ACTION OF RIBONUCLEASE ON NEOPLASTIC GROWTH

I. CHEMICAL ASPECTS OF NORMAL TUMOUR GROWTH: THE LANDSCHÜTZ ASCITES TUMOUR

by

L. LEDOUX* AND S. H. REVELL

The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London (England)

With the discovery by LOEWENTHAL AND JAHN[1] of the Ehrlich ascites cell carcinoma, a new type of neoplastic growth became available for biochemical studies. Following on the work of LETTRÉ[2] and KLEIN[3], a large number of such tumours has been produced and described ([4,5,6,7] and others). Several authors have pointed out that these tumours are of special interest because they provide free suspensions of mammalian cells which are homogeneous[8] and of low chemical variability[8].

One of us (L.L.) during a study of the action of ribonuclease on solid and ascites tumours[9,10,11,12] has been prompted to estimate the nucleic acids and the protein content of both ascitic fluid and ascites cells with regard to the growth of the latter.

KLEIN[4] and GOLDBERG *et al.*[8] had previously determined the RNA and DNA content of an ascites tumour (Ehrlich), at a fixed moment of the growth, that is, the median survival time. But results of other authors[12,13] indicated that various metabolic and chemical changes occurred during the growth of an ascites tumour.

We have therefore attempted to follow the history of the nucleic acid and protein content of an ascites suspension during its growth and to correlate the characteristics of this growth with the chemical properties. Of the ascites cell tumours available, the Landschütz offered several advantages from the cytological point of view: it has a low percentage of mitotic abnormalities[17,14] but is very similar in other respects to the Ehrlich ascites cell carcinoma[14]. For this reason, we have chosen it for the present investigation.

The present paper describes experiments with this tumour in several groups of mice. The results indicate that the changes observed in the ribonucleic acid content of the cells can be correlated with changes in their growth rate.

These results also suggest a possible interpretation of the effects obtained with different chemicals[3,15,16] and particularly with ribonuclease on solid[11] or ascites tumours[10] *in vivo*.

* Chargé de recherches du Fonds National belge de la Recherche scientifique.
Permanent address: Laboratoire de Morphologie animale, Université libre de Bruxelles, Belgique.

References p. 426.

MATERIAL

a. *Tumour cells*

The Landschütz* tumour cells were produced by injecting 6 week old C^+ strain white mice intraperitoneally with approximately 10^7 cells per mouse. The physiological age of the first* and all the subsequent inocula was 10–12 days. The Landschütz tumour is very similar to the Ehrlich ascites carcinoma used by other investigators[8,15] but has a narrower distribution of chromosome numbers and fewer mitotic abnormalities[14]. The chromosome modal number of the Landschütz is slightly higher than that of the Ehrlich strain[14].

b. *Products*

Ribonucleic acid (RNA) Hopkins and Williams preparation, purified by reprecipitation and dialysis: This product is free of DNA contaminant.

Deoxyribonucleic acid (DNA): Thymus DNA obtained from calf thymus, purified. No RNA contaminant could be detected in the samples used.

Protein: Ribonuclease purified by chromatography[19].

All inorganic and organic reagents were pro-analysis products.

METHODS

a. *Chemical*

The ascitic fluid was withdrawn with a hypodermic syringe, fitted with a No. 12 needle, from an animal killed by neck dislocation.

This withdrawal was made as quickly as possible after killing the animal and a given volume of fluid was immediately centrifuged at 3,500 r.p.m. for 3 min. The supernatant was separated and stored. Both cells and supernatant were consecutively treated according to SCHNEIDER[18] and different fractions were obtained: acid-soluble, trichloro-acetic acid (TCA) extract (DNA and RNA) and proteins. The acid-soluble was analysed by the U.V. spectrophotometric method, using the difference $E_{260}-E_{300}$ as a measure of the free nucleotide content. Numerous tests have proved that the results so obtained were identical with those obtained by the orcinol colorimetric method[18].

The TCA extract was analysed according to SCHNEIDER: for RNA by using the modification of LUSENA[20]; DNA was estimated by the usual technique[18].

