

Studies on Amino Acid Incorporation into Protein of Tumors Induced by Rous Sarcoma Virus and Hyperplasia Induced by Fowl Pox Virus in Chorioallantoic Membrane of Chicken Embryos*

SHREEPAD R. WAGLE, JAMES ASHMORE, AND ALVIN S. LEVINE

(Departments of Pharmacology and Microbiology, Indiana University School of Medicine, Indianapolis, Indiana)

SUMMARY

Comparisons were made of the rate of incorporation of C¹⁴-labeled L-amino acids into protein of chicken embryo chorioallantoic membrane (CAM) infected with viruses that induce cell proliferation. A tumor virus (Rous sarcoma virus) and a nontumor virus (fowl pox virus) were used in these studies. *In ovo* studies of glycine-C¹⁴ incorporation into the protein of Rous sarcoma virus-induced tumors showed a progressive increase in protein specific activity from the 3d to the 6th day following infection of the CAM of 10-day-old embryos, whereas uninfected CAM yielded a marked decrease in activity during the same time period. *In vitro* incubation of tissue slices with C¹⁴-labeled glycine, glutamate, and leucine showed a one- to twofold increase in amino acid incorporation into protein of Rous tumors and fowl pox hyperplasia when compared with uninfected (control) CAM from embryos of the same age. Comparisons of fractions from cell-free homogenates showed the pH-5 enzyme-microsome system of Rous tumors to have a three- to fourfold increase in amino acid incorporation into protein over that observed with similar preparations from CAM or fowl pox virus-infected tissues. Species specificity of the pH-5 enzymes was observed. Rous tumor pH-5 enzymes increased the amino acid incorporation activity of CAM and chick embryo liver microsomes but were ineffective when combined with rat liver microsomes.

Studies in these laboratories are concerned with the sequence of events involved in viral induction of neoplastic growth (1, 6, 15, 16). The present study compares the chorioallantoic membrane (CAM) of the chicken embryo with tumors and hyperplasia of CAM with respect to rates of incorporation of C¹⁴-labeled L-amino acids into protein. Tumors were induced by infection of CAM with Rous sarcoma virus, and hyperplasia of CAM was induced by infection with fowl pox virus. It is believed that the specific virus-induced cellular response offers the investigator an approach to the sequence of changes in the evolution of neoplastic growth. Virus-induced neoplasia as an experimental system has not been a favored medium for

biochemical studies. Although much knowledge exists regarding the biochemistry and metabolism of transplantable tumors (2, 4), there is a paucity of information of the biochemistry of virus-induced tumors. Studies of the amino acid incorporation process of virus-induced malignant tissues have not appeared in the literature; however, numerous studies of amino acid incorporation of normal tissue and transplantable tumors have been reported (7, 12, 17).

MATERIALS AND METHODS

Virus.—Rous sarcoma virus, lot number CT 895, was obtained from the laboratory of Dr. W. Ray Bryan at the National Institutes of Health. The fifth CAM passage of Rous sarcoma virus was stored frozen at -72°C . as a seed-pool for all experiments. An inoculum of 10^5 – 10^6 pock-forming units was dropped onto the CAM of 10-

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day embryos in a 0.2-ml. volume saline diluent as a standard procedure in these studies as previously described (6).

Fowl pox virus was obtained from the laboratory of Dr. Vincent Groupé at the Institute of Microbiology of Rutgers University. The fifth CAM passage of fowl pox virus was stored frozen at -72°C . as a seed-pool for all experiments. An inoculum of 10^5 – 10^6 pock-forming units in 0.2 ml. saline diluent was routinely used as described for Rous sarcoma virus.

Tissues and biochemical systems.—*In ovo* experiments compared Rous sarcoma virus-infected CAM with uninfected tissue for rates of incorporation of glycine- C^{14} into protein. The CAM of 10-day-old embryos was dropped, and 1 mg. of glycine containing 1 μc . of C^{14} activity was included in each 0.2 ml. of Rous sarcoma virus inoculum. An equal amount of glycine and C^{14} activity, but without virus, was injected onto the CAM of experimental control embryos. The inoculated eggs were incubated at 36°C . Rous sarcoma virus-infected and control eggs were taken from the incubator at 3, 4, 5, and 6 days, respectively, for determination of specific activity of proteins according to the method of Manchester and Krahl (9). Tissue proteins were isolated by trichloroacetic acid precipitation and then extracted with hot trichloroacetic acid. The precipitate was digested with performate and then reprecipitated with trichloroacetic acid.

