A STUDY ON THE METABOLIC STATE OF PROTEINS IN THE CELLS OF TWO ASCITES TUMORS

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INTRODUCTION

In connection with our quantitative studies on the multiplication of neoplastic cells *in vivo* we have previously[1] investigated the metabolic stability of deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA) and the total proteins of Ehrlich ascites tumor cells labeled with glycine-2-^{14}C and adenine-8-^{14}C. Provided that incorporated ^{14}C is not released from these cell fractions, the specific activity of DNA, PNA and proteins would be expected to decrease as an inverse function of population growth after the inoculation of labeled tumor cells. This was found to be true for the case of DNA measured in the course of one week after inoculation. After the inoculation of $17 \cdot 10^6$ tumor cells, this period corresponds to about 6 cell generations. The PNA fraction was found to lose part of its activity initially, but during the subsequent major part of the growth period it showed a behaviour similar to the DNA fraction. In contrast, ^{14}C was continuously released from the glycine-labeled proteins, indicating a degradation of the protein molecule. The results of numerous investigations[2-7] on different animal tissues have been interpreted in a similar way. However, recent studies on microorganism systems demonstrated metabolic stability of the proteins[8,9,10].

The question arises whether the differences in the relative stability of the proteins obtained in the various studies reflect characteristic differences between different biological materials or whether they are at least partly due to differences in the experimental conditions, such as in the precursor used, etc. To approach this problem, we have decided to repeat our earlier studies with the same ascites tumor but using another precursor. The findings were also compared with the behaviour of another ascites tumor line under similar experimental conditions.

MATERIAL AND METHODS

Mice: In all experiments heterozygous male albino mice of our own laboratory breed were used. The animals were 2–3 months old and had a body weight of 20 to 22 grams. They were kept on a standard pellet diet. Food and drinking water were available *ad libitum*.

Tumors: Two tumor lines were used:

(1) The Ehrlich-Stockholm ascites tumor (referred to as E-St tumor) was produced in 1948 by Dr. KLEIN from a solid Ehrlich carcinoma he received from Dr. A. FISCHER (Carlsberg Foundation, Copenhagen, Denmark). In FISCHER's laboratory the tumor had been maintained in tissue culture for several years. After its transformation to the ascitic form, it was carried by serial ascitic fluid transfers (over 400 passages) in hybrid mice. The modal chromosome number was found to be in the tetraploid range[11]. Detailed studies on the DNA content and growth characteristics of this tumor line have been published[12,13].

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(2) The Ehrlich-Landschütz ascites tumor (referred to as E-L tumor) was sent to us from Munich by Dr. CH. LANDSCHÜTZ in 1953 and has been carried since then by serial intraperitoneal transfers in hybrid mice. The chromosome analysis of this tumor together with other evidence makes it probable that it represents a subline of the hyperdiploid Ehrlich-carcinoma extensively used in Germany[14]. It differs in several characteristics from the Ehrlich-Stockholm line and it is doubtful whether the tumor lines ever did have a common origin.

Both lines are carried routinely by intraperitoneal transfer of 0.2 ml of ascitic fluid 8 to 10 days after the previous inoculation.

Labeled compounds: ^{35}S-methionine, with a specific activity of about 5 mc per mmole, ^{14}C-8-adenine and ^{14}C-2-glycine, both with a specific activity of 1 mc per mmole were used. They were obtained from the Radiochemical Centre, Amersham, England. All compounds were dissolved and administered in saline at pH 7.4.

Labeling the tumor cells: The procedure of labeling the tumor cells with glycine and adenine has been described previously[1]. An essentially similar method was used with methionine. With the exception of one series, it was administered alone in two intraperitoneal injections with an interval of 24 hours. The total amount of ^{35}S-methionine was 3–5 μc. In one series, doubly labeled tumor cells were used. Labeled methionine and labeled adenine were administered simultaneously in this case until a total dose of 5 μc ^{35}S-methionine and 3 μc ^{14}C-adenine per mouse was given. The animals were killed and the labeled cells were collected two days after the last injection. The size of the inoculum was the same with the E-St tumor as in the previous experiments ($17 \cdot 10^6$ cells). In the experiments with the E-L tumor 0.1 to 0.3 ml of the undiluted ascites (containing $20–60 \cdot 10^6$ tumor cells) was used for inoculation. Samples of ascitic fluid were collected at regular intervals after inoculation, as described previously[1]. The chemical preparations, measurements of radioactivity, and tumor cell counts, were carried out in the same way as in previous studies[13,15].