The proteins were determined by the application of MEHL's method[21], after digestion of the TCA residue by *N* KOH.

b. *Cytological*

A small part of the ascitic fluid withdrawn was fixed and stained by dilution with about two parts of a solution of orceine in 60% acetic acid. Estimates of the mitotic indices (totals of cells in prophase, metaphase, anaphase and telophase per 1000 cells), and of proportions of polyploid cells and blood cells in the total population, were all made from these samples.

RESULTS

a. *Chemical results*

The first three graphs (Figs. 1, 2 and 3) show the variation in concentrations (per unit volume) of different components in a typical group of experiments.

The points represent means of duplicate determinations made on a mixture of the pooled fluids of one or several animals (according to the available quantity of fluid).

The graphs also show the mortality curve of the group used. This curve is expressed in terms of percentage of numbers of surviving mice, plotted against time.

It can be seen from these graphs that the DNA content per unit volume remains approximately constant throughout the growth period. Similarly KLEIN had observed, in the case of Ehrlich ascites[23] that the concentration of cells per unit volume

* This strain has been provided by Dr. KLEIN, to whom the authors are very grateful.

References p. 426.

was constant during the growth. These graphs also demonstrate that the amounts of RNA, free nucleotides and protein in the external fluid increase slightly during growth, although being always comparatively small. The viscosity of this fluid also increases greatly, which is probably due to the accumulation of a component or components, not studied during this investigation.

Table I sums up the observed mean contents of both ascites fluid and ascites cells at different moments of the growth. The concentrations are expressed in mg per unit volume.

Instead of expressing the concentrations per unit volume, it is more useful to express them per amount of DNA, thus obtaining a picture of their relative proportions in the cells. The validity of this procedure will be discussed below. Figs. 4, 5 and 6 represent the variation of the ratio RNA/DNA, free nucleotides/DNA, protein/DNA respectively, during tumour growth. These results have been obtained from five groups of 50 animals, each group representing a new transfer of inoculum.

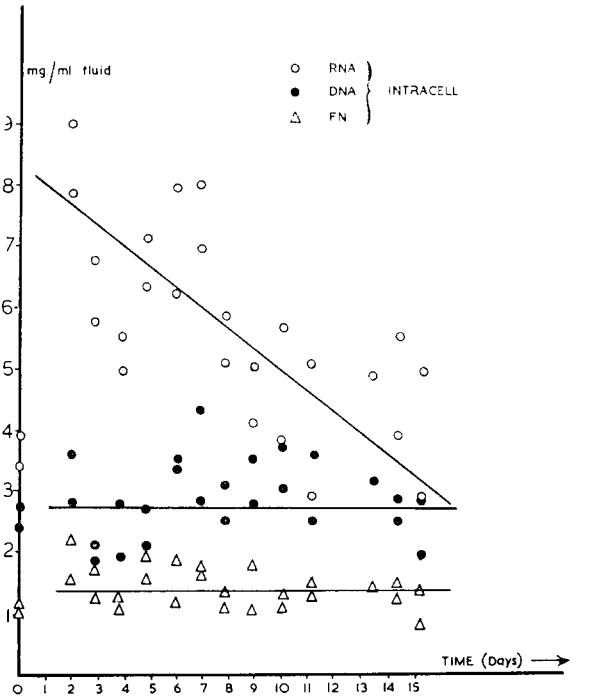

Fig. 1. Intracellular content in different nucleotide-containing substances at various times of growth of a Landschutz ascites tumor (concentrations per unit volume of fluid).

TABLE I

Mean content (mg)/ml ascitic fluid

Time (MST units)*	*0–0.25*	*0.25–0.50*	*0.50–0.75*	*0.75–1.00*	*1.00–1.50*
a. Intracel. RNA	7.75	7.55	5.16	4.95	4.26
DNA	2.46	2.90	2.58	2.75	2.79
F.N	1.48	1.68	1.47	1.65	1.54
Protein	44.4	52.0	46.5	49.5	49.3
No. of samples	(14)	(34)	(27)	(32)	(29)
b. Extracel. RNA	0.34	0.37	0.35	0.40	0.43
F.N	0.32	0.35	0.38	0.38	0.40
Protein	10.2	11.3	13.0	14.2	16.1
No. of sample	(5)	(10)	(12)	(11)	(10)

* Median survival time unit: the period between tumour implantation and the time when 50 % of the animals were dead.

