Metabolic aspects of growth in HU-treated crown-gall tissue cultures

II. *Helianthus annuus*

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Abstract

The dynamics of growth and changes in nucleic acid and protein contents in sunflower calluses and tumours cultured in hydroxyurea (HU) containing media were examined. HU-induced changes in healthy tissues ran in parallel always in the same direction, in tumourous ones however an uncoupling between DNA synthesis and tissue growth on one hand and RNA and protein synthesis on the other took place. A detailed analysis of the results allows to suppose that the specific activity of HU on tumourous tissue could be an index of: 1) quantitative disturbances in its genes function (2) degree of the loss of sensitivity to the factors of regulation.

INTRODUCTION

Previously the effects of HU on a tobacco tumourous tissue of bacterial origin and a homologous callus tissue were investigated (R e n n e r t, 1976). A considerable reduction of growth and DNA level appeared in both tissues. Apart from that an inhibition of protein synthesis was found in the callus tissue. In the tumour however HU accelerated protein biosynthesis, inhibiting at the same time protein degradation processes. This caused a great accumulation of protein in the tumour. It could be supposed that this different response of the tobacco tumour tissue is an expression of the modification of its cell metabolism, showing simultaneously a particular role of translation processes in tumourous growth.

However the results of B i l e c k a's investigation (1975 a, b) per-
formed with another strains of tobacco tissues are in part contradictory
with such a conclusion. This incompatibility is mainly concerned with
the biochemical analysis of tissues. In this scope the changes induced
by HU in tumourous tissue did not vary from a model typical of the
callus. However the tissues used by B i l e c k a were cultured on a poor
medium for many years and they lost some features distinguishing
them mutually. Both tissues: callus and tumour showed approximate
biochemical features and they responded to the same range of HU
concentrations.

Independently from that on the basis of autoradiographic examina-
tion of B i l e c k a (1975a, b) it can be thought that HU, in the way
specific for the tumour, perturbs the synthesis of histone proteins and
the transfer of nucleolar RNA to cytoplasm.

In the light of these data a question arises, whether the specific
effects of HU activity in tobacco tumourous tissues are really con-
ditioned on the specificity of tumour metabolism and which of them
are characteristic in general for a crown-gall metabolism or to which
extent. An answer to this question requires to examine the tissues of
other plant species, because specific tumour features differentiate them
markedly as regards to their anatomical structure and degree of po-
lyploidy (K u p i l a, 1958). These features could influence a response
to HU. This research deals with sunflower tissues. Sunflower tumours
unable to redifferentiate in contrast with tobacco tumours contain only
diploid cells and show a uniform cytological structure (K u p i l a-
-A h v e n n i e m i, T h e r m a n 1968).

MATERIAL AND METHODS

Tissue cultures of sunflower were isolated from fragments of a healthy
tissue and tumours induced by *Agrobacterium tumefaciens* (strain
CCM 1037, Brno) on the stems of *Helianthus annus*, variety Borowski
Prążkowany grown in a greenhouse. The tissues were cultured on
a L i n s m a i e r and S k o o g medium (1965) containing 50 mg/l of case-
in hydrolysate. Callus tissue was supplemented with IAA and kinetin
(2.0 and 0.2 mg/l), tumours however were grown without these regula-
tors. pH of the media were adjusted to 6.0. Other details of tissues
culturing and the methods of experiments with HU were analogous to
those applied previously (R e n n e r t, 1976). Quantitative determinations
were also performed identically as those with tobacco tissues (R e n-
n e r t, 1977).

RESULTS

Growth

Low concentrations of HU induce a stimulation in young callus tissues, in elder tissues however this stimulation decreases gradually (Fig. 1). At this stimulation the tissues show a higher percentage of dry matter (Fig. 2). Within a range of HU concentrations 22.5—60 mg/l successive reduction of an increase in fresh matter of tissues takes place.
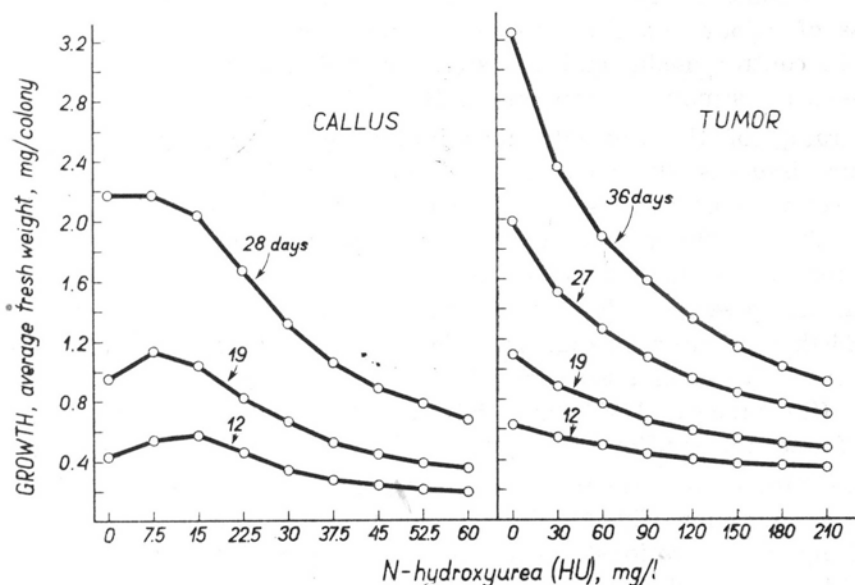

