distance. The change in slope in the last third of the line is indicative of aggregation, common with liver arginase.

Text-fig. 3. Again the papilloma enzyme resembles the domestic rabbit papilloma arginase and differs from rabbit liver arginase.

To test for possible polymerization of a smaller subunit into a larger active unit, papilloma and liver arginase were exposed to tetra-*n*-butyl ammonium (TNBA) ions (known to be quite effective in the depolymerization of tobacco mosaic virus protein, reference 15). 0.05 M TNBA ions had no influence upon the turbidity of either liver or papilloma arginase solutions when incubated at room temperature (25°C) for 2 hours. This treatment thus did not appear to depolymerize the enzymes.

Partial Specific Volumes.—The partial specific volumes were determined for the purified enzymes by equilibration in density gradients at 125,000 *g* for 60 hours. Table VI shows the results. Again, wild and domestic rabbit papilloma arginase proved alike, while differing from the liver or kidney arginases.

Is the Higher Molecular Weight and Density of Papilloma Arginase due to Absorbence of Nucleic Acid or Polysaccharides?—Since the work of Landsteiner many years ago, it has been known that an animal might make antibodies against its own protein if it is combined with new chemical groupings. However, the ultraviolet absorbence spectrum failed to show nucleic acid absorption, and a highly sensitive test for polysaccharides (the Molisch reaction) revealed no polysaccharide in papilloma arginase, though it was positive when as little as 0.01 per cent of the protein present was added as sucrose.

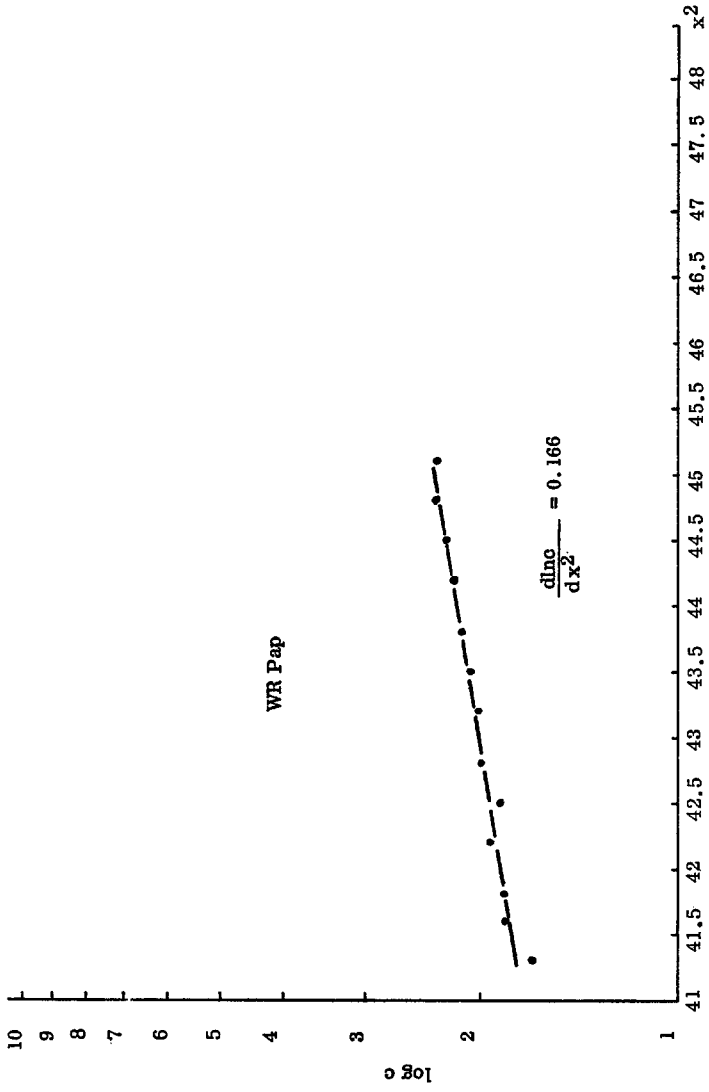
TABLE V

Molecular Weight Determination of WR Pap.

Components of equations	Value	Equation	Molecular weight
R	8.313×10^7 ergs/degree mole		
T	283 degrees Kelvin		
w^2	8440×10^2 radians/sec.		
$\overline{V}$	0.77		
ρ	Assumed 1.003		
$(1 - \overline{V}\rho)$	0.228		
		$\mathrm{MW} = \frac{2RT \frac{d\ln c}{dx^2}}{(1 - \overline{V}\rho)w^2}$	41,000
$\frac{d\ln c}{dx^2}$	0.166		

Are There Differences in the Activation of the Enzymes?—Domestic rabbit liver arginase contained 13 per cent nitrogen and, after 60 hours' dialysis against water, 5 per cent manganese; papilloma arginase contained 16 per cent nitrogen and no manganese. The absence of manganese in the enzyme or any evidence of manganese binding by papilloma arginase, (as judged from the lack of influence of manganese on the sedimentation velocity together with the elemental analysis) made it seem worthwhile to find whether the papilloma arginase required the presence of manganese for activation. All previously described arginases (16–18) have required either manganese or cobalt, and similar requirements were found for the other rabbit enzymes prepared in the present study. As is usual, manganese proved about twice as effective as cobalt. With manganese activation, the purified liver enzyme had an activity of 5400 arginase units (13) per mg protein nitrogen.

Papilloma arginase again proved very different. Papilloma arginase was



TEXT-FIG. 3. Plot of log of concentration in increments of curvature of middle fringe against square of the radial distance of wild rabbit papilloma arginase.

active even after treatment with 1 per cent EDTA to eliminate traces of divalent ions. Papilloma arginase, without activation, had an activity of 2500 arginase units per mg protein nitrogen. Wild and domestic rabbit papilloma arginase were similar. It is noteworthy that the manganese in liver arginase is bound to the degree that treatment with 1 per cent EDTA causes its precipitation while no precipitation occurred with papilloma arginase. Since it has been reported (19) that the sedimentation velocity of liver arginase is influenced by the amount of bound manganese, the movement of papilloma arginase in sucrose gradients was measured with and without manganese. No effect was found.

TABLE VI

Partial Specific Volumes of Arginases*

	Sucrose	PSV
	per cent	
DR liver	56	0.79
WR liver	56	0.79
DR kidney	56	0.79
Horse liver	56	0.79
DR Pap	62	0.77
WR Pap	62	0.77

* Determined as the reciprocal of the density of sucrose giving zero sedimentation of the enzyme following 60 hours at 39,000 RPM using the Spinco SW-39 rotor.

Peptide Patterns.—A change in the position of a single amino acid in the hemoglobin molecule results in a discernible difference in peptide pattern (20). The peptide patterns of liver and papilloma arginase derived from wild and domestic rabbits were therefore compared.

10 mg of each arginase was denatured by heating at 96°C for 4 to 6 minutes at pH 7. Following this, they were incubated 60 minutes at 32°C with 1 per cent trypsin at pH 8.2, chromatographed in one direction, and electrophorized in the second direction.

The peptide patterns of wild and domestic rabbit liver arginase are shown in Fig. 1. It is evident that though there are differences they roughly resemble each other, and further are very different from the wild or domestic rabbit papilloma arginases (Fig. 2) which are remarkably alike.

Amino Acid Analysis.—The amino acid analysis of domestic rabbit liver and papilloma arginase is shown in Table VII. The previously reported results of Greenberg with horse liver arginase (21) and of Grassman with calf liver arginase (19) are included for comparative purposes. The absence of sulfur-containing amino acids in papilloma arginase was confirmed by elemental analysis which showed that the protein was sulfur-free. 3 mg of protein was

used. Liver arginase also contained no detectable sulfur. Arginase derived from calf liver has been reported to contain no proline. On several occasions in these studies of rabbit liver and papilloma arginase there has been no detectable proline when the columns were eluted at 30°C. These same preparations however, were found to contain proline when eluted at 50°C or when checked using paper chromatography. The apparent absence of proline at 30°C results from the frequent elution of proline and glutamic acid together at this temperature.

TABLE VII

Amino Acid Composition of Purified Arginase

Amino acid	Horse liver* Amino acid residues 100 gm protein	Calf liver‡		DR liver		DR pap.	
		μmoles found	μmoles per cent	μmoles found	μmoles per cent	μmoles found	μmoles per cent
Aspartic	6.46	1.0	9.49	0.6930	9.4	0.2919	7.1
Glutamic	8.98	0.96	9.11	0.7940	10.8	0.3200	10.3
Glycine	4.61	1.62	15.38	0.5950	8.1	0.3890	9.3
Alanine	1.85	0.99	7.40	0.5430	7.4	0.3800	9.2
Serine	4.49	0.80	7.60	0.2321	3.1	0.2620	6.3
Threonine	4.53	0.68	8.46	0.3958	5.4	0.2650	6.4
Valine	7.36	0.85	8.07	0.4270	5.8	0.2530	6.1
Isoleucine	11.81	0.52	4.94	0.5020	6.8	0.2185	5.3
Leucine		0.98	9.30	0.7030	9.5	0.3630	8.7
Phenylalanine	3.66	0.51	4.84	0.3175	4.3	0.1429	3.3
Histidine	NR	0.10	0.95	0.1948	2.6	0.1309	3.1
Tyrosine	0.87	0.20	NR	0.2351	3.2	0.1001	2.4
Lysine	6.91	0.92	8.74	0.7840	10.6	0.2910	6.9
Arginine	4.30	0.40	3.82	0.3750	5.1	0.1252	3.0
Proline	5.65	0.1	0	0.5610	7.6	0.2775	6.7
Methionine	1.43	0.05	0	0.0105	0.14	NR	0.0
Cystine	NR	NR	NR	0.0135	0.18	NR	0.0

NR, not recorded.

* Greenberg, 1956.

‡ Grassman, 1958.

Whether this artefact occurred in the amino acid analysis of calf liver arginase is not known. The amount of proline detected in rabbit liver and papilloma arginase could not be derived from contamination in view of the homogeneity tests.

Does Purified Papilloma Arginase have other Biological Activity?—The purified papilloma arginase was tested to find whether it has detectable activity against other amino acids. 2 mg of arginase was put in 3 cc of tissue culture media 199 and incubated at 37°C at pH 7 or 18 hours. No effects were found on amino acids other than the conversion of arginine to ornithine by arginase.

Will Anti-Rabbit Globulin React with Arginase?—The above data, together

with that previously reported (2), provide evidence that papilloma arginase is derived synthetically from virus rather than rabbit information. The influence of anti-rabbit globulin on rabbit liver and papilloma arginase was therefore tested.

A 1 per cent solution each of the two arginases was layered over a normally reconstituted solution of anti-rabbit globulin in saline. A strong precipitin occurred at the globulin-liver arginase interface while none appeared with papilloma arginase. The incubation period was 2 hours at 37°C and overnight at 4°C.

Though not conclusive in itself, this experiment provides supporting evidence that the papilloma arginase is not of rabbit origin.

Is There an Incorporation of Wild Rabbit Genetic Information in the Virus DNA?—The following experiment was designed to find whether the virus, derived from tumors of the wild rabbit, might be bringing wild rabbit genetic information along with it.

Three domestic rabbits were immunized with a 10 per cent saline extract of normal wild rabbit skin. The immunization procedure was to inject each of the animals subcutaneously, intramuscularly, and intraperitoneally once weekly for 3 weeks. After an interval of 1 week the animals were bled from the heart. The sera were heated at 65°C for 30 minutes, then precipitin reactions were carried out against 10 per cent saline extracts of the following domestic rabbit tissues: Shope papillomas, tar-induced papillomas, the Vx-2 transplantable carcinoma (originally arising in a Shope papilloma, reference 22), normal skin, and normal kidney. Purified Shope papilloma arginase, "purified" virus, and an extract of normal wild rabbit skin were also tested. The virus was purified by centrifugation three times at 90,000 *G* for 1 hour in 0.05 M phosphate buffer and, finally, by separation of the single band sedimenting at 120,000 *G* after 48 hours in 80 per cent sucrose. The Shope papilloma virus used to provide the domestic rabbit papilloma material used for antigen was from the batch purified as outlined. A measured of its purity was provided by the negative precipitin test with the DR anti-WR skin sera. In addition, it is well known that repeated centrifugations alone provide homogeneous preparations which do not contain other virus-like particulates observable with the electron microscope (23). Sera from domestic rabbits carrying Shope papillomas and that from normal rabbits were also tested as indicated.

The results are shown in Table VIII. It is plain that the Shope virus–induced papillomas of the domestic rabbit contain an antigen not found in other domestic rabbit tissues tested. This antigen is not related to the virus protein coat, the virus–induced arginase, or Vx-2 antigen. It appears therefore that in addition to virus information, the DNA of the virus brings along information for the synthesis of an antigen reacting immunologically as if of wild rabbit origin. The nature of the antigen is unknown.