The points represent means of duplicates obtained from the pooled fluid of two different mice.

References p. 426.

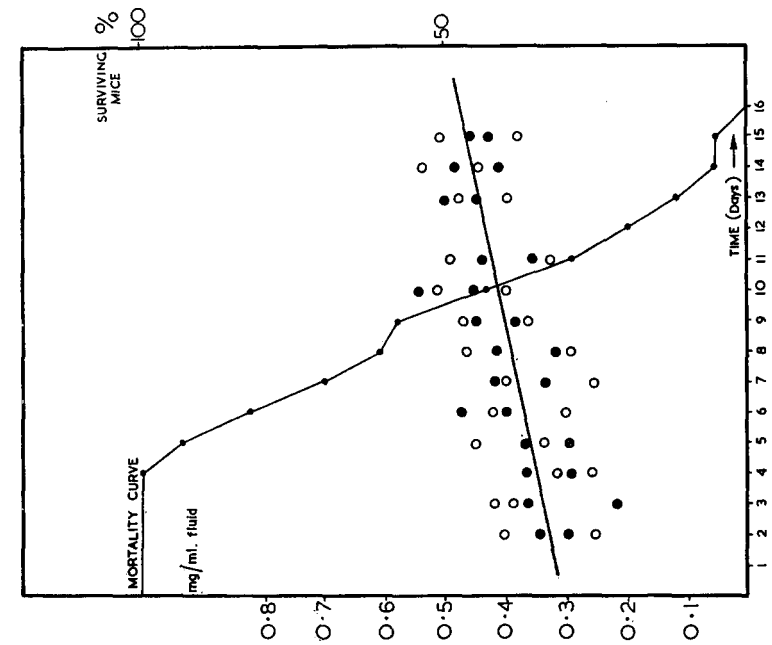

Fig. 3. Extracellular content in RNA (O) and free nucleotides (●) of the ascites suspension (concentrations per unit volume of fluid) and mortality curve of the corresponding animals.

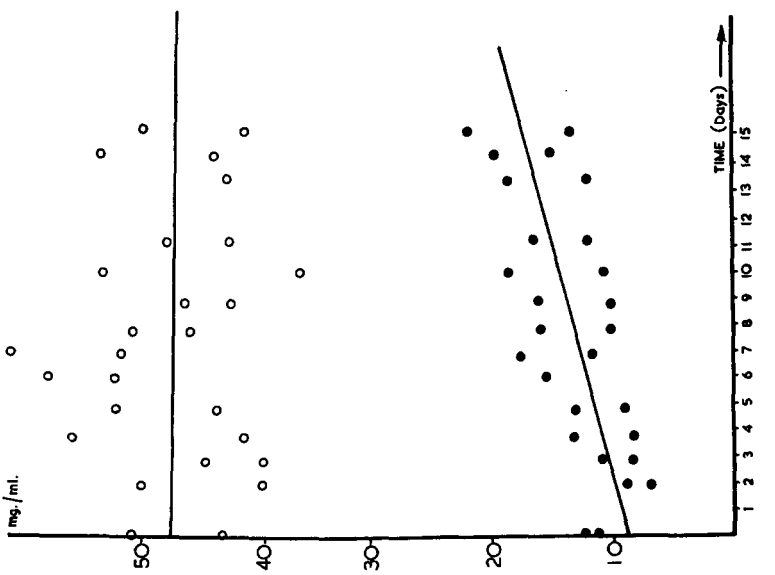

Fig. 2. Intra- and extracellular protein content of Landschutz ascites suspension (concentrations per unit volume of fluid).

The abscissae represent time expressed in units of median survival time (MST)*. This unit is used because the MST has varied from 10–14 days (mean = 11.5 ± 0.8) although the same amount of inoculum has been used in several groups.