In vitro experiments were concerned with comparisons of incorporation of glycine, glutamate, and leucine into protein by tissue slices and cell-free homogenates of tissue. Rous tumors and hyperplasia induced by fowl pox virus were excised 5–6 days following virus inoculation and incubation at 36°C . At this time Rous tumors were well developed, coalesced, and minimal necrosis was observed. Fowl pox virus-induced hyperplasia involved the total exposed dropped CAM after 5–6 days' incubation. Saline diluent was injected onto the dropped CAM of control eggs and incubated for 5–6 days, as was the case with virus-infected tissue.

Tissue slices: Tissue slices were made of virus-infected tissue with a Stadie-Riggs hand microtome to give sections comparable in thickness to control CAM. The tissue slices were random sections of selected tumors. Rous tumors were selected as follows: (a) tumor-bearing CAM was excised from embryonated eggs and placed in a Petri dish filled with saline; (b) the Petri dish was placed over a black surface; (c) a bright light was placed over the tissue, and the tumors appeared as opaque grayish-white areas in contrast to trans-

parent CAM; and (d) the opaque areas were dissected free of transparent CAM. Both ectodermal and mesodermal cells are malignant (11). Hyperplasia induced by fowl pox virus was excised in a similar manner. The tissues were incubated in 6 ml. of Ringer-bicarbonate medium to give an initial concentration of 1 mg/ml and a total of 2.0 – 2.5×10^5 counts/min radioactivity/flask as described previously (14). Approximately 0.5 gm. of wet tissue slices was placed in each flask. After 90 minutes' incubation at 36°C . the tissues were analyzed for C^{14} activity in protein according to the method of Manchester and Krahl (9).

Homogenates: Approximately 15 gm. each of Rous sarcoma virus- or fowl pox virus-infected tissue and normal CAM were homogenized, 5 gm. at a time, with 2.5 times their volume of buffered medium as described by Hoagland *et al.* (5). The homogenate was centrifuged at $10,000 \times g$ for 15 minutes at 0°C . The supernatant fluid, containing the microsomal and soluble fractions, was removed and centrifuged at $105,000 \times g$ for 1 hour. The supernatant fluid containing the soluble cell fractions was decanted for preparation of the pH-5 enzymes. The microsomal pellets were homogenized with the same volume of buffered medium, and the resulting homogenate was centrifuged again at $105,000 \times g$. The microsomes obtained after the centrifugations were homogenized with buffered medium to give a microsomal suspension containing 25 mg. of protein/ml. The second washing of the microsomes was required to reduce the concentration of pH-5 enzymes in the microsomes.

To prepare pH-5 enzymes, the supernatant fluid from the first centrifugation at $105,000 \times g$ was diluted with an equal volume of unbuffered medium (0.9 M sucrose, 0.004 M MgCl_2 , and 0.025 M KCl), and the pH was carefully adjusted to 5.2 by adding acetic acid drop by drop with constant stirring. The solution was kept at 4°C . during this procedure. The precipitate was collected by centrifugation in the cold. The precipitate was then dissolved in buffered medium to give a solution of the pH-5 enzymes containing 10 mg of protein/ml.

The microsomes and pH-5 enzymes thus prepared were used in all the studies reported here. The incubation mixture, containing 6 mg. of microsome protein and 2.5 mg. of pH-5 enzyme-protein, was then incubated with a C^{14} -labeled amino acid, adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenol pyruvate (PEP), and pyruvate kinase (PK). The mixtures were incubated (under 5 per cent CO_2 in N_2) for 1 hour at 37°C . After the incubation, the protein was precipitated with 10 per cent trichloroacetic acid as described previously (9).

RESULTS

Data given in Table 1 show a progressive increase in protein-specific activity by Rous sarcoma virus-infected CAM from the 3d day after infection through 6 days. The C^{14} -labeled glycine was introduced *in ovo* onto the CAM at the time of virus inoculation. In experimental control studies C^{14} -labeled glycine was injected onto CAM of 10-

TABLE 1

In Ovo INCORPORATION OF GLYCINE- C^{14} INTO PROTEIN BY CAM AND ROUS SARCOMA VIRUS-INFECTED CAM*

Day after injection of glycine- C^{14} and virus†	Uninfected CAM (counts/min/mg protein)‡	Rous sarcoma virus-infected CAM (counts/min/mg protein)‡
3	210 ± 15	200 ± 12
4	180 ± 10	300 ± 10
5	140 ± 16	380 ± 12
6	120 ± 8	420 ± 18

* CAM: chorioallantoic membrane of chicken embryo.