The Influence of Arginine Supplements on the Growth of the Tumors.—The postulated relation of the enzyme to the metabolism of the papilloma is to deplete cellular arginine, interfere in nuclear histone synthesis, and thereby remove a cell growth control mechanism (2). Should it be possible to by-pass the enzyme and make more arginine available, the growth of the tumor should be slowed. The following experiments were designed to cast light on this point.

Simple supplementation of the diet or parenteral injection of amino acids will not suffice to raise the blood level of amino acids, as any such influence is countered by adaptive changes in the metabolism of the animal (24). Because of this, doses of the arginine antagonist, canavanine, which acts to tie up arginase (25), were given to rabbits in amounts insufficient to cause the animals to

TABLE VIII

A Wild Rabbit Antigen in DR Papilloma

		DR Shope pap.	DR tar pap.	DR skin	DR kidney	V x-2 tumor	WR skin	Shope pap. arginase	Shope virus*
DR anti-WR skin sera	1	+	−	−	−	−	+	−	−
	2	+	−	−	−	−	+	−	−
	3	+	−	−	−	−	+	−	−
DR sera from rabbits with pap.	4	+	−	−	−	−	+	+	+
	5	+	−	−	−	−	+	+	+
Normal DR sera	6	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−

* 1 mg/cc saline.

TABLE IX

Inhibition of Arginases by Canavanine

	Amount of arginine remaining*		
		Arginine only	Arginine + canavanine‡
Incubation time	0	2 hrs.	2 hrs.
	per cent	*per cent*	*per cent*
DR liver	100	25	50
DR pap	100	20	75

* 0.1 mg arginase per 1.2 cc of solution pH 8.6. Liver arginase activated with manganese. Papilloma arginase without manganese.

‡ Equal amounts of arginine and canavanine.

lose weight. In addition, they were given large parenteral supplements of arginine. That canavanine inhibits papilloma as well as liver arginase *in vitro* is shown in Table IX.

In parallel work (26) we have found that the nucleoside, adenosine, is used preferentially over adenine by the papilloma. This is not specific for the papilloma but characteristic of the rabbit. An antagonist of adenosine, 6-mercaptopurine riboside, was found an effective palliative chemotherapeutic agent for the papilloma, but in higher doses causes considerable weight loss.

TABLE X

Canavanine-Arginine Pap.

II

Rabbits		Canavanine	Canavanine, continued	6-MPR	6-MPR, continued
		mm/day	*mm/day*	*mm/day*	*mm/day*
5	Av. aggregate growth of paps.	0.02	0.22	0.11	0.25
5	Wt. loss av. gm	120	None	300	None

Canavanine = 2.5 gm arginine, 0.75 gm canavanine 2 times per day beginning 4th day after inoculation.

6-MPR = 10 mg/kilo 2 times per day.

TABLE XI

Canavanine-Arginine Pap.

I

Rabbits	Days postinoculation							
	9	10	12	15	17	19	21	36
Experimental								
6-44	N	N	N	N	N	+	+¼	
6-45	N	N	N	+	+	+½	+½	
6-37	N	N	N	N	N	+	+¼	+++3½
6-42	N	N	N	N	N	+	+¼	+++3½
6-43	N	N	N	N	+	+¼	+½	++2½
Control								
6-40	N	N	+	+	+¼	+½	+1	
6-41	N	+	+	+¼	+¼	+½	+1	++2½
6-47	N	+	+H	+H	+H	+H	+1	+2
6-38	N	N	+	+H	+H	+½	+1	++2½
6-39	N	+	+H	+H	+H	+H	+¾	++2

	Experimental	*Control*
Av. aggregate growth of paps./day since inoculation	0.07 mm/day	0.17 mm/day
Weight loss	Av. 20 gm	None

N, no tumors; H, hair.

2.5 gm. arginine, 0.38 gm. canavanine 2 times per day beginning 4th day after inoculation.

No injection between 21st and 36th day.

In the following experiment, the relative effectiveness of the canavanine-arginine treatment is compared with that of the more classic metabolic antagonist, 6-MPR, Table X. Though both inhibited the growth of the papillomas, 6-MPR was not nearly as effective even though the animals receiving it lost

considerable weight. There was little weight loss by the canavanine-arginine group. In the next experiment, Table XI, the dose of canavanine was reduced and the experimental animals manifested no non-specific toxic effect. Again, the inhibitory effect upon the growth of the papillomas was manifest by the increased incubation period and decreased growth rate after gross appearance. It is noteworthy that the growth rate of the hair of the rabbits receiving canavanine and arginine was greatly slowed. This particularly is of interest in that in certain other species the hair follicles have been shown to be made up in part of cells containing an arginase (27).