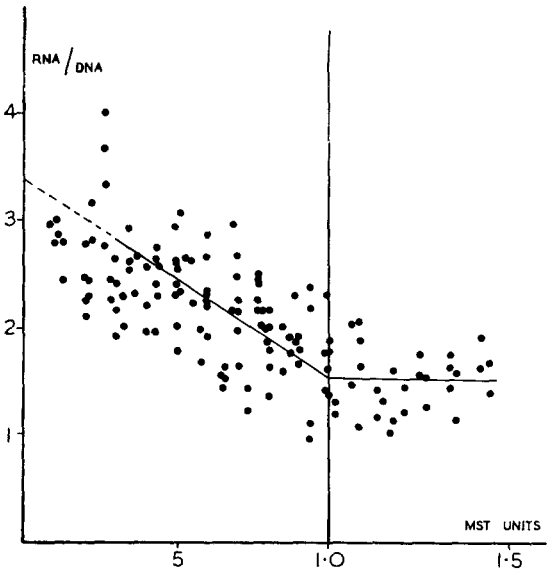

Fig. 4. Changes in intracellular RNA/DNA ratios during the growth of the ascites tumor. Time in M.S.T. units (*cf.* text).

It can be deduced from these results that the RNA/DNA ratio decreases by 50% during tumour growth while none of the other ratios observed change appreciably.

By using a U.V. microphotographic method, which will be described in detail elsewhere, R. J. KING[22] of this Institute has followed the same phenomenon in living cells. Briefly, the method consists in measuring the U.V. cytoplasmic absorption, at 257 mμ, of a large number of individual cells taken at random from the suspensions used in the chemical analysis. A very good agreement was found between the results obtained by the two methods.

It should be pointed out that there was a regular adjustment of the cellular content by transfer to a fresh animal: the values of the RNA/DNA ratio in different inocula could vary greatly, but after a very short period in the new host, they all reached the same level. Table II (columns 1 and 2) shows the observed results.

The occurrence of such adjustment agrees with the observation by KLEIN that the mortality curve depends only on the number of cells implanted and is independent of their physiological age[8,23]. It can also be seen (Table II, column 3) that the differences in the inocula have no influence on the RNA content at the median survival time (MST).

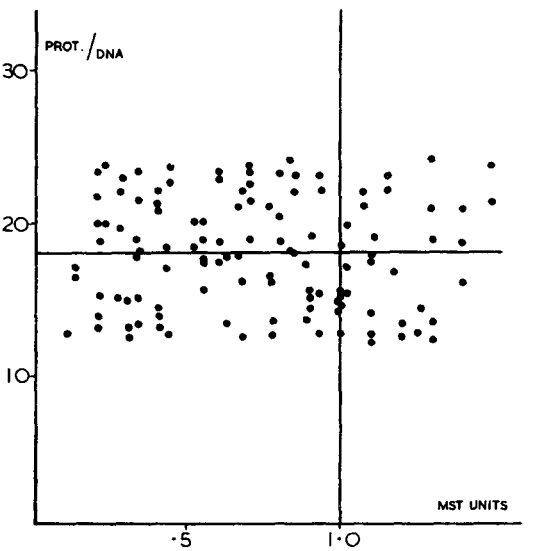

Fig. 5. Intracellular Protein/DNA ratios during the growth of the tumor.

* Determined from a probit/log time curve.

References p. 426.

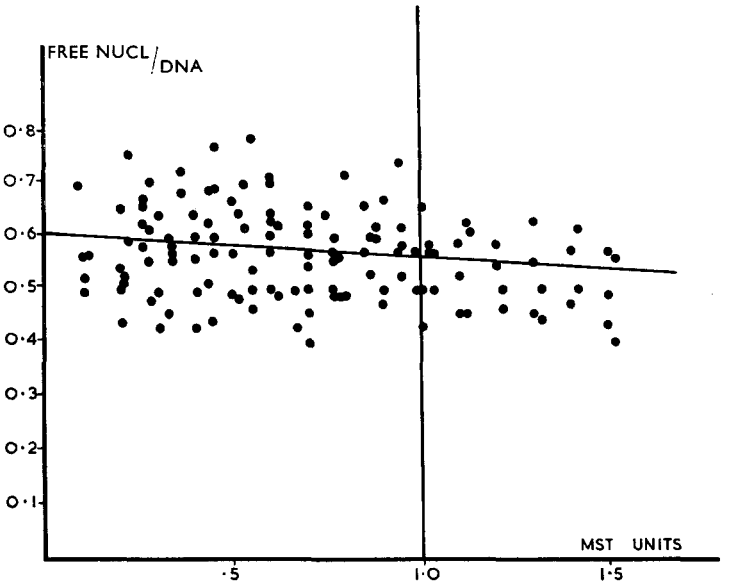

Fig. 6. Intracellular free nucleotides/DNA ratios during the growth of the tumor.