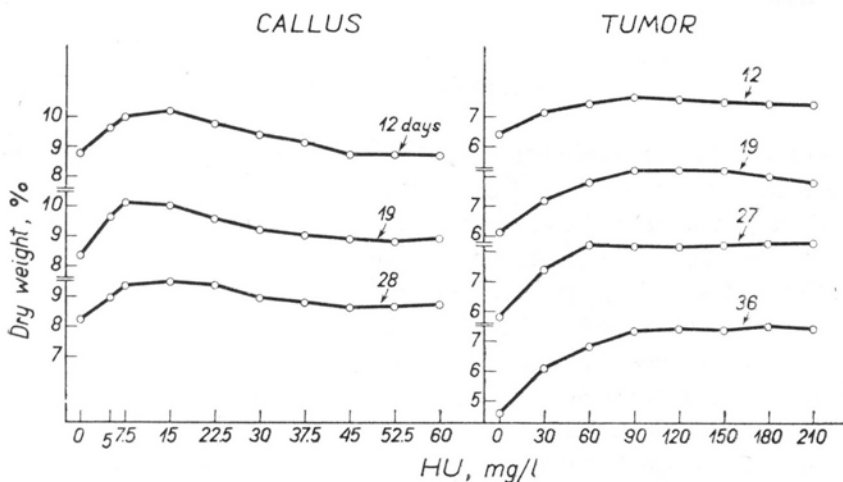Fig. 1. Run of sunflower tissue cultures growth in the media containing HU

Fig. 2. Dry matter content in sunflower tissues treated with HU

28 day old callus tissues at the highest HU concentration (60 mg/l) show an inhibition of the order of 70%. Longer keeping of these tissues in the culture leads to their deep necrosis and death. Therefore there are no data available in this work about the influence of HU on older callus tissues of sunflower. This remains in connection with their specific dynamics of growth. A strain of sunflower callus tissue used in present experiments is characterized by a short enough duration of one passage. Its maximum growth phase falls on the period between 19 and 28-th day of culturing, then the senescence begins. Formerly green tissues become fairly quick yellow and they brown here and there. A loss of colour and then browning and necrosis — becoming earlier than in control media and the stronger the longer is a culture duration — are distinctive symptoms of HU influence on this tissue.

A range of HU concentrations inhibiting the growth of sunflower tumour tissue is 30—210 mg/l of a medium (Fig. 1). The inhibition increases with an increase in HU concentration and in time up to 70% value. Though the rate of sunflower tumour tissue growth is higher, as compared to that of callus tissue, a period of the tumour growth during one passage is longer and the maximum phase falls within 28 and 36-th day of culturing. Therefore it was possible to perform experiments with elder tissues. Similarly, as in the case of tobacco tumour tissue (Rennert, 1977), any HU growth stimulating dose has not been found for sunflower tumour.

HU-induced increase in dry matter content was observed in tissues under examination (fig. 2). This is slight in the callus (at the growth inhibiting concentrations) in the tumour however rather considerable. It could be justified by a high degree of hydratation in a control tumourous tissue in comparison with a relatively low hydratation in a control callus tissue.

Metabolic changes

C a l l u s. Lower concentrations of HU (7.5—22.5 mg/l) induce an increase in protein level in fresh weight of tissues. By 12th and 19th days of culturing this stimulation reaches the values of 40 and 30% in elder tissues (28 days) however this decreases till 14%. As HU concentration increased irrespective of the age of tissue the protein content gradually decreased and at the highest concentrations of inhibitor, showed lower values as related to control by 10—15% (Fig. 3A). Calculation of the results as related to dry matter causes some lowering of protein peaks (Fig. 3B).

Curves illustrating RNA and DNA contents show nearly the same relations (Fig. 5 and 7). The only difference consists in the changes
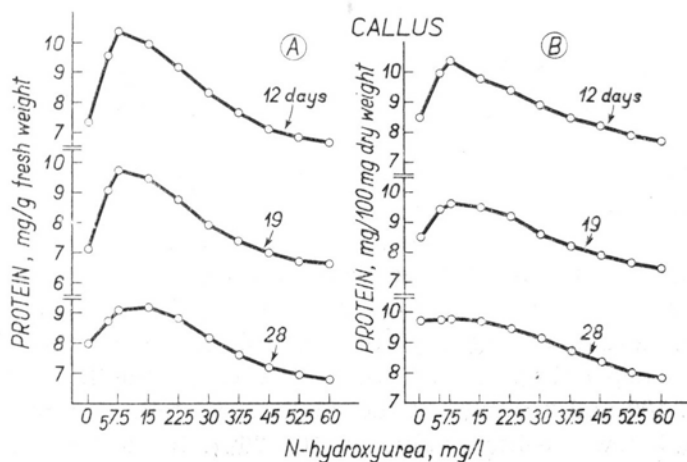
Fig. 3. Changes of protein content in sunflower callus tissue treated with HU
Protein in fresh matter (A) and dry mater (B). The same concerns fig. 4

quantity; HU-stimulated DNA increase is the lowest one; at the successive stages of culturing this amounts respectively: 30, 26 and 6%.

Higher concentrations of HU (30—60 mg/l) markedly decrease a level of both nucleic acids. At the highest HU concentration a decrease of
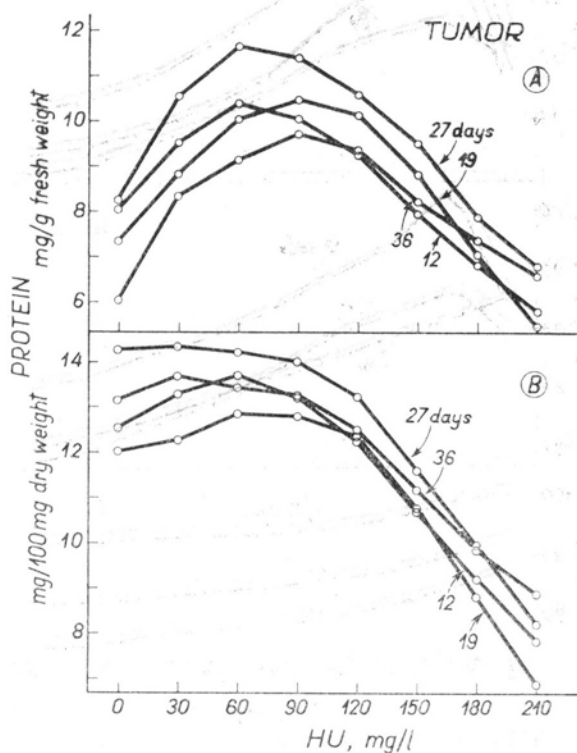

Fig. 4. HU-induced protein increase in sunflower tumour tissue

DNA content is 40—45%, that of RNA however — 16—27%. All these changes are closely related to the growth intensity and they are of the same direction. As long as lower HU concentrations-induced stimulation of parameters under examination declines with the age of tissue, so an inhibition — at higher HU concentrations — increases with the culture duration.

T u m o u r. Initial HU concentrations (30, 60, 90, 120 mg/l) at which the growth of tissue is inhibited cause an increase in protein content in this tissue, as greater as elder it is. The protein content increases the most at 60 and 90 mg/l of HU and this is higher than in control by 30—60% depending on the age of tissue. At the highest HU concentrations: 180 and 210 mg/l of the medium, protein content in younger tissues falls below a control value by 10—26%. In 36 day aged cultures however this inhibition disapears completely (Fig. 4A). Such an ac-
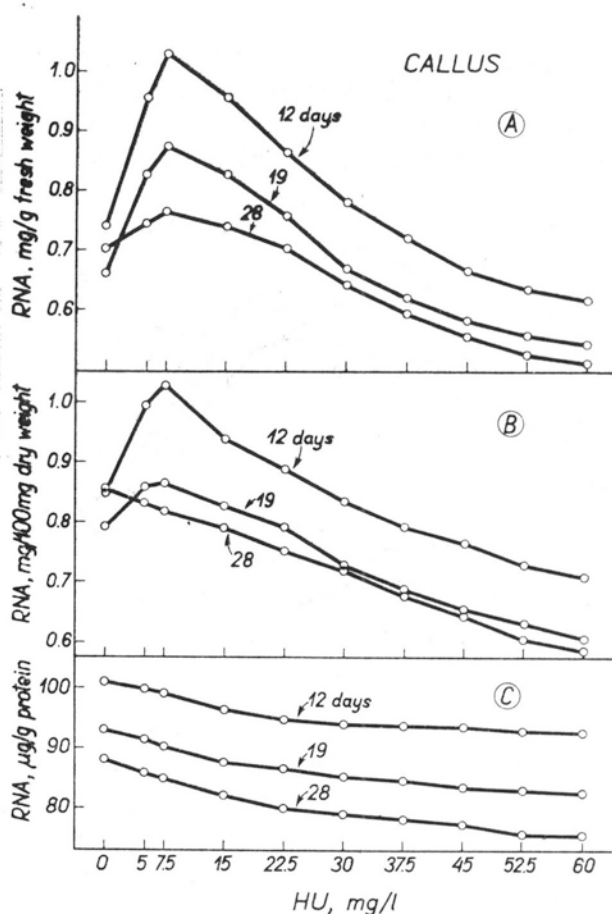

