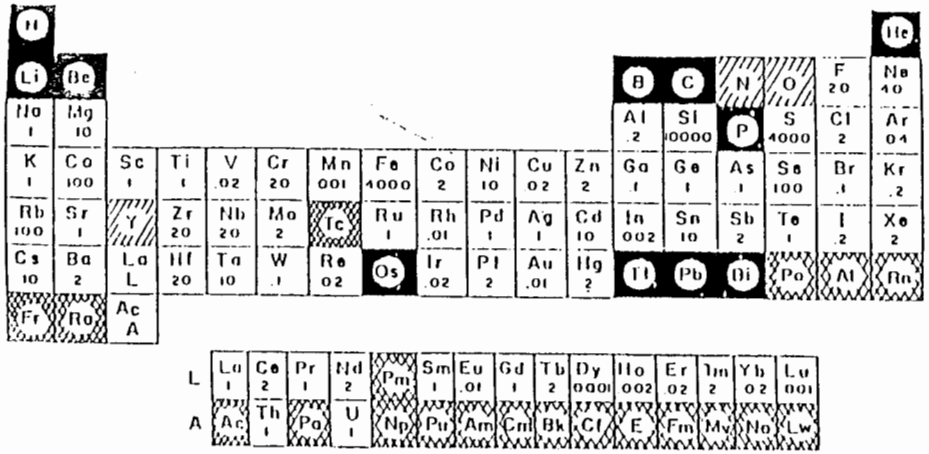


☐ Sensitive to instrumental thermal neutron activation analysis (smallest amount that can be detected is given in micrograms)

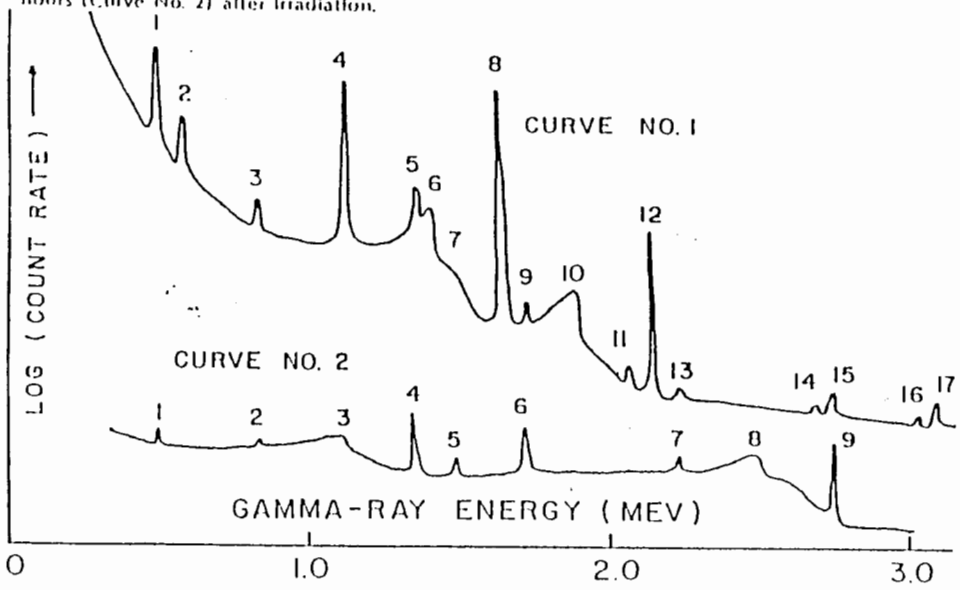
▨ Insensitive to thermal neutron activation, but sensitive to fast neutron activation analysis.

▩ Not detectable by instrumental neutron activation analysis

▨ Artificial or naturally radioactive element

Figure II – Sensitivity of Instrumental Neutron Activation Analysis using a Lithium-drifted Germanium detector system.

Figure III – Gamma-ray energy spectra from *Aloe vera* juice at 1 minute (Curve No. 1) and at 10 hours (Curve No. 2) after irradiation.



BIOSTYