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THE EARLY INTERFERENCE OF LIVER CARCINOGENS WITH PROTEIN SYNTHESIS AND ITS POSSIBLE BEARING ON THE PROBLEM OF TUMOR INDUCTION

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Abstract—Inhibition of protein synthesis and disaggregation of polyribosomes are characteristic, early effects of many liver carcinogens. These effects may in special cases depend on direct, structural interaction of the added compounds with ribosomes. However, the inhibition is more typically associated with the metabolic oxidation of the compounds in the liver endoplasmic membranes. Reactive metabolites, formed in this process, may interfere with the controlled handling of information-carrying macromolecules at various cellular levels. The experimental data sugggest that the inhibition of the ribosomal activity under these conditions is not primarily due to a reduced synthesis or life-span of messenger RNA, but rather to a disturbed peptide chain initiation. The relation between early and late events in the carcinogenic process is discussed in terms of a gradual alienation of the cytoplasm under the influence of carcinogens.

EXPERIMENTS with chemically induced liver tumors indicate that the neoplastic transformation is associated with alterations at the protein level, including a selective deletion of enzymes and other protein species.[1–3] The irreversibility of this process suggests that it may be of genotypic nature and basically different from the various reversible effects that usually accompany the primary cellular interaction of carcinogens. Cytological data are compatible with this assumption.[4]

Like the ultimate neoplastic transformation, the primary interaction has its characteristic selectivity, depending on the chemical nature of the carcinogen, and the properties of the treated animal. In certain instances, striking analogies have been demonstrated between the pattern of selectivity of the primary interaction and that of the ultimate neoplastic transformation.[2] Analogies of this kind have favored the notion that primary (reversible) and ultimate (irreversible) events in the carcinogenic process may be connected by direct links of causal relationship. Elaborate "circuits" have been constructed for making the transition biologically intelligible.[5] It must be emphasized, however, that constructions of this kind should be adopted with caution as long as nothing is known with certainty about the length and complexity of the intermediate chain of events.

Irrespective of these analogies, the selectivity of the primary cellular interactions of carcinogens is of interest in itself. It may help us to specify, by what kinds of functional imbalance cells are brought into a state that enables them to simplify their biological pattern towards irreversible dedifferentiation and independent growth.

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Selective interaction of carcinogens with components of the protein-synthesizing system of liver cells

The ability of liver carcinogens to interact with cell constituents is either a direct function of their molecular constitution, or arises inside the target cells as a result of metabolic activation. In either case, the cellular interaction may be selective, and may directly or indirectly affect the synthesis of cell proteins.

(1) Many liver carcinogens (e.g. carcinogenic amines, CCl_4) are oxidatively detoxicated by a multi-enzyme system in the liver endoplasmic membranes.[1] In the course of this process reactive derivatives, probably free radicals related to metabolic intermediates,[6] may dissociate from the system.[7] The membranes themselves are most severely affected by these products,[7–11] and by progressive disorganization of the detoxicating system the lesion may deteriorate autocatalytically.[12,13] The reactive metabolites must be expected to have a limited life-span in their cellular environment, and may form steady-state diffusion gradients in the vicinity of the membranes. The membrane-attached polyribosomes, which are responsible for a great part of the protein synthesis in liver cells,[14] are in a particularly exposed position.[8] Since the endoplasmic reticulum is continuous with the outer nuclear envelope,[15] a gradient of reactive molecules may also extend into the nucleo-cytoplasmic interspace, and probably reach into the periferic parts of the nuclei.[16,17]

(2) Aminoacridines may serve as an example of carcinogens with inherent ability to specific interaction with components of the protein synthesizing system. The carcinogenic potency of this interesting group of aromatics has recently been established.[18] Their ability to interfere with the helical structure of polynucleotides has mainly been discussed with reference to DNA, but their interaction with RNA is of a similar nature, and they are effective inhibitors of ribosomal functions.[19–24]

Possible effects of carcinogens on the supply of RNA templates

Although carcinogens may have mutagenic properties, this seems not to be a general rule. Quantitative interference with the RNA synthesis occurs, but is difficult to demonstrate in short-term experiments, except *in vitro* by use of liver slices.[12,25] *In vivo* it appears later, and is less marked than the inhibition of amino acid incorporation.[17] For instance, the pulse-labelling of mouse-liver RNA *in vivo* is not markedly decreased 2–3 hr after the administration of a sublethal dose of the highly hepatotoxic mouse liver carcinogen, CCl_4, although the amino acid incorporating activity of the ribosomes is strikingly reduced at this period (Table 1).

This does not mean that liver carcinogens are without influence on the RNA-synthesizing system. It has for instance been shown that the administration 4′-F-DAB* to rats produces alterations in the pattern of rapidly synthesized RNA, supposedly mRNA.[26] These alterations are at first readily reversible, but later become more permanent. After prolonged administration of carcinogenic amines, there may also be a shift in the proportion between euchromatic and heterochromatic material in the nuclei, and the activity of isolated DNA as a template for added RNA-polymerase is reduced.[27,28]

Nucleoli are particularly sensitive indicators of the early interference of carcinogens with nuclear functions. Several carcinogens give rise to a partial segregation of the

* Abbreviations used: DAB, *p*-dimethylaminoazobenzene; DOC, sodium deoxycholate; poly U, polyuridylic acid; mRNA, rRNA, tRNA, messenger, ribosomal and transfer RNA, respectively.

TABLE 1. DISTRIBUTION OF RAPIDLY LABELLED RNA AMONG LIVER FRACTIONS FROM NORMAL AND CCl_4-TREATED MICE*

	Distribution of labelled RNA				
	Total counts/min		Per cent		Relative deviation of CCl_4 from controls (per cent ± S.D.)
	Controls	CCl_4	Controls	CCl_4	
Whole homogenates	37,030	39,630	(100)	(100)	(100)
15,000 *g* sediments, minus cell debris	27,020	29,840	73	75	108 ± 6 (5)
Purified nuclei	24,070	26,400	65	67	115 ± 13 (5)
15,000 *g* supernatants	7070	6350	19	16	76 ± 5 (5)
Microsomes	1332	1776	3·5	4·5	120 ± 9 (5)
Ribosomes	713	1010	2	2·5	116 ± 10 (5)
Microsomal supernatants	4950	3780	13·5	9·5	72 ± 8 (5)
Postmicrosomal particles	711	641	2	1·5	79 ± 7 (5)

* Groups of four mice were given 2·5 ml/kg CCl_4 by stomach tube. Two hr later the treated and control animals were pulse-labelled for 60 min with [^{14}C] orotic acid (20 μc per animal, i.v.). The livers from each group were pooled and homogenized in 0·25 M sucrose containing 5 mM $MgCl_2$. Nuclei were prepared from the 15,000 *g* sediment (freed from debris) by the Chauveau method, but in the presence of 0·2% Triton X 100. Ribosomes were prepared from the 150,000 *g* (40 min) microsomal pellets by treatment with 1% DOC and centrifuging for 3 hr at 150,000 *g*. Postmicrosomal particles were prepared from the microsomal supernatants by centrifuging for 3 hr at 150,000 *g*. The fractions were washed extensively in ice-cold 5% TCA and ethanol, and the labelled RNA was extracted with 5% TCA at 90°. The figures represent the means of five experiments.

particulate and fibrillar components of the nucleoli, reminding of that produced by certain inhibitors of RNA and protein synthesis.[29–31] In later pre-carcinogenic stages the nucleolar volume may increase.[32] With thioacetamide a striking increase in the nucleolar size is observed from 24 to 48 hr, and the RNA synthesis by the nucleolus-associated chromatin is enhanced.[30,33–34] The extensive accumulation of nucleoprotein in the nucleoli has been interpreted in terms of an impaired draining of the newly synthesized products.[33]

Besides their well-established role in the manufacture of ribosomes, nucleoli seem to have some relay function in the transportation of protein-bound informative RNA from the nucleus to the cytoplasm.[35] Particularly clear-cut evidence for this assumption has been provided by experiments with virus-induced cell hybrids containing bird erythrocyte nuclei in mammalian cytoplasm.[36] The appearance of bird-specific proteins in these hybrids is closely correlated with (a) the formation of nucleoli in the activated nuclei and (b) the appearance of newly synthesized RNA in the cytoplasm. We have observed that the proportion of 40–60 min pulse-labelled RNA in the cytoplasmic fraction of mouse liver is moderately reduced 2–3 hr after a sublethal dose of CCl_4 (Table 1). In conformity with the data just discussed, this may indicate a decreased efficiency of the nucleo-cytoplasmic RNA-transportation, correlated with incipient nucleolar dysfunction.