Fig. 5. Curves of RNA content in sunflower callus tissue treated with HU RNA contents in (A) mg per g of fresh matter; (B) mg per dry matter and (C) µg per mg of protein
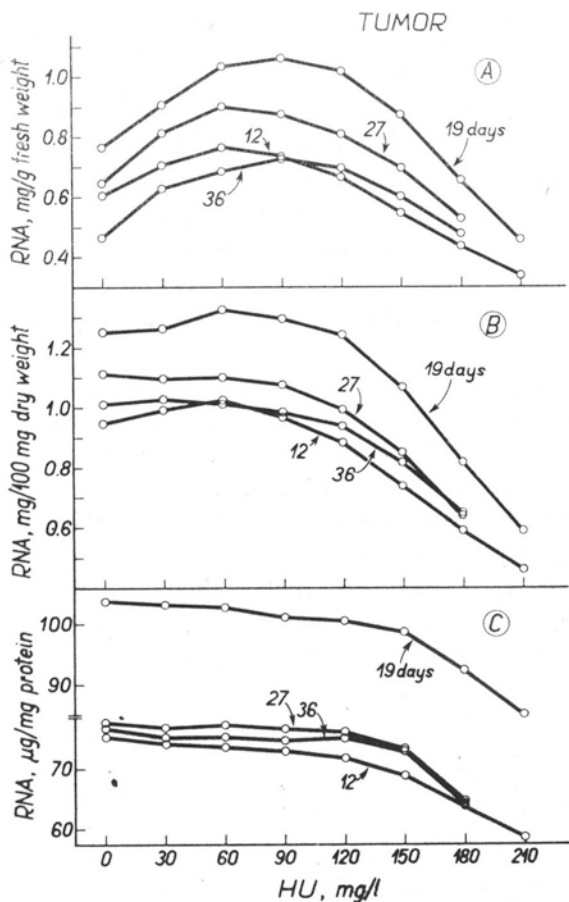
Fig. 6. HU-induced RNA increase in sunflower tumour tissue
Explanations as in fig. 5

cumulation of protein compounds could point to the stimulation of their synthesis, but certainly this proves an inhibition of their degradation.

Analogous relationships were observed in the case of RNA content in tissues under examination (Fig. 6). It is characteristic that when protein and RNA contents will be calculated to dry matter, the stimulation found in relation to fresh matter disappears almost entirely, the inhibition however markedly increases (Fig. 6B). In contrast to proteins and RNA, DNA content decreases in the tissue at all HU concentrations applied as stronger as higher is its concentration and as longer is a time of its activity duration (Fig. 8A). Maintenance of a constant RNA/protein ratio (Fig. 6C) at a considerable decrease (20—50%) of DNA/protein ratio (Fig. 8B) is a reflex of HU-induced metabolic disproportion in this tissue.

Effects of HU-activity depending on the type of tissue

Low concentrations of HU induce a growth stimulation in the sunflower callus tissue. This is accompanied by an increased level of all metabolic indices under examination (Figs. 2, 3A, 5A, 7A).

At an extended culture duration this total stimulation gradually declines. An increase in HU concentration has similar effect. This leads initially to the decay of stimulation symptoms and then to growth inhibition the stronger the higher is HU concentration. An inhibition of this tissue growth is always connected with DNA, RNA and protein contents decrease.