RESULTS

The data obtained in eight series of experiments with both the E-St and E-L ascites tumors, respectively, are summarized in Tables I and II. In all cases the products of the specific DNA and protein activities and the total tumor cell number were calculated. Within one series, the decrease of the numerical value of the products is a measure of the release of the incorporated activity from the cell components, while their constancy can be interpreted to indicate metabolic stability. In order to make the products comparable between the different experimental series and to express deviating values in percent, relative products were calculated so that the value of the product at zero time was taken as equal to 100.

Glycine, adenine and methionine were separately used as precursors to label the proteins and DNA of the E-St tumor (Series 1 to 5). The values of the glycine and adenine series have been computed from data previously published[1]. The values of the relative products showed only minor variations and indicated that essentially no or relatively little activity was released from the DNA fraction when labeled either with glycine or adenine. A similar constancy is also shown by the proteins labeled with methionine. In contrast, activity was lost from the protein fraction when glycine was used as label. The release had a significant correlation with the time after inoculation of the tumor cells (Fig. 1, Table III).

The results with the E-L tumor labeled with methionine and adenine, together or separately, were slightly different (series 6 to 8). In all the three cases studied, the proteins released about 30 to 40% of their incorporated activity between the collection of the labeled cells for inoculation and the 3rd day of their subsequent growth in the new host. After this initial decrease of activity, the proteins of the cells of the E-L tumor showed a behaviour similar to that of the E-St tumor cells with apparently no further loss (Fig. 1 and Table III). The values obtained in the series with both methionine and adenine-labeled cells (series 6) indicate that the initial release is restricted to the protein fraction only and DNA is stable straight on from the inoculation. In

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TABLE I

CHANGES IN THE CELL NUMBER AND THE SPECIFIC ACTIVITY OF PROTEINS AND DNA PURINES DURING THE GROWTH OF THE E-St ASCITES TUMOR AT DIFFERENT TIMES AFTER THE INOCULATION OF LABELED CELLS

Series number	Labeled precursor	Day after inoculation	Total* number of free tumor cells × 10^6	Activity** (c min mg)		Product of total cell number and specific activity		Relative products:	
				proteins	DNA purines	proteins × 10^9	DNA purines × 10^9	cells × proteins	cells × DNA
		0	17	619	6710	105	114	(100)	(100)
		2	98	65.7	1070	64	105	61	92
1	glycine	3	175	34.7	660	61	116	58	102
		5	473	10.4	274	49	130	47	114
		6	533	7.1	221	38	118	36	103
		7	693	4.9	139	34	96	32	85
		0	17	417	6960	71	118	(100)	(100)
		2	90	42.7	1520	39	137	54	116
2	glycine	3	160	25.0	780	40	125	56	106
		4	195	11.6	540	23	105	32	89
		5	330	5.9	289	20	95	27	81
		6	398	6.0	231	24	92	34	78
							Mean:	43.7 ± 4.1	96.6 ± 4.3
		0	17		33300		566		(100)
		2	106		6820		724		128
		3	135		3500		473		84
3	adenine	4	265		2290		607		107
		5	300		1640		423		75
		6	600		1410		847		150
		7	760		1060		805		142
								Mean:	114.3 ± 12.6
		0	17	394		67		(100)	
		2	68	91.7		62		91	
4	methionine	3	140	47.3		66		96	
		4	242	41.9		102		146	
		5	258						
		6	388	19.2		75		107	
		0	17	602		106		(100)	
		2	94	115		108		102	
5	methionine	3	128	88.2		113		107	
		4	280	47.3		131		124	
		5	306	30.0		92		87	
		6	560	17.9		100		94	
							Mean:	106 ± 6.3	

* Mean of 4 mice.
** Mean of 6 to 15 mice.

this series, the constancy shown by the product of cell number and specific activity is further corroborated by the ratios of specific activities of protein and DNA, which could be calculated without the errors connected with the determinations of total cell number during tumor growth. After an initial decrease of about 30%, the ratios varied between comparatively narrow limits.