Several mechanisms could be imagined as involved in a nucleolar control of mRNA transport. Newly synthesized mRNA occurs in the nucleus in a stabilized form, attached to a species of 30 S protein particles, "informofers".[37] Where these are assemblied is not known, but the nucleolus is an obvious candidate. Informofer

aggregates[37] seem to transport the newly synthesized mRNA from the active gene sites to the pores in the nuclear membrane.[38] Conditions of nucleolar disorder may possibly lead to a shortage of active informofers in the nuclei.

Possible mechanisms of ribosomal inactivation in short-term experiments with liver carcinogens

When the newly synthesized mRNA appears in the cytoplasm, it is associated with particles, "informosomes", presumably derived from the nuclear informofers.[38,39] The initiation of new protein chains seems to involve the association of informosome strands[38] with ribosomal subunits.[39–41] The detailed mechanism of this association is still unknown, but will be of great importance to elucidate, as available evidence strongly suggests that chain initiation constitutes a major regulatory site for protein synthesis in eucharyotic organisms. Under various conditions of impaired cell activity (e.g. reduced oxidative phosphorylation, amino acid deficiency, or in the presence of NaF) polysomes reversibly disaggregate to endogenously inactive monomers.[42–44] A similar effect has been observed in the liver of animals treated for a few hours with a single dose of various liver carcinogens.[12,17,45] At the same time there is an extensive detachment of the particles from the structurally disarranged endoplasmic reticulum.[10]

The inactivation and disaggregation of the ribosomes are apparently caused by reactive metabolites formed in the endoplasmic membranes. Although the detailed mechanism is unknown, several possibilities can be visualized:

(1) Damage to the ribosomes by reactive metabolites[17,46] may render them incapable of forming a functional initiation complex. For initiation the ribosomes probably must dissociate into subunits[41] and the smaller subunit interact with mRNA-carrying informosomes[39,40] and aminoacyl tRNA under the influence of soluble factors,[47] including aminoacyltransferase I.[48] To fit with experimental data the postulated ribosomal lesion must be specifically restricted to the initiation proper. Even when the endogenous activity of the ribosomes in amino acid incorporation is strikingly reduced, the particles are perfectly capable of utilizing poly U as an artificial template

TABLE 2. INABILITY OF CCl_4-INDUCED RIBOSOMAL MONOMERS TO UTILIZE POLY U AFTER PRETREATMENT WITH K^+-FREE MEDIUM

Medium of pretreatment	CCl_4-induced monosomes		Normal ribosomes	
	With poly U	Without poly U	With poly U	Without poly U
With KCl	546 (100%)	32 (100%)	655 (100%)	155 (100%)
Without KCl	84 (15%)	29 (91%)	314 (48%)	139 (90%)

Starved rats were treated for 3 hr with 2·5 ml/kg CCl_4. The 15,000 *g* supernatant liver fraction (in 150 mM sucrose, 75 mM KCl, 9 mM $MgCl_2$ and 35 mM tris–HCl pH 7·7) was mixed with 1% DOC and fractionated by 0·26–1·1 M sucrose gradient centrifugation. The monosome fraction (containing some dimers) was pelleted and suspended in a medium containing 150 mM sucrose, 1·5 mM $MgCl_2$ and 35 mM tris–HCl pH 7·7. KCl was 75 mM or absent. The suspensions were passed through Sephadex G-25 columns equilibrated with the same media and dialyzed for 2 hr (0°) against the same media. Whole ribosomes from control animals were treated in the same way. The activity of endogenous and poly U-dependent phenylalanine incorporation was determined at different $MgCl_2$ concentrations.[23] The data represent optimum values (counts/min per 0·5 O.D.U. of ribosomes)

for phenylalanine incorporation *in vitro*, provided that the Mg^{2+} concentration of the system is properly controlled.[49,50] This is also observed with isolated, disaggregated monosomes (Table 2).

When preparations of disaggregated monosomes from CCl_4-treated rats or mice were treated with K^+-free media, their ability to utilize polyuridylic acid as a template was lost (Table 2), and they were at the same time transformed into particles with lower sedimentation velocity, approximating that of 60 S ribosomal subunits (Fig. 1). Normal liver ribosomes with endogenous amino acid incorporation were appreciably more resistant under these conditions.[51] It was originally believed that the lability of the disaggregated ribosomes from CCl_4-treated animals was indicative of a carcinogen-induced structural damage to the particles. However, further experiments with isolated monosomes and polysomes from Ehrlich ascitic tumor cells and rabbit reticulocytes indicated that the striking structural and functional dependence of disaggregated ribosomes on K^+ ions is a normal, although previously overlooked, feature of mammalian ribosomal monomers.[52]

So far, attempts to demonstrate conformational abnormalities in the surface of carcinogen-induced ribosomal monomers by means of molecular probes[23,24] (*vide infra*) have been negative. Convincing evidence is thus lacking that metabolites of liver carcinogens seriously interfere with the basic structural or functional properties of the particles.