Any stimulation of growth cannot be observed in sunflower tumour tissue. The presence of HU in a medium induces the rate of growth reduction proportional to its concentration. This is inseparably connected with DNA content decrease (Fig. 8A). However it is characteristic that these changes are not accompanied by any decrease of RNA and protein contents. On the contrary, within HU concentrations 30—120 mg/l, protein and RNA contents in the fresh tissue weight markedly increase. Though, if related to dry weight, this stimulation becomes less clear (Fig. 4B). but a disproportion between the growth of tissue and
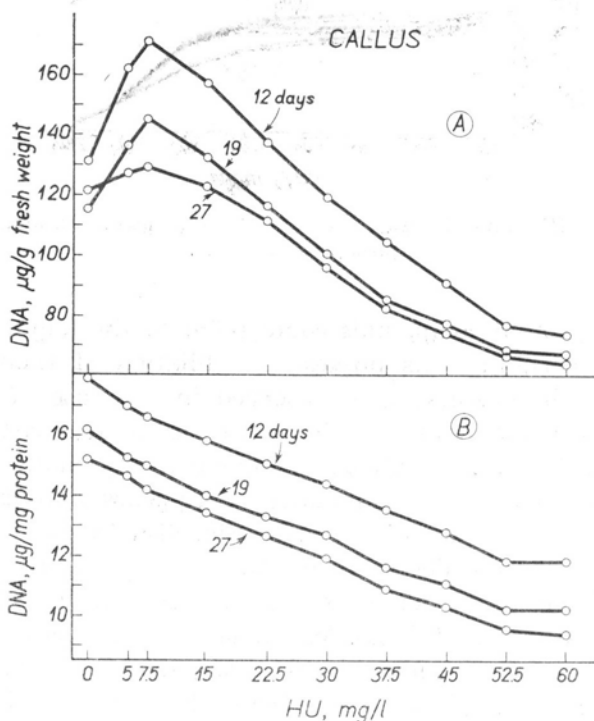

Fig. 7. Changes of DNA content in sunflower callus tissue treated with HU DNA in fresh matter (A) and as related to protein (B)
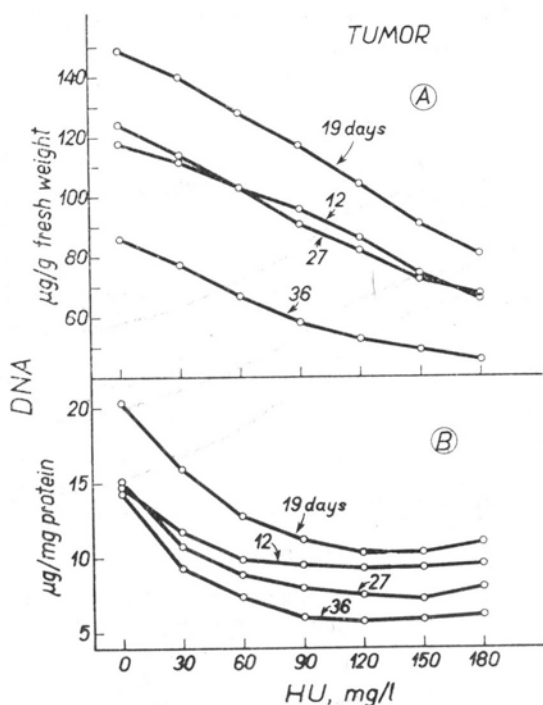
Fig. 8. Reduction of DNA content in sunflower tumour tissue at varying HU
concentrations
Explanations as in fig. 7

its DNA content on one hand and RNA and protein on the other is
continually striking. This protein level decreases only at two the highest
HU concentrations (180 and 210 mg/l) and just in younger tissues
(Figs. 4A, 6A).

A comparison of the effects of varying HU concentrations on younger
and elder tissues allows to assume that an increase in RNA and protein
contents found in a tumour is the result of both: their increased bio-
synthesis and the inhibition of degradation processes. An increase in
these compounds levels in younger tissues attests — as seems — to
intensification of the processes of synthesis. However an inhibition de-
cay in the old tissues (Figs. 4A, 6A, 36 days) and RNA and protein
accumulation progressing in time at lower HU concentrations (30—90
mg/l) are unquestionable symptoms of the block in degradation pro-
cesses.

Thus HU produces considerable changes of all the parameters under
examination in both tissues. An intensity of these changes depends on
the age of tissues and the concentration of compound. As long as changes
in DNA, RNA and protein contents and in the growth rate of callus
tissue run paralelly and are always of the same direction (Fig. 9) so

in the tumour tissue an uncoupling between DNA synthesis and growth on one hand and RNA and protein synthesis on the other takes place (Fig. 10).
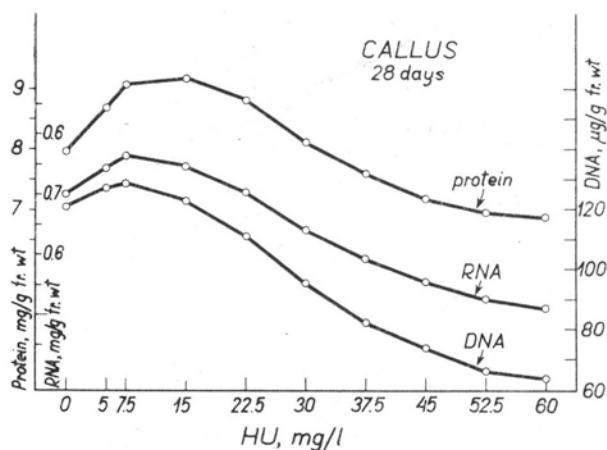
Fig. 9. Convergence of HU-induced changes of DNA, RNA and protein contents in sunflower callus tissue