TABLE II
(RNA/DNA)*

Inoculum	*After 0.1 unit MST*	*After 1.0 unit MST*
4.23	2.94	1.45
1.87	2.54	1.34
1.55	2.96	1.55
1.34	2.82	1.55
1.29	2.50	1.50

* Means of duplicate determinations made on 4 different ascitic fluids.

b. *Cytological study*

Results obtained by previous workers with different ascites tumours[23,24] indicated that during the growth of the tumour the mitotic index decreases, the mean cell generation time increases and the rate of growth decreases continuously.

In the present work mitotic index was measured in two or three animals every day (taken from a group of animals implanted together) by the examination of 1000 tumour cells from each, and some estimates were also made of the proportions of these mitoses which were aberrant, of the proportion of tumour cells which were polyploid and of the relative numbers of blood cells in the ascites population.

Mitotic index. In the measurement of mitotic index all prophases, metaphases, anaphases and telophases were counted (each telophase being counted as one cell). The index of the tumour cell population used for inoculating all the animals (itself a mixture of the tumours from two animals) was 3.6%. Each animal received about 10^7 cells. Table III shows the results obtained.

A variable number of the mitoses scored appeared to be abnormal in various ways (*e.g.* metaphase degeneration, multipolar anaphase). The numbers observed ranged from 0 to 40% of the total mitotic count (average 13.5%), but this variation

References p. 426.

TABLE III

Time* (days)	No. of animals	"Diploid"			Polyploid			Total** mitosis (per 1000 cells)	Av. % mitosis	*** RNA/DNA
		Mitosis	Resting stage	Total	Mitosis	Resting stage	Total			
Implant	2	34	939	973	2	25	27	36	3.6	1.82
1st day	1	50	939	989	1	10	11	51		2.20
	2	53	930	983	1	16	17	54		2.03
	3	54	926	980	1	19	20	55	5.3	
2nd day	1	79	912	991	1	8	9	80		2.65
	2	95	890	985	5	10	15	100		3.30
	3	68	924	992	3	5	8	71	8.2	2.50
3rd day	1	35	952	987	0	13	13	35		2.20
	2	53	939	992	1	7	8	54		2.35
	3	48	948	996	1	3	4	49	4.6	2.00
4th day	1	28	958	986	2	12	14	30		1.80
	2	30	966	996	1	3	4	31		
	3	17	970	994	0	6	6	17	2.6	1.70
5th day	1	16	979	995	0	5	5	16		
	2	31	958	989	1	10	11	32		2.08
	3	23	970	993	0	7	7	23	2.4	1.80
6th day	1	24	971	995	0	5	5	24		1.66
	2	26	970	996	0	4	4	26	2.5	1.70
7th day	1	31	950	981	0	19	19	31		1.57
	2	20	970	990	0	10	10	20		1.70
	3	26	967	993	0	7	7	26	2.6	
8th day	1	20	979	999	0	1	1	20		1.50
	2	28	957	985	0	15	15	28		
	3	28	956	984	2	14	16	30	2.6	1.80
9th day	1	11	986	997	0	3	3	11		1.30
	2	27	964	991	3	6	9	30	2.0	1.60
10th day	1	10	980	990	0	10	10	10		1.28
	2	14	978	992	2	6	8	16	1.3	1.38
11th day	1	31	933	964	5	31	36	36		1.83
	2	24	949	973	0	27	27	24	3.0	1.67

* MST = 13.5 days — 1 day = 0.74 units MST.
** Probable error: ± 20%.
*** Probable error: ± 8%.

seemed not to be correlated with age of the tumour or any of the other parameters measured. However, a close study was not made of this side of the problem and these figures should be regarded as approximate.

Fig. 7 expresses the relationship existing between the RNA/DNA ratio and the actual mitotic index of the population. As seen from this graph, a very good correlation is obtained. The equation of the regression line is $y = 1.97 + 0.189\ (x - 3.56)$ where x indicated the percentage of mitosis and y the RNA/DNA ratio. The two

References p. 426.

exterior lines indicate the limits of probable error ($P = 0.05$). The correlation coefficient thus is very significant.