DISCUSSION

The results now available relating to the Shope virus–induced arginase, inclusive of that previously published, are shown in Table XII. It is evident that in every respect tested the virus–induced arginase from wild and domestic rabbit papilloma is the same. Further, it differs strikingly from the other arginases of wild or domestic rabbits and that derived from the horse liver. Papilloma arginase is unique among reported arginases in not requiring divalent ions such as manganese or cobalt for activation (16–19, 21). It follows that the splitting off of urea from arginine by this enzyme works through a different mechanism than that of enzymes of this sort previously studied. On comparing it with liver arginase, the slower movement of papilloma arginase in sucrose gradients and its lower sedimentation velocity is noteworthy in view of its greater density and higher molecular weight. This comparison suggests a helical or rod-like shape for papilloma arginase as opposed to a more spherical shape for liver arginase. The high partial specific volume, 0.79 ml/gm, of liver arginase is indicative of the presence of a lipid component. The positive Molisch reaction, indicating the presence of polysaccharide, together with the high partial specific volume, suggests that liver arginase is attached to a membrane. Its 13 per cent nitrogen content is an indication of the extent to which it is non-protein. Papilloma arginase, in contrast, is more a typical soluble protein. Marked differences in solubility are also noteworthy in this relation. Papilloma arginase is relatively easily dissolved in water. Liver arginase requires a greater ionic strength. This finding relates perhaps to the probable lipid component of liver arginase, which may relate in turn to its activation by the divalent ions, manganese or cobalt. Taken together, these results provide evidence that the virus–induced arginase is synthesized from virus DNA information rather than being a rabbit protein.

The 42,000 molecular weight of papilloma arginase has particular pertinence when viewed in the light of the molecular weight of the Shope virus DNA. This is on the order of 4 to 6 million, and it is double-stranded (28). Information responsible for the induction of the papilloma is derived from the virus DNA (29). It has been estimated that a protein with molecular weight on the order of 380,000 may be coded on a DNA of this size (30).

In addition to the arginase and the wild rabbit antigen associated with Shope virus–induced papillomas, there are three other antigens previously known to be associated. These are the protein coat of the virus (3), the Vx-2 antigen (first found in a transplantable carcinoma arising from a Shope papilloma (22) and

TABLE XII

Comparative Arginase Studies

	DR liver	WR liver	Tar carcinomatoid	Tar pap.	WR or DR skin	DR pap.	WR pap.	Vx-7	Vx-2
Arginase	+	+	+	−	−	+	+	+	−
Antigenicity*	−	−	−		−	+‡	+‡	+	−
Sheep anti-rabbit globulin	+					0			
Homogeneity	S_{20}^{w} Electrophoresis Sucrose x^2: Inc	S_{20} Sucrose				S_{20} Electrophoresis Sucrose x^2: Inc	S_{20} Sucrose x^2: Inc		
Solubility	Fair	Fair				Excellent	Excellent		
Tube sucrose gradient	41	41	41			46	46		
S_{20}^{w}	4.4	4.6				3.7	3.8		
PSV	0.79	0.79				0.77	0.77		
MW	37,000					42,000	41,000		
Peptide analysis	A	A±				B	B		
Amino acid analysis	Yes					Yes			
Mn activation	Yes	Yes	Yes			No	No		
Co activation	Yes	Yes				No	No		
Mn content	5 per cent					0			
Sulfur content	0					0			
N^2 content	13 per cent					16 per cent			
Molisch	Positive					Neg.			

PSV, partial specific volume.
MW, molecular weight.
±, only roughly similar to A.
* Virus does not absorb antibodies against arginase.
‡ Serum taken from animals several months after virus induction of tumors.

since found in another carcinoma), and the Vx-7 of similar origin (31), and Evans' antigen which is related to the retrogression of papillomas (32). The experiments reported herein, together with those previously reported, (22, 2) make clear that arginase, the protein coat of the virus, the Vx-2 antigen, and the wild rabbit antigen are antigenically distinct. There is no direct information on whether Evans' antigen is one of the above or is distinct. Should the

Vx-2 and Evans' antigen, like the protein coat, arginase, and (presumably) the wild rabbit antigen, be derived synthetically from information brought in with the virus DNA, we may well be approaching the limit of 380,000 molecular weight suggested for the Shope virus DNA. The presence of the wild rabbit antigen in the papillomas, furthermore, raises the question as to the actual size of the virus DNA. Its presence seems indicative of integration of the virus DNA in the host genome of the wild rabbit, judging from what is known of lambda phage and the gene GAL in bacteria (33). The possibility that such integration might occur with tumor viruses has been suggested by Dulbecco and Vogt (34).