Fig. 10. HU-induced disproportion in DNA RNA and protein contents in sunflower tumour tissue

DISCUSSION

A general picture of changes induced by HU in calluses and tumours of sunflower confirms entirely the results obtained for tobacco tissues (Rennert, 1977). Comparing these data it could be stated that characteristic features of HU activity common for both plant species are:

1) higher effectiveness of concentrations as related to normal tissues
2) stimumlation of the callus growth at subthreshold concentrations
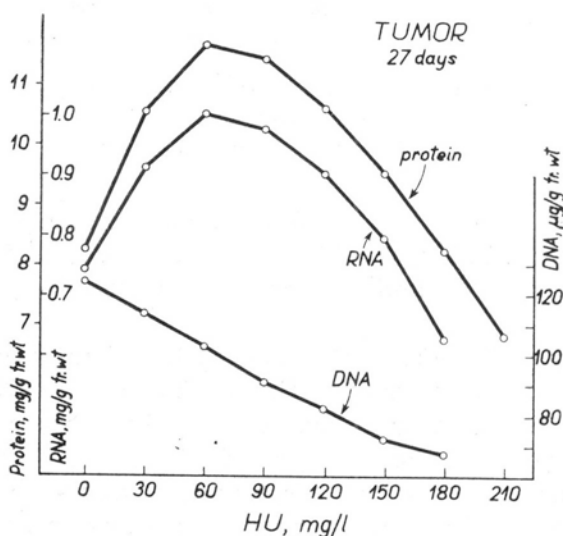3) uncoupling between DNA synthesis and growth, and RNA and protein synthesis in tumours.

HU concentrations effective for mammalian cells are in the range $\geqslant 10^{-4}M$. A similar sensitivity show calluses of sunflower and tobacco (20—60 mg/l). A necessity of applying several times higher concentrations of HU to tumourous tissues approaches them to bacteria where the concentrations needed for DNA synthesis inhibition are at least by two orders of magnitude higher than for mammalian cells (R o s e n- k r a n z et al., 1967). Though molecular basis of differentiated sensitivity of *Eucaryota* and *Procaryota* is still unknown, it seems that an intensity of the synthesis of nucleic acids and proteins is just this basis of various sensitivity of sunflower calluses and tumours to HU. It is thought that HU inhibits a conversion of ribonucleotides to the corresponding deoxyribonucleotides (S k o o g et al., 1971; O d m a r k, 1971) due to inactivation of protein B_2 in the complex of ribonucleosidediphosphate reductase (K r a k o f f et al., 1968). Thus the more precursors and active enzyme protein are formed the more HU molecules are needed for the system saturation. It was found that tumourous tissues show a higher rate of the synthesis of purines, pyrimidines, nucleic acids and proteins than homologous "normal" tissues (S r i v a s t a v a 1968, 1973; N i l e s, M o u n t, 1973; N o v a k, G a l s t o n, 1975). However a degree of these changes is different in various tissues. HU quantity necessary to induce the first appreciable symptoms of DNA inhibition in a tumourous tissue of sunflower is 1.5 times higher than that for homologous callus tissue. In the latter for the initiation of DNA and protein synthesis inhibition it is necessary respectively 1.5 and 2.5 times more HU than for that of DNA. In the tumour however an inhibition of RNA and protein synthesis requires 5—6 times higher HU concentrations than that of DNA. It proves that a sensitivity of DNA synthesis in sunflower tumour to HU decreases relativity sligthly as compared to the callus but a sensitivity of RNA and protein synthesis lowers by nearly whole order of magnitude. Similar relations were observed in tobacco callus and tumourous tissues though any distinct RNA accumulation has not been found in this case. It seems that a degree of the reduction of sensitivity to HU is positively correlated with a degree of respective processes of synthesis intensification.

Previously observed stimulation of the growth of tobacco callus tissue was poorly marked and it could seem to be doubtful. However in the sunflower callus this appeared clearly resulting in equally distinct metabolic changes. A kind of these changes proves that HU- induced growth stimulation shows regular physiological attributes. It is difficult to explain why a strong inhibitor is able to promote stimula-