Polyploidy. Polyploidy was estimated in two ways. Resting cells which were obviously highly polyploid (as judged by the size or number of their nuclei) were scored separately: their numbers ranged from 1 to 31 per thousand (average 10.6). No consistent trend was apparent in this variation. In addition, mitoses which were obviously above the modal chromosome number of 46[14, 25] were also separately scored: these varied from 0 to 5 per thousand (average: 1.1), apparently at random. There was thus no detectable tendency for the proportion of polyploid cells to change during the development of the tumour (Fig. 8).

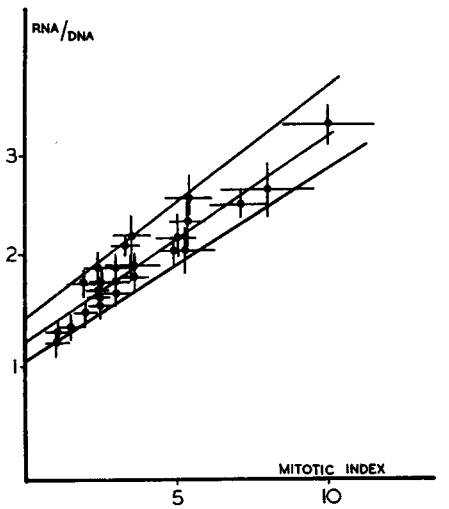

Fig. 7. Relation between mitotic indices of the cell suspensions and their actual RNA/DNA ratios.

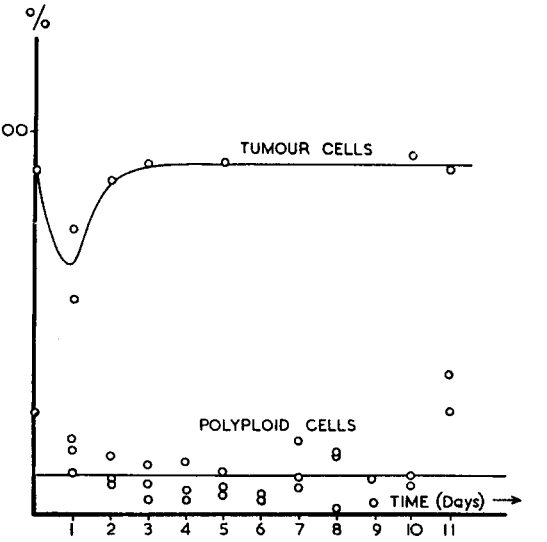

Fig. 8. Variation in the percentage of tumor and polyploid cells of the cell suspensions used.

Blood cells. The proportion of blood cells in the ascites population was highest at 1 day after inoculation but declined rapidly to an average figure of below 10%. Fig. 8 also shows the variation in percentage of tumour cells during the growth of the tumour. The results obtained here agree perfectly with those described by other authors[23]. Bearing in mind that the RNA content of non-tumour cells is much lower than that of the tumour cells, one can conclude that the values of RNA/DNA observed during the first period of the growth are underestimated.

DISCUSSION

The study of the variations of the ratios: RNA/DNA, free nucleotide/DNA and protein/DNA throughout the growth of the Landschütz ascites tumour reveals that RNA is the only one of these constituents to vary during the ageing of the tumour.

The decrease observed amounts to 50% of the initial value and reaches its end point at the median survival time (MST).

References p. 426.

Of course, the use of the ratio RNA/DNA as a measure of the ratio RNA/cell might be criticised: the ratio could decrease by increase of the DNA content. Under these conditions, however, the protein or free nucleotide content should increase at the same rate.

The careful study made on an ascites tumour by KLEIN[26] indicates that the DNA content of these cells can be used instead of the actual number of cells for chemical determinations, as a measure of the number of cells.