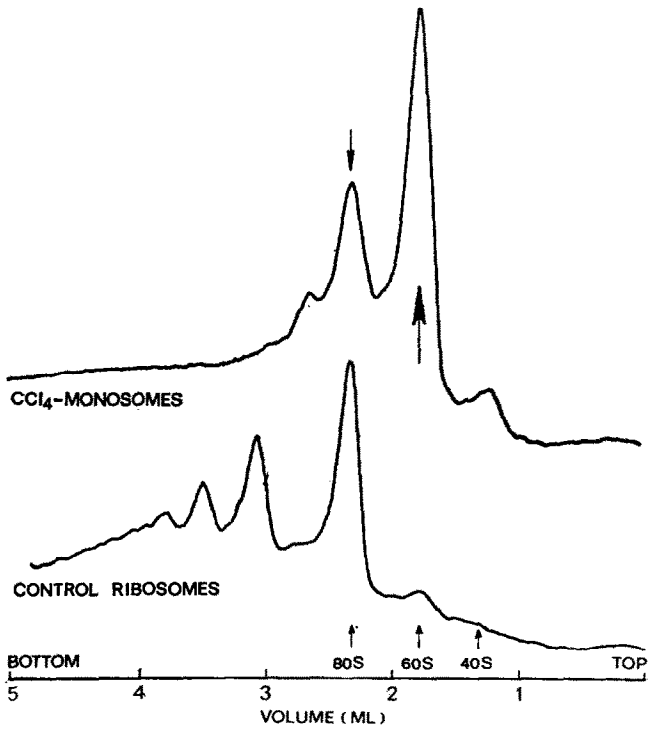

FIG. 1. Structural alteration of CCl_4-induced rat liver ribosomal monomers in K^+-free medium. The particles were prepared, and treated with K^+-free medium, as described in Table 2. The profiles were obtained by centrifugation in 0·5–1·5 M sucrose gradients containing 5 mM KCl, 1·5 mM $MgCl_2$ and 35 mM tris–HCl (Spinco SW 39).

(2) Under the influence of reactive metabolites there may be an acute shortage of active informosomes in the cytoplasm. Because of the poor biochemical characterization of these particles, this possibility cannot yet be conclusively tested. As is shown in Table 1, the proportion of rapidly labelled RNA in the fraction of postmicrosomal particles of mouse liver was significantly reduced 3 hr after CCl_4 administration. This observation is compatible with a decreased supply of informosomes from the nuclei. However, the validity of this interpretation is open to some question because of the unexpectedly high proportion of rapidly labelled RNA in the microsomal and ribosomal fractions.

(3) The active life-span of mRNA may be decreased. This possibility is not supported by the following set of experiments: Groups of mice were injected intravenously with [^{14}C]-orotic acid. One hour later CCl_4 (2·5 ml/kg) was given to one group. At the same time actinomycin D (2 mg/kg) was injected intraperitoneally into all animals to block further RNA synthesis. The distribution of labelled RNA was investigated 2 hr later by the same fractionation procedure as in Table 1. In comparison with the controls there was no decrease in the isotope content of any of the RNA fractions from the CCl_4-treated mice (not illustrated).

It may be concluded that of the various alternatives for explaining the reduced activity of liver ribosomes in the carcinogen-treated animals an impaired peptide chain initiation seems at present to be most readily compatible with available data. Whether this should be ascribed to a ribosomal, informosomal or membranous[53] defect is uncertain.