The fact that the growth of the papillomas may be repressed by large supplements of arginine in the presence of canavanine supports the hypothesis that the action of the arginase is to deplete cellular arginine and reduce in turn the synthesis of arginine-rich nuclear histones (2). Judging from the work of Allfrey and Mirsky (4), and more recently that of Huang and Bonner (35), this may release the nucleus from histone repressor effects, with results in more rapid growth of the papilloma cells. The large amount of adenosine utilized by the papilloma (26) and its low histone (2) is consistent with the report that histone acts as a supressor of chromosomal RNA synthesis (35).

Arginase may also be implicated in the growth of human warts. This is suggested from the increase in amounts of the enzyme in warts over that found in normal human skin (17). Human epidermis, in contrast to that of the rabbit[2] (18, 1) has, however, readily detectable arginase, and the epidermal enzyme, like liver arginase, requires manganese for activation.

SUMMARY AND CONCLUSION

These studies make plain that the Shope papilloma virus induces the production of an arginase in rabbit squamous epithelium, and provide evidence that the information for the synthesis of the enzyme is derived from the virus rather than the rabbit. This form of induction is therefore different from that brought about by chemicals such as galactosides (36).

Striking differences were shown between the physiochemical properties of Shope virus-induced arginase and other arginases of domestic and Kansas cottontail rabbits.

The absence of a requirement for manganese suggests the mechanism of

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[2] In a recent report (Rothberg and van Scott, *Nature*, 1961; **189** 892) arginase was reported as being in rabbit epidermis. However, it was not found extractable and the suspension system used contained large numbers of bacteria (estimated from the amount of skin used to be on the order of 5 to 10 million per cc), which could account for the enzyme activity. It is also noteworthy that, in contrast to papilloma arginase, manganese was required for activation. Further, the existence of an arginase in rabbit epithelium does not bear on the question of whether papilloma arginase is derived from viral information, but rather, if present, on metabolic interrelations within the virus infected cells.

splitting off urea from arginine by papilloma arginase probably differs from that of previously described arginases.

These findings (particularly the experiments in which it was demonstrated that papilloma growth could be greatly reduced by giving animals supplemental arginine in the presence of small amounts of the arginase inhibitor, canavanine) provide evidence that suggests the following mechanism of action for the virus: viral DNA introduces into rabbit epithelium the information for the synthesis of an arginase for which the cells have no control mechanism. The arginase depletes cellular arginine and, in turn, the synthesis of arginine-rich nuclear histones (2), thereby freeing the nucleus for greater synthetic activity (4, 35), which results in more rapid growth of the papilloma cells.

Domestic rabbit papillomas induced with purified virus derived from wild rabbit papillomas were shown to contain an antigen which reacts immunologically like a wild rabbit antigen. It was distinct from the arginase, the protein coat of the virus, and the Vx-2 antigen. The presence of the wild rabbit antigen seems indicative of integration of the virus DNA in the host genome of the wild rabbit.

Other incidental findings include the description of a simple method, accurate to within 1 per cent, for determining the partial specific volume of a protein, and the description of a modification of the Richards and Schachman method (9) enabling the determination of the molecular weight and homogeneity of a protein with as little as 0.25 mg and with an accuracy of 2 per cent.