On the other hand, the results obtained by KING[22] with direct U.V. observation on living cells indicate that, in the present experiments, the cytoplasmic U.V. absorption (at 260 mμ) per unit thickness and per cell decreases in the same way as the ratio RNA/DNA. In consequence one can assume that the RNA content of the cells really decreases during growth. As a further proof, it is worth mentioning the constancy in the low percentage of the polyploid cells (which is in agreement with previous observations[23]) and the low variation in the number of degenerating cells. Moreover, the fact that the percentage of tumour cells is the lowest during the first days following implantation even causes the maximal values for RNA/DNA, observed at this period, to be underestimated. While there is no absolute correlation between the RNA/DNA ratio and the mitotic index of different types of tumours[1] such a correlation seems to exist when considering one type of tumour: this is borne out by the present observation that the RNA content of normal Landschütz ascites cells was found to be correlated to the mitotic index of the cell suspension. One must also record the results of KLEIN showing that the mean generation time increases linearly and that the relative growth rate decreases during the growth period[25]. KLEIN attributes this effect to the increasing shortage of available nutrients per cell and per unit of time.

However, under the conditions of our experiments, the extracellular content seems to increase during the growth of the tumour, in RNA, free nucleotides, proteins, *etc.* (as suggested also by the large increase of viscosity). This indicates that the supply reaching the tumour cell should be sufficient to permit the maintenance of the cellular composition. Experiments with ribonuclease[9,12] show on the other hand that the content in free nucleotides of the internal and external fluid are in dynamic equilibrium. They also disclose the possibility of renewing the increase (*in vitro*) of the RNA content of the cells by adding to the medium enzymes stimulating the cellular metabolism. Obviously, the necessary precursors are available for the tumour cells throughout their growth and it is therefore at present a puzzle why the RNA content decreases when protein, DNA and free nucleotide content remain constant. In any case this decrease in RNA is accompanied by a decrease in the growth rate.

This parallelism is in complete agreement with all observations suggesting a relationship between the RNA content and the rate of growth[27,28,29,30], principally those by BRACHET who always emphasized the importance of RNA for the life of the cell[31,32].

Our own results, reported here, strongly support this concept. While it has been previously evolved from comparison of normal with proliferating and embryonic with adult tissues, in the present case it is confirmed by the work with an homologous cell suspension during its evolution *in vivo*. This means that *the decrease in growth rate is associated with a parallel decrease in RNA content under normal conditions of cellular growth.*

References p. 426.

It has also been observed in the course of our investigations that cells of low RNA and slow growth rate could be rejuvenated again and again by transplanting them into fresh animals when both RNA content and growth rate increase again. The adjustment by the host of the cellular content of the implanted tumour cells agrees with the results of KLEIN *et al.*[8] who observed that whatever the amount of implanted cells, the nucleic acid content was constant at the median survival time and that variation in physiological age of inocula had no discernible effect on the proliferation of Ehrlich ascites[23].

On the other hand, this author has shown that by decreasing considerably the RNA content of the cells (by storage at 4° C[15]), the infectivity of the inoculum was considerably decreased. It seems therefore that an RNA threshold for this adjustment process exists. In consequence, if one could by chemical means decrease sufficiently the content of normal RNA below its critical limit, one might expect to modify greatly the rate of the neoplastic growth. In fact, this has been found in the case of ascites tumours treated by acridines[3], where the slowing down of the tumour is accompanied by a drop in the RNA content (the DNA content remaining unchanged). This also seemingly occurs in either ascites or solid tumours treated by ribonuclease[10,11], the nucleolytic enzyme directly concerned with RNA metabolism.

The authors wish to thank Professors A. HADDOW, F. BERGEL, P. C. KOLLER AND J. BRACHET for criticisms and suggestions.

SUMMARY

During the normal growth of an ascites tumour (Landschütz) the intracellular concentration of the ribonucleic acids (RNA) decreases by about 50%. At the same time, the amounts of protein, free nucleotides and deoxyribonucleic acids (DNA) do not vary appreciably. The depletion of cellular RNA is accompanied by a corresponding decrease of the mitotic index and by a general slowing down of the tumour growth. This depletion is not due to the disappearance, from the ascitic fluid, of RNA precursors. It is reversible by transplanting the tumour into a fresh animal. There is presumably a relation between these facts and the chemotherapeutic action of certain anti-tumour agents.

RÉSUMÉ

Au cours de la croissance normale d'une tumeur d'ascites (Landschütz), la concentration intracellulaire des acides ribonucléiques décroît de 50% environ. Pendant ce temps, la teneur en protéines, en "nucléotides libres", et en ADN ne varie pas sensiblement. L'appauvrissement en ARN cellulaire est accompagné d'une diminution correspondante de l'index mitotique et d'une ralentissement général de la croissance tumorale.