Effects of aminoacridines on liver ribosomes

After a sublethal dose of acridine orange or proflavine to rats or mice an increasing proportion of the liver ribosomes undergo a characteristic conformational alteration, which can be demonstrated *in vitro* by treating the isolated particles with suitable molecular probes, e.g. chymotrypsin.[23,24] Figure 2A illustrates a normal disc-electrophoretic pattern of rat liver ribosomal proteins extracted with 0·2 M HCl. A very similar pattern is obtained with mouse liver ribosomes. When the suspension of intact particles was treated for 20 min at 0° with 5 μg/ml chymotrypsin, some proteins were attacked by the enzyme (Fig. 2B, white arrows) while others were protected by structural shielding. Within the section of the electrophoretic pattern shown in Fig. 2B, C and D, the ribosomes from aminoacridine-treated animals showed a characteristic alteration in the shielding pattern. A previously protected protein ("10") located in the larger ribosomal subunit, was rendered susceptible to the enzyme (Fig. 2D). Unlike the effects discussed in the previous section, this alteration was caused by the carcinogen as such, and could be reproduced *in vitro* with isolated ribosomes (Fig. 2C). The *in vitro* alteration was strictly time and temperature dependent.

The effect of aminoacridines on the ribosomes should most likely be interpreted in terms of an intercalation of these molecules in double-stranded folds of the RNA chain, as has been suggested for the interaction of acridine dyes with DNA,[19,21] tRNA[54] and rRNA.[22] At the temperature of the mammalian body this may lead to a conformational rearrangement of superficially located RNA-helices, detectable by the unmasking of underlying proteins.

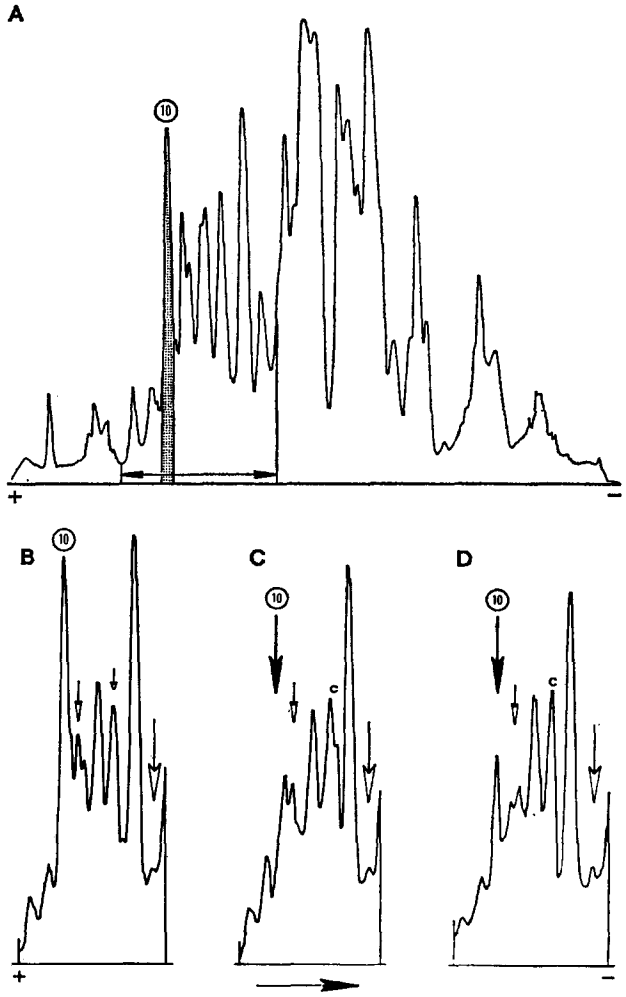

FIG. 2. Conformational alteration of liver ribosomes under the influence of amino acridines *in vivo* and *in vitro*. (A) Disc-electrophoretic pattern[23] of normal rat liver ribosomal proteins extracted with 0·2 M HCl. (Horizontal arrows indicate the section included in B, C and D.) (B) Alteration of the protein pattern after exposure of the particles for 20 min (0°) to 5 μg/ml chymotrypsin (white arrows). (C) Unmasking of a previously shielded protein ("10") by preincubation of the particles for 10 min (35°) with 0·5 mM acridine orange before the chymotrypsin test. (D) Partial unmasking of the same protein *in vivo* 60 min after a peroral dose of 300 mg/kg proflavine to mice. The isolated ribosomes were immediately tested with chymotrypsin as in B and C.

It should be recalled that aminoacridines are potent inhibitors not only of RNA-synthesis, but also of ribosomal functions, including the puromycin-induced release of nascent peptides.[20] This reaction reflects the functions of the larger ribosomal subunit in peptide chain elongation.