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TABLE II

CHANGES IN THE CELL NUMBER AND SPECIFIC ACTIVITY OF PROTEINS AND DNA PURINES DURING THE GROWTH OF THE E-L ASCITES TUMOR AT DIFFERENT TIMES AFTER THE INOCULATION OF LABELED CELLS

Series number	Labeled precursor	Day after inoculation	Total* number of free tumor cells × 10^6	Activity** (c/min/mg) proteins	Activity** (c/min/mg) DNA purines	Product of total cell number and specific activity: proteins × 10^8	Product of total cell number and specific activity: DNA purines × 10^8	Ratio of specific activities: proteins / DNA	Relative products: cells × proteins	Relative products: cells × DNA	Relative ratio: proteins / DNA
		0	46	687	733	316	337	0.94	(100)	(100)	(100)
		3	260	98.0	153	255	398	0.64	81	118	68
		4	340	73.5	116	250	394	0.63	79	117	67
6	methionine	5	392	43.7	86.5	171	339	0.51	54	101	54
	and adenine	6	720	38.2	46.1	275	331	0.83	87	98	88
		7	840	24.1	36.1	202	302	0.67	64	90	72
		8	—	—	—	—	—	—	—	—	—
		9	1000	18.0	31.5	180	315	0.57	57	93	61
		10	1180	16.0	28.7	189	339	0.56	60	100	60
		0	78	903		722			(100)		
		3	140	33.6		470			65		
		4	170	23.2		394			55		
7	methionine	5	281	13.4		376			52		
		6	354	11.7		414			57		
		7	433	8.5		369			51		
		8	522	8.6		475			66		
		0	24	790		190			(100)		
		3	201	54.7		110			57		
		4	252	47.9		120			62		
8	methionine	5	333	33.7		112			58		
		6	457	22.8		104			54		
		7	686	15.3		105			55		
		8	746	14.7		109			57		
									Mean:	61.6 ± 2.3	102 ± 4.1	67 ± 4.1

* Mean of 4 mice.
** Mean of 6 to 15 mice.

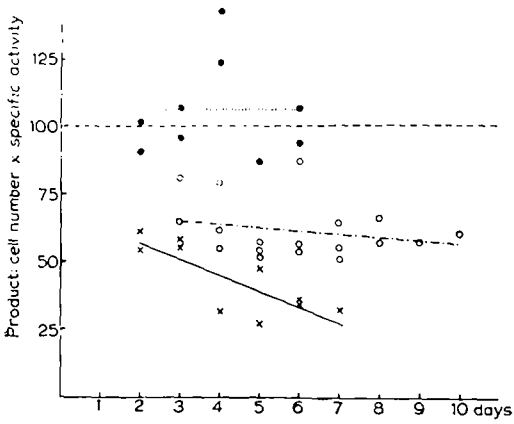

Fig. 1. Products of total number of free tumor cells and specific activity of proteins of ^{35}S-methionine-labeled E-St (o -· -o) ^{14}C-glycine-labeled E-St (×- -×) and ^{35}S-methionine-labeled E-L (●·····●) ascites tumors, respectively, at different times after inoculation. The initial value is taken as equal to 100. The points represent data pooled from two (E-St) or three (E-L) separate experimental series. The regression lines have been fitted by the method of least squares. Regression and correlation data are shown in Table III.

TABLE III

REGRESSION AND CORRELATION DATA OF THE PRODUCTS OF TOTAL TUMOR CELL NUMBER AND SPECIFIC ACTIVITY OF PROTEINS DEPICTED IN FIG. 1

Tumor	*Label*	*Number of products*	*Regression coefficient* $b \pm \sigma b$*	*Correlation coefficient r*	*Level of significance** P*
E-St	^{14}C-glycine	10	-5.89 ± 1.50	−0.811	~ 0.001
E-St	^{35}-S-methionine	9	$+0.31 \pm 4.56$	+0.026	> 0.1
E-L	^{35}-S-methionine	19	-1.18 ± 1.10	−0.239	> 0.1

* Denotes the daily procentual increase or decrease of the value of the products.

** Probability that numerical changes of the products at different times after irradiation are due to chance.

DISCUSSION

The data of this study indicate that during the growth of two ascites tumors the release of previously incorporated radioactivity from total proteins is different when two different amino acids are used as labels.

While glycine-labeled proteins lost activity continuously, proteins labeled with methionine retained their activity during the whole period of growth of the E-St tumor. In the E-L tumor there was an initial loss of activity followed by constancy during the major part of the growth period.

The loss of activity from the multiplying ascites tumor cells can be interpreted as evidence for an intracellular degradation of the protein molecule. A direct demonstration of the turnover of individual proteins in the homogeneous cell population of the muscle tissue was presented by VELICK *et al.*[6,7]. In their experiments, the different incorporation rates of amino acids into different enzymes of the intact muscle tissue gave evidence for the independent metabolism of individual proteins. By specific inhibition of the release of labeled amino acids, STEINBERG[5] demonstrated a true dynamic degradation of proteins in rat-liver and kidney slices. In the experiments of GREENLEES AND LE PAGE[2], radioactivity was continuously released from the ^{14}C-glycine-labeled cells of the TA3 ascites tumor during the growth in the peritoneal cavity of the mouse. Opposed to these findings are the results obtained with *E. coli*:

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isotopic studies on the induced synthesis of an enzyme[8,9] brought proof that, once formed, the enzyme is stable within the cell and does not undergo any appreciable degradation and resynthesis during growth. These results were recently confirmed and the conclusions about the metabolic stability were extended for all or at least the bulk of the proteins in the growing bacterial cell[10].