tion symptoms, but a similar phenomenon was observed in many cases. This is — as it seems — of regular character (R e j o w s k i, G r z e s i u k, 1967). It concerns both: inhibitoros and stimulators. The compounds of stimulatory character if used in sub-threshold concentrations, could induce inhibition, those however of inhibitor type — stimulation. It would be an expression of natural adaptation of the living system in response to definite doses of a given compound (I v a n o v a, 1966). Inhibiting DNA synthesis HU leads in effect to the inhibition of cell division. A similar antimitotic effect show maleic hydrazide and azauracil though the mechanisms of their activity are different. Both these compounds at low concentrations produced a significant stimulation of the growth of tissue cultures derived from several tobacco species (S c h a e - f f e r, S o r o k i n, 1966). These authors supposse that this stimulation could prove the presence of control system involving the nucleic acid precursors synthesis. Whatever is the mechanism of HU-induced stimulation of growth, such a mechanism does not exist in tumourous tissues under examination, because neither of them (sunflower and tobacco) responded to low HU concentrations by the growth acceleration. It seems to be inconsistent with the results of B i l e c k a (1971) who found HU-influenced growth stimulation in both: callus and tumour of *Nicotiana*. However it could be supposed that in this case HU was used by tissues as a source of reduced nitrogen because White's medium contains suboptimum for tobacco tissue nitrogen amounts in the only form of nitrates (M u r a s h i g e, S k o o g, 1962). Under such conditions low concentrations of HU can stimulate growth (S i n g h, K u m a r, 1968, 1969; R e n n e r t, 1975). However it is unspecific activity and it does not reveal, at using adequate amounts of reduced N, which are present in the L i n s m a i e r and S k o o g medium (1965) applied in the present experiments.

The rate of a tissue mass increase is the resultant of two various processes: growth and cell division. Each of these processes show a characteristic course of macromolecules synthesis and their characteristic ratio. HU induces an arrest (limitation) of DNA synthesis non influencing directly RNA and protein synthesis (Y o u n g, H o d a s, 1964; S i n c l a i r, 1967, G a l e et al., 1964; R o s e n k r a n z et al., 1966; S c h w a r t z et al., 1965).

For this reason within a certain range of concentrations and certain limited duration of HU activity the synthesis of RNA and protein in a biological system under examination will run at a normal rate in the way characteristic for this system. Thus in both tissues appears HU-induced gradual reduction of mitotic activity in the cells but they retain their enlargement ability. There is an evidence that this type of growth requiring RNA and protein synthesis can take place at a lack of cell division in the presence of HU. (B a r l o w 1969; R e n n e r t, K n y p l,

1972). Then the different influence of HU on protein and RNA in both tissue types shows that both these tissues realize a different, individual program of growth. So far as HU-induced changes in callus tissues are similar to those in many other biological systems examined, the reaction of tumour tissue is a less typical one. Therefore some questions arise: (I) which features of tumourous growth decide on a specific, HU-induced accumulation of protein and RNA and (II), in which way the intensification of RNA synthesis can he held at DNA synthesis blocked.

Ad. I. Under normal physiological conditions cell enlargement and divisions are mutually conditioned. If at the lack (or limitation) of mitotic activity RNA and protein contents decrease in one of tissues under examination, in the other however — increase, it means, that the latter tissue is able to form greater quantities of named components and that their synthesis runs in uncontrolled way, contrary to outstanding need. Basing on a detailed analysis of similarities and differences between the influence of HU on "normal" and tumourous tissue the following interpretation could be proposed.

As the course of DNA synthesis in both tissues seems to be approximate, a fundamental difference responsible for the realization of tumourous growth is a modification of the run of protein and RNA synthesis. An increased rate of synthesis and a lack of sensitivity to regulatory factor or a loss of them are characteristic features of this modification. This is in agreement with data that the rate of RNA synthesis in crown-gall tissues is considerably higher than that in homologous healthy tissues (S r i v a s t a v a, 1968; N i l e s, M o u n t, 1973). A run of protein synthesis undergo also considerable modifications (N o v a k, G a l s t o n, 1975). The present state of investigations on the metabolism of plant tumours shows univocally, that due to a constitutive synthesis of hormones the cells of crown-gall are continuously forced to an excessive production of compounds needed for intensive growth and cell division. A continuous activation of such a program makes impossible any shifting of metabolic patterns towards other direction.

Ad II. HU-induced increase in RNA content is — as it seems — a result of its increased synthesis; in elder tissues however — of inhibited degradation. This indicates that under certain conditions of concentration and time of activity duration, HU suppresses DNA synthesis in cells activating at the same time RNA synthesis. This phenomenon is difficult to explain. However it could be supposed that HU produces a break of normal relationships between DNA and RNA polymerases resulting in competition for DNA template (B e r g et al., 1965). A similar situation was observed in several transplatable animal tumours influenced by alkyl derivatives of urea. They block DNA synthesis in the way approximate to HU. (G o r b a c h e v a, K u-