CONCLUDING REMARKS

In their primary cellular interactions many liver carcinogens selectively interfere with cytoplasmic and nuclear functions related to protein synthesis. Despite the vital importance of the disturbed functions it is not possible at present to indicate a direct

relationship between these effects and the ultimate neoplastic transformation. A key role in chemical carcinogenesis has in the past been ascribed to various molecular interactions and metabolic inhibitions. It is felt that if a specific key mechanism actually occurs, it may be of a complexity beyond present-day biochemical resolution.

From a biological standpoint it is conceivable that cells under the continuous, selective pressure of liver carcinogens may be diverted into regulatory states beyond the normal range of well-controlled homeostatic balance.[55] In addition to their ordinary functions, these cells have to respond to various emergency stimuli, like stress hormones[12] and inducers of tissue regeneration and cell repair. Some of the injured cells may not be able to integrate the partially conflicting response patterns into adequately co-ordinated reaction sequences. Because of its great structural complexity the mitotic part of the cell cycle is particularly critical in this respect. Occasional errors in the redistribution of genetic material may occur,[4] and the discrimination between euchromatic and heterochromatic regions may become less strict.[27] Direct damage to DNA by reactive metabolites[16] may contribute to these effects. Unsuccessful attempts to compensate for the primary genomic abberations may further increase the range of variability.

The important functions of cell nuclei in regulation and differentiation are ultimately dependent on factors mediated through the cytoplasm. This suggests that the neoplastic transformation is no isolated nuclear phenomenon. A cytoplasmic involvement in spontaneous and chemically induced growth abnormalities in microorganisms has been clearly indicated.[56,57] Experiments with transplanted embryonic nuclei[58] and with tissue explants[59] suggest that inheritable defects may arise in nuclei exposed for a number of cell cycles to an inadequate cytoplasmic environment. An essential effect of the primary cellular interaction of carcinogens may be to alienate the cytoplasm and make it unsuitable for supporting integrated nuclear functions. The interference of liver carcinogens with protein synthesis may be of interest in this context in view of the continual protein exchange between nucleus and cytoplasm,[60] and its possible essentiality for a normal nucleo-cytoplasmic interrelationship.

REFERENCES

1. G. C. MUELLER and J. A. MILLER, *J. biol. Chem.* **202,** 579 (1953).
2. E. WEILER, *Z. Naturforsch.* **11,** 31 (1956).
3. S. SOROF, E. M. YOUNG and M. G. OTT, *Cancer Res.* **18,** 33 (1958).
4. R. W. BEGG, H. F. STICH and M. M. MAINI, *Proc. Am. Ass. Cancer Res.* **3,** 93 (1960).
5. H. C. PITOT and C. HEIDELBERGER, *Cancer Res.* **23,** 1694 (1963).
6. A. J. VITHAYATHIL, J. L. TERNBERG and B. COMMONER, *Nature, Lond.* **207,** 1246 (1965).
7. T. HULTIN, *Expl. Cell Res.* **13,** 47 (1957).
8. T. HULTIN, *Expl. Cell Res.* **10,** 71, 697 (1956).
9. J. C. ARCOS, *Bull. Tulane med. Fac.* **20,** 133 (1961).
10. K. R. PORTER and C. BRUNI, *Cancer Res.* **19,** 997 (1959).
11. E. A. SMUCKLER, E. ARRHENIUS and T. HULTIN, *Biochem. J.* **103,** 55 (1967).
12. T. HULTIN and E. ARRHENIUS, *Adv. Enzyme Regulation* **3,** 389 (1965).
13. E. ARRHENIUS, *FEBS Symposium* **16,** 209 (1969).
14. E. C. HENSHAW, T. B. BOJARSKI and H. H. HIATT, *J. molec. Biol.* **7,** 122 (1963).
15. G. E. PALADE, *J. Biophys. biochem. Cytol. Suppl.* **2,** 85 (1956).
16. M. B. SPORN and C. W. DINGMAN, *Nature, Lond.* **210,** 531 (1966).
17. S. VILLA-TREVINO, *Biochem. J.* **105,** 625 (1967).
18. A. MUNN, in *Bladder Cancer* (Ed. W. B. DEICHMANN and K. F. LAMPE), p. 187, Aesculapius, Birmingham, Alabama (1967).
19. L. S. LERMANN, *J. molec. Biol.* **3,** 18 (1961).
20. T. HULTIN and K. A. ABRAHAM, *Biochim. biophys. Acta* **87,** 232 (1964).