Cet appauvrissement n'est pas dû à la disparition dans le milieu nutritif (plasma ascitique) des précurseurs de l'ARN. Il est réversible si on transplante la tumeur dans un organisme sain. Il existe vraisemblablement une relation entre ces faits et l'action chémotherapeutique de certains agents anticancéreux.

ZUSAMMENFASSUNG

Während des normalen Wachstums einer Aszit-Geschwulst (Landschütz) sinkt die intrazellulare Ribonukleinsäurekonzentration um etwa 50%. Gleichzeitig kann keine bemerkbare Veränderung des Gehaltes an Proteinen, freien Nukleotiden und DNA beobachtet werden. Das Verschwinden des zellularen RNA wird von einem entsprechenden Herabsinken des mitotischen Index und einer allgemeinen Verlangsamung des Geschwulstwachstumes begleitet. Der RNA-Schwund wird nicht durch das Verschwinden der RNA-Vorgänger aus der Aszitischen Flüssigkeit verursacht. Der Schwund kann durch Umpflanzung der Geschwulst in ein neues Tier rückgängig gemacht werden. Es darf angenommen werden, dass zwischen diesen Tatsachen und der chemotherapeutischen Wirkung von bestimmten geschwulstheilenden Mitteln eine Beziehung besteht.

References p. 426.

REFERENCES

1 N. LOEWENTHAL AND G. JAHN, *Z. Krebs.*, 37 (1932) 439.
2 H. LETTRÉ, *Z. physiol. Chem.*, 268 (1941) 59.
3 G. KLEIN, *Cancer*, 3 (1950) 1052.
4 G. KLEIN, *Exptl. Cell Research*, 2 (1951) 518.
5 H. GOLDIE AND M. D. FELIX, *Cancer Research*, 11 (1951) 73.
6 A. LEVAN AND T. J. HAUSCHKA, *Hereditas*, 38 (1952) 18.
7 G. KLEIN AND E. KLEIN, *Cancer Research*, 11 (1951) 466.
8 L. GOLDBERG, E. KLEIN AND G. KLEIN, *Exptl. Cell Research*, 1 (1950) 5431.
9 L. LEDOUX AND E. BALTUS, *Experientia*, 10 (1954) 500.
10 L. LEDOUX, *Nature*, 175 (1955) 258.
11 L. LEDOUX, *Nature* (in press).
12 L. LEDOUX, *Biochim. Biophys. Acta* (in press).
13 G. A. LE PAGE, *Cancer Research*, 13 (1953) 178.
14 J. HIN TJO AND A. LEVAN, *Lunds Univ. Arsskr.*, 15 (1954) 50.
15 E. KLEIN, N. B. KURNICK AND G. KLEIN, *Exptl. Cell. Research*, 1 (1950) 1127.
16 H. E. SKIPPER, *Cancer Research*, 13 (1953) 545.
17 P. C. KOLLER, personal communication.
18 N. I. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
19 C. N. W. HIRS, J. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
20 C. V. LUSENA, *Can. J. Chem.*, 29 (1951) 107.
21 J. W. MEHL, *J. Biol. Chem.*, 157 (1945) 173.
22 R. J. KING, personal communication.
23 G. KLEIN AND L. REVESZ, *J. Natl. Cancer Inst.*, 14 (1953) 229
24 H. M. PATT AND M. E. BLACKFORD, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 520.
25 K. BAYRENTREE, *Z. Naturforsch.*, 10 (1952) 7.
26 E. KLEIN, *Exptl. Cell Research*, 8 (1955) 188.
27 J. BRACHET, *Enzymologia*, 10 (1941) 87.
28 R. JEENER, *Nature*, 163 (1949) 837.
29 J. BRACHET AND R. JEENER, *Enzymologia*, 13 (1944) 196.
30 T. CASPERSSON, *Symposia Soc. Exptl. Biol.*, 2 (1947) 127.
31 J. BRACHET, *Chemical embryology*, Interscience, New York, 1945.
32 J. BRACHET, *Le Rôle des acides nucléiques dans la vie de la cellule et de l'embryon*, Desoer, Liège, 1952.

Received May 2nd, 1955