k u s h k i n a, 1970). To explain this specific response the activity of
RNA polymerase in the nuclei isolated from the Ehrlich ascites tumour
cells was examined (G o r b a c h e v a et al., 1972). At a low ionic
strength in the presence of $MgCl_2$ these nuclei form a product ap-
proximate to ribosomal RNA (system I), instead at high ionic strength,
in the presence of $MnCl_2$ a product with the base composition approx-
imate to DNA is formed (system II). Low concentrations of N-methyl-
and N-propyl-N-nitrosourea (MNU and PNU) caused and increase in
RNA-polymerase activity in both systems. An increase in concentrations
caused an inhibition of Mn^{2+}-dependent reaction but did not influence
any RNA synthesis activated by Mg^{2+}. The authors think that this
system is responsible for the stimulation of RNA synthesis appearing
at DNA block under the influence of PNU and MNU. If, as N i l e s
and M o u n t suggest (1973), the chromatin of crown-gall cells contains
much higher quantity of RNA polymerase than that in normal cells,
so an activating effect of HU can conduce to greater increase in RNA
content in these cells.

Such an explanation seems to be the most probable, but there are
also some other probabilities.

1. The observed reaction could be a result of HU-perturbed syn-
thesis of histone repressors. It was shown in many cases that HU
induces selective changes in the synthesis of respective histone frac-
tions (B u t l e r, M u e l l e r, 1973; R u d e r m a n, G r o s s, 1974). It
should be emphasized, that these changes in tobacco tumour tissue were
different in a characteristic manner from those appearing in a normal
type of tissue (B i l e c k a, 1975 b).

2. HU causes the exhaustion of deoxyribonucleoside triphosphates
pool, therefore DNA of the whole genome cannot be reproduced. It is
possible that a residual synthesis of DNA in the presence of HU still
allows for a partial, differential replication, assuring in this way con-
tinuation of RNA and protein synthesis needed for the growth support.
This phenomenon would correspond to amplification or extra replica-
tion of DNA observed in the region of nucleolus organizers, where
ribosomal genes are present. In regular systems the cycle of amplifica-
tion is characteristic for differentiating tissues (N a g l, 1974). There is
an evidence that HU completely inhibits the extra replication of DNA
connected with cell differentiation in *Cymbidium* cultures (N a g l et
al., 1972). A phenomenon of genes amplification was also noted in cancer
tissues (G r e e n b e r g, U h r, 1967; K r u e g e r, M c C a r t h y, 1970).
Some results suggest that crown-gall DNA contains a satelitary frac-
tion which has not been found in healthy tissues DNA. This is
characterized by a high content of G-C pairs and an ability to amplifica-
tion (G u i l l é, G r i s v a r d, 1971). Thus it is possible that HU does not
disturb any differentiated amplification of some DNA sequences in

a tumour and this conditions a characteristic increase in RNA and protein content.

3. Maybe a high intensity of the processes of RNA and protein synthesis maintained in the presence of HU is the result of defect on the level of biological organization. In the nuclei of parenchyma cells in *Cymbidium* protocorms HU caused atypical accumulation of chromatin on the nuclear envelope suggesting an injury of DNA bonds with membrane (N a g l, 1973). It cannot be excluded that such a change of the spatial organization causes an uncoordinated response of the system. If some structural factors are responsible for the coordination of enzymes connected with nucleic acids synthesis (H a r l a n d, 1973) so any change of these factors properties and function leads to disorder in the regulation mechanisms.

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Metaboliczne aspekty wzrostu tkanki tumora crown-gall traktowanej hydroksymocznikiem (HU) w hodowli in vitro

II. Helianthus annuus

Streszczenie

Porównywano wpływ różnych stężeń HU na przebieg wzrostu oraz zmiany poziomu kwasów nukleinowych i białek u kalusowej i tumorowej tkanki słonecznika w czasie trwania kultury. Niskie stężenia inhibitora wywołują zróżnicowane reakcje u obu typów tkanek. W tkance „normalnej" występuje stymulacja wzrostu oraz zwiększenie poziomu wszystkich mierzonych parametrów. W tumorze natomiast obserwuje się zwiększanie poziomu RNA i białek przy równoczesnej

redukcji tempa wzrostu i obniżaniu się poziomu DNA. Ze wzrostem stężenia
i wydłużaniem się czasu znika specyficzność działania HU. W obu tkankach na-
stępuje ogólna inhibicja. W tkance tumorowej, dla obniżenia poziomu DNA po-
trzeba 1.5×, a dla obniżenia poziomu RNA i białek 5-6× więcej HU jak w tkance
„normalnej".

Cechy metabolizmu komórek crown-gall, które zarysowały się w obecności
HU, pozwalają przypuszczać, że zasadniczą różnicą odpowiedzialną za realizację
wzrostu patologicznego jest modyfikacja przebiegu procesów syntezy białek i RNA.
Charakterystycznym rysem tej modyfikacji jest zwiększona szybkość syntez oraz
brak wrażliwości na czynniki regulacji.