21. N. J. Pritchard, A. Blake and R. R. Peacocke, *Nature, Lond.* **212,** 1360 (1966).
22. R. I. Cotter, P. McPhie and W. B. Gratzer, *Nature, Lond.* **216,** 864 (1967).
23. T. Hultin and A. Sjöqvist, *Biochim. biophys. Acta* **182,** 147 (1969).
24. T. Hultin, *Europ. J. Biochem.* **9,** 579 (1969).
25. T. Hultin, E. Arrhenius, H. Löw and P. N. Magee, *Biochem. J.* **76,** 109 (1960).
26. C. Kidson and K. S. Kirby, *Cancer Res.* **25,** 472 (1965).
27. E. Harbers, B .Lederer, W. Sandritter and U. Spaar, *Virchow's Arch.* **1B,** 98 (1968).
28. W. Troll, S. Delman, E. Berkowitz, Z. Chmielewicz, J. L. Ambrus and T. J. Bardos, *Biochim. biophys. Acta* **157,** 16 (1968).
29. E. A. Smuckler and E. P. Benditt, *Lab. Invest.* **14,** 1699 (1965).
30. D. Svoboda, A. Racela and J. Higginson, *Biochem. Pharmac.* **16,** 651 (1967).
31. C. Harris, H. Grady and D. Svoboda, *J. ultrastruct. Res.* **22,** 240 (1968).
32. E. Grundman and W. Fechler, *Z. Krebsforsch.* **67,** 80 (1965).
33. R. G. Kleinfeld, *Cancer Res.* **17,** 954 (1957).
34. J. G. Villalobos, W. J. Steele and H. Busch, *Biochim. biophys. Acta* **91,** 233 (1964).
35. R. P. Perry, *J. nat. Cancer Inst., Monog.* **14,** 73 (1964).
36. H. Harris, E. Sidebottom, D. M. Grace and M. E. Bramwell, *J. cell Sci.* **4,** 499 (1969).
37. O. P. Samarina, E. M. Lukanidin, J. Molnar and G. P. Giorgiev, *J. molec. Biol.* **33,** 251 (1968).
38. A. Monneron and W. Bernhard, *J. ultrastruct. Res.* **27,** 266 (1969).
39. E. C. Henshaw, *J. molec. Biol.* **36,** 401 (1968).
40. P. Mandel and J. Kempf, *Life Sci.* **8,** 165 (1969).
41. R. Kaempfer, *Nature, Lond.* **222,** 950 (1969).
42. T. Hultin, *Dev. Biol.* **10,** 305 (1964).
43. P. A. Marks, E. R. Burka, F. M. Conconi, R. A. Rifkind and W. Perl, *Proc. natn. Acad. Sci. U.S.A.* **53,** 1437 (1965).
44. E. Eliasson, E. Bauer and T. Hultin, *J. cell Biol.* **33,** 287 (1967).
45. E. A. Smuckler and T. Hultin, *Expl molec. Path.* **5,** 504 (1966).
46. J. J. Roberts and G. P. Warwick, *Int. J. Cancer* **1,** 179 (1966).
47. B. Cohen, *Biochem. J.* **110,** 231 (1968).
48. L. Skogerson and K. Moldave, *J. biol. Chem.* **243,** 5361 (1968).
49. M. E. Weksler and H. V. Gelboin, *Biochim. biophys. Acta* **145,** 184 (1967).
50. E. A. Smuckler, B. Parthier and T. Hultin, *Biochem. J,* **107,** 151 (1968).
51. E. A. Smuckler and T. Hultin, unpublished.
52. P. H. Näslund and T. Hultin, *Biochim. biophys. Acta* **204,** 237 (1970).
53. P. G. W. Plagemann, *J. biol. Chem.* **243,** 3029 (1968).
54. H. Grosjean, J. Werenne and H. Chantrenne, *Biochim. biophys. Acta* **163,** 616 (1968).
55. R. C. Shank, *Biochem. J.* **108,** 625 (1968).
56. G. L. Dorn, G. M. Martin and A. M. Purnell, *Life Sci.* **6,** 629 (1967).
57. S. E. Hawkins and L. R. Willis, *Nature, Lond.* **222,** 86 (1969).
58. S. Hennen, *Genetics* **46,** 869 (1961).
59. L. Hayflick and P. S. Moorhead, *Expl. Cell Res.* **25,** 585 (1961).
60. A. Zetterberg, *Expl. Cell Res.* **43,** 526 (1966).