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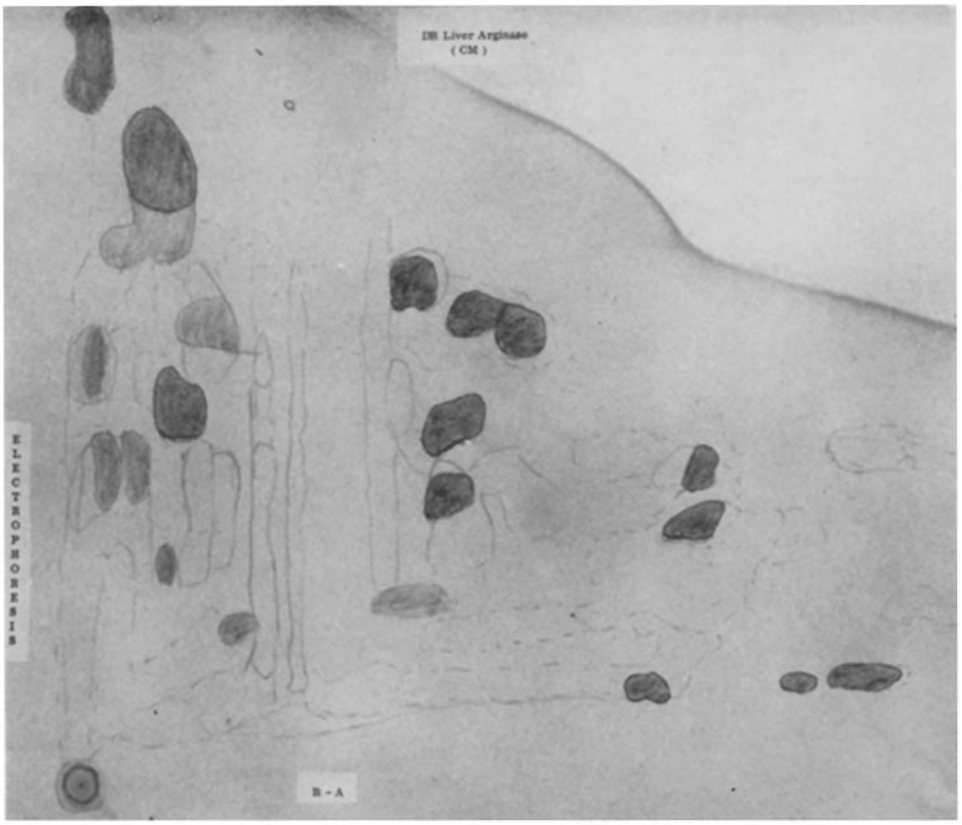
EXPLANATION OF PLATES

PLATE 24

FIG. 1. A comparison of peptide patterns of wild rabbit and domestic rabbit liver arginase. Following tryptic digestion, the digested protein was chromatographed in one dimension in butanol–acetic acid, and in another electrophorized using a puridine–glacial acetic acid–water buffer. Although the patterns resemble each other in general configuration, a number of differences are discernible. $\times \frac{3}{30}$.