† 1 μ c. of glycine-2- C^{14} was injected onto the CAM of 10-day-old embryos, with or without 10^6 - 10^8 EID₅₀ of virus.

‡ Data represent average results of three experiments.

TABLE 2

INCORPORATION OF C^{14} -LABELED AMINO ACIDS INTO PROTEIN BY CAM AND TISSUE SLICES OF VIRUS-ALTERED CAM*

VIRUS INFECTION OF CAM†	No. OBSERVATIONS	C^{14} -LABELED L-AMINO ACIDS		
		Glycine (counts/min/mg protein)	Glutamate (counts/min/mg protein)	Leucine (counts/min/mg protein)
Control	5	120 ± 8	125 ± 8	110 ± 6
Fowl pox	5	205 ± 20	251 ± 12	235 ± 20
Rous sarcoma	6	260 ± 15	215 ± 10	202 ± 15

* CAM: chorioallantoic membrane of chicken embryo.

† Tissue was excised from chicken embryos 5-6 days following virus infection. Saline-injected embryos served as experimental controls. All embryos were inoculated at 10 days of age. Approximately 0.5 gm. of each tissue was incubated in 6 ml. of medium containing 1 mg/ml of the respective amino acid and a total of 240,000 counts/min/flask. The incubation was for 90 minutes at 36° C. in a shaking water bath.

day-old embryos in the absence of Rous sarcoma virus. These data show a progressive decrease in specific activity of control CAM when assayed after 3, 4, 5, and 6 days, respectively.

Table 2 summarizes the data on the *in vitro* incorporation of radioactive glycine, glutamate, and leucine into protein by slices prepared from Rous tumors, fowl pox hyperplasia, and control CAM from embryos of comparable age. The re-

sults suggest that there is an increase in the incorporation of amino acids into protein by virus-altered tissues when compared with normal control CAM.

Table 3 gives data on the amino acid incorporation into protein by the cell-free reconstituted pH-5 enzyme-microsome system prepared from various tissues. The pH-5 enzymes from Rous tumors incorporated three- to fourfold more label into protein than did the control tissues, whereas similar preparations from fowl pox hyperplasia showed no difference in incorporation of C^{14} activity from amino acids into protein when compared with control CAM. The increased protein biosynthetic activity recovered from Rous tumor was further studied by recombination experiments with fractions from homogenates of other tissues. Results of this study are given in Table 4. The data show

TABLE 3

INCORPORATION OF C^{14} AMINO ACIDS INTO PROTEIN BY RECONSTITUTED FRACTIONS OF TISSUE HOMOGENATES OF CAM AND VIRUS-ALTERED CAM*

VIRUS INFECTION OF CAM†	No. OBSERVATIONS	C^{14} -LABELED L-AMINO ACIDS		
		Glycine (counts/min/mg protein)	Glutamate (counts/min/mg protein)	Leucine (counts/min/mg protein)
Control	7	107 ± 5.5	149 ± 7.5	180 ± 10
Fowl pox	5	118 ± 8.0	136 ± 11.0	190 ± 15
Rous sarcoma	7	416 ± 20	460 ± 26	512 ± 30

* CAM: chorioallantoic membrane of chicken embryo.

† Tissue was excised from chicken embryos 5-6 days following virus infection. Saline-injected embryos served as experimental controls. All embryos were inoculated at 10 days of age. Tissues were homogenized as described in "Materials and Methods." The complete system was incubated with 6.0 mg. of microsomes, 2.0 mg. of pH-5 enzymes, 1.0 μ moles adenosine triphosphate, 0.25 μ M guanosine triphosphate, 0.25 μ moles amino acid (200,000 counts/min), 0.25 μ moles phosphoenol pyruvate, and 0.25 mg. pyruvate kinase.

that activity for the increased amino acid incorporation into protein in the Rous tumor was localized in the pH-5 enzymes. In addition, the data also indicate species specificity of pH-5 enzymes, since this preparation from Rous tumor was ineffective with rat liver microsomes and vice versa. The data further indicate that Rous tumor pH-5 enzymes stimulate the incorporation of amino acid into protein in embryonic chicken liver preparations. The results observed were similar for all three amino acids studied.