The retention of the activity of the methionine-labeled tumor cells in the present study is in line with a similar concept of metabolic stability and seems to indicate that the proteins that had been labeled with this precursor were irreversibly synthesised and stable within the cell. This, together with the results of the glycine labeling, might suggest the existence of two types of proteins, each being in a static state or in a dynamic equilibrium. Nevertheless, our data do not exclude the possibility of a process of slow breakdown which can be obscured by a predominating high rate of protein synthesis (*cf.* the discussion in ref.[16]). Another explanation would be a more strict and economical reutilisation of liberated activity after methionine labeling as compared to labeling with glycine. It is hoped that analyses in progress on the specific activity of different separated amino acids within the labeled tumor cells (*e.g.* methionine and cysteine in the case of methionine-^{35}S labeling) and probably the use of carbon and sulfur-labeled methionine will give more information on this point.

The initial loss of the activity of proteins in the E-L cells compared to the stability of the proteins in the E-St tumor indicates that different results can be obtained even with the same precursor when two different tumors of similar (perhaps common) origin are used for the assay. The possibility that the failure to recover all the administered isotope in the protein fraction might be due to the death of a part of the transplanted cells was considered by several authors[3,4]. This possibility may, however, be excluded in our case since the results of the doubly labeled series show that no loss of the DNA activity occurred, which would have been expected if a great number of the cells would have died and lysed. An initial release of some protein molecules from the cells owing to the trauma involved in transplantation and establishment of contact with the new host could be a possible explanation.

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SUMMARY

The cells of two Ehrlich ascites tumors of similar, perhaps common, origin, the tetraploid E-St and the hyperdiploid E-L lines, were labeled *in vivo* with glycine-2-^{14}C, adenine-8-^{14}C and methionine-^{35}S and inoculated intraperitoneally in known numbers. The subsequent increase in the number of free tumor cells and the decrease of the specific activity of the DNA and protein fractions were determined at regular intervals and their products calculated. The products obtained with the adenine- and glycine-labeled DNA fraction did not vary considerably. Similarly, the products of cell number and methionine-labeled protein remained practically unchanged during the growth cycle of the E-St and, with the exception of an initial decrease during the first three days after inoculation, of the E-L ascites cells. In contrast to this, glycine-labeled proteins lost activity progressively. The constancy of the values of the products was taken as suggesting a metabolically stable state, while a decrease of these values was regarded as indicating a turn-over process. It seems that both the nature of the precursor and the biological characteristics of the tumor used may be important factors that determine the relative stability of the system.

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THE GUANIDINATION OF SOME BIOLOGICALLY ACTIVE PROTEINS*

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S-Methyl isothiourea was first employed by SCHÜTTE[1] to convert the free amino groups in proteins to guanidino groups, and has subsequently been used for a similar purpose by CHRISTENSEN[2] and by ROCHE *et al.*[3,4]. The reaction of O-methyl isourea with amino acids and peptides was investigated by GREENSTEIN[5,6], and later this reaction was employed by HUGHES *et al.*[7] for the preparation of guanidinated human serum albumin. Recently CHERVENKA AND WILCOX[8] reported the complete guanidination with O-methyl isourea of the free ε-amino groups in chymotrypsinogen.

Although a number of biologically active substances have been guanidinated, with the exception of the aforementioned study on chymotrypsinogen[8] no reports have appeared on the effect of guanidination on biological activity. It is the purpose of this communication to present information relative to the guanidination of two pituitary hormones, lactogenic hormone (prolactin) and growth hormone (somatotropin), and of the enzyme lysozyme.

MATERIALS AND METHODS

O-Methyl isourea acid sulfate was prepared from O-methyl isourea hydrochloride[9] by the method of HUGHES *et al.*[7]. O-Methyl isourea neutral sulfate was synthesized by the method of BELLO[10]. In the initial experiments, the acid sulfate was employed; it was converted to the free base with $Ba(OH)_2$, and used according to the conditions employed by HUGHES[7]. Other experiments were

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