DB Liver Arginase
(CM)
ELECTROPHORESIS
B - A

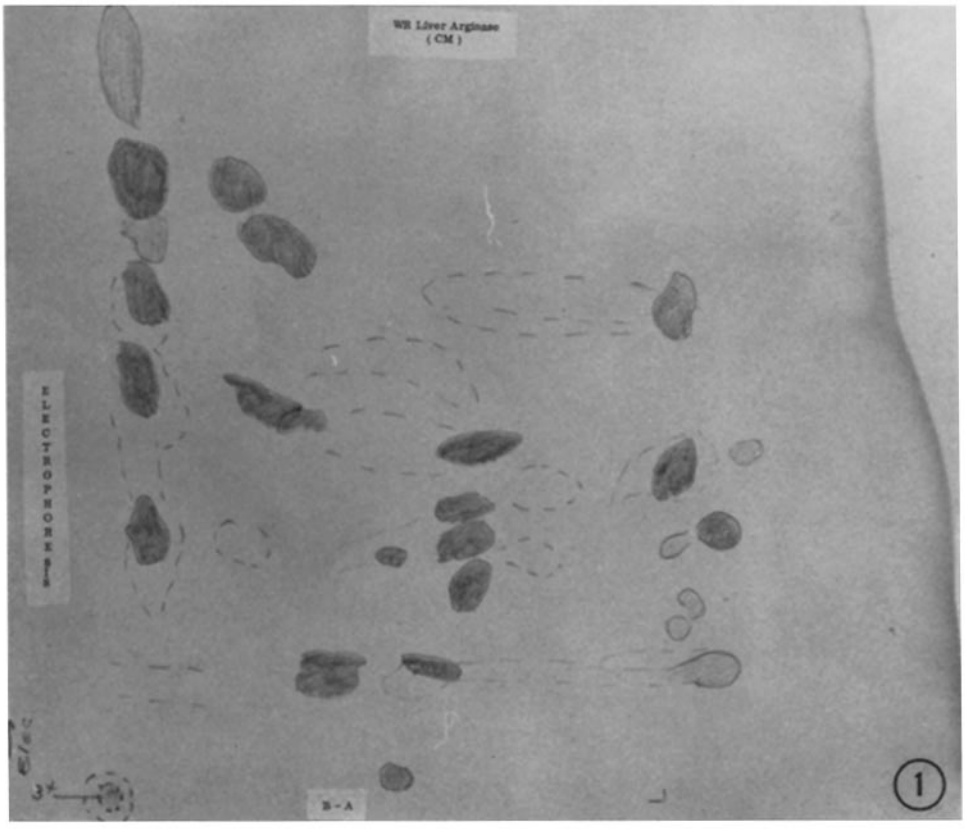
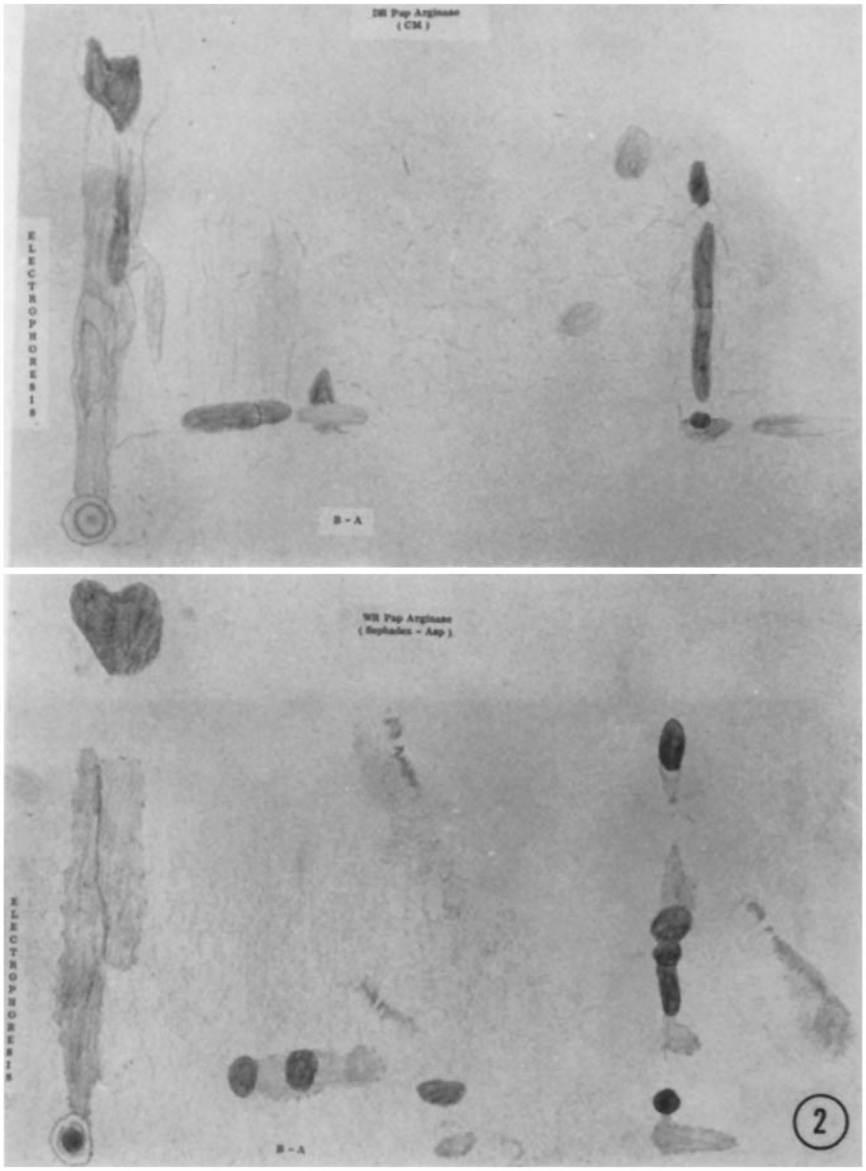

WB Liver Arginase
(CM)
ELECTROPHORESIS
B - A
1

PLATE 25

FIG. 2. A comparison of peptide patterns of wild rabbit and domestic rabbit papilloma arginase using the same system as used in Fig. 1. The remarkable resemblance of the preparations is evident, and particularly the striking difference from the patterns of liver arginase in Fig. 1. $\times \frac{13}{50}$.



(Rogers and Moore: Shope virus mechanism)