DISCUSSION

The significance of these data is the dramatic change in protein biosynthesis following infection

of CAM with Rous sarcoma virus. Since both Rous sarcoma virus and fowl pox virus induced proliferation of CAM cells, the increased amino acid-incorporating activity of Rous tumor but not of fowl pox hyperplasia indicates that increased cellular growth per se is not significant. Further, it appears that increased protein biosynthesis begins 3–4 days after the inoculation of Rous sarcoma virus. This observation coincides with the first appearance of scattered round cells identifiable with certainty as Rous sarcoma after inocula-

infected cells later increased to almost double that in the uninfected cells by the 7th day. This change took place following the plateau of maximum virus production. At this time, virus-infected cells continue to accumulate nucleotides and proteins, whereas the noninfected cells do not. It appears unlikely that the increase in protein synthesis observed is due to viral replication. The evolution of the transformed cell is believed to be the important factor. It also appears that the stimulated rate of protein synthesis in the tumor system results from

TABLE 4
INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEIN BY pH-5 ENZYME-MICROSOME SYSTEMS FROM ROUS SARCOMA VIRUS-INDUCED TUMORS AND OTHER PREPARATIONS*

SOURCE OF MICROSOMES	SOURCE OF pH-5 ENZYMES	C ¹⁴ -LABELED L-AMINO ACIDS		
		Glycine (counts/min/mg protein)	Glutamate (counts/min/mg protein)	Leucine (counts/min/mg protein)
Tumor†	Tumor	415 ± 17 (5)#	408 ± 21 (6)	502 ± 25 (5)
CAM†	CAM	124 ± 11 (5)	155 ± 9 (5)	173 ± 13 (4)
Tumor	CAM	196 ± 10 (4)	142 ± 15 (4)	182 ± 15 (4)
CAM	Tumor	285 ± 31 (4)	270 ± 24 (4)	312 ± 30 (4)
R. liver§	R. liver	120 ± 10 (4)	130 ± 8 (4)	151 ± 11 (4)
R. liver	Tumor	48 ± 8 (4)	41 ± 6 (4)	56 ± 11 (4)
R. liver	CAM	50 ± 6 (4)	55 ± 6 (4)	43 ± 6 (4)
C. E. liver	C. E. liver	128 ± 6 (4)	136 ± 14 (4)	163 ± 10 (4)
C. E. liver	Tumor	191 ± 8 (4)	188 ± 10 (4)	230 ± 8 (4)
C. E. liver	CAM	118 ± 6 (4)	136 ± 8 (4)	136 ± 8 (4)

* Tissue homogenates were prepared as described in "Materials and Methods." The reconstituted system of 6.0 mg. of microsomes and 2.0 mg. of pH-5 enzymes was incubated with 1.0 μ moles adenosine triphosphate, 0.25 μ moles guanosine triphosphate, 0.25 μ moles amino acid (200,000 counts/min), 0.25 μ moles phosphoenol pyruvate, and 0.05 mg. pyruvate kinase for 60 minutes.

† Tumor: Rous sarcoma virus-induced tumor of chorioallantoic membrane.

‡ CAM: chorioallantoic membrane from 16-day-old chicken embryos.

§ R. liver: rat liver from young adults.

|| C. E. liver: chicken embryo liver from 16-day-old embryos.

Number of observations.

tion of chicken wing-web with Rous sarcoma virus (10) and the first changes in cell morphology appearing as foci of altered chicken embryo fibroblast cells in tissue culture (8). It appears that transformation of CAM cells to tumor cells is conditional for increased protein biosynthesis in CAM. Earlier studies (13) of Rous sarcoma virus infection when compared with fibroma, herpes simplex, vaccinia, swine influenza, and Newcastle disease viruses after 48 hours' infection of CAM did not show marked metabolic differences. Studies of chemical changes in chicken embryo cells infected with Rous sarcoma virus *in vitro* (3) show that, from the time of infection to the 3d day, the average total RNA per infected cell was not different from that of uninfected cells; however, RNA in

increased activity of the pH-5 enzymes, and the data suggest evidence of species specificity in cell-free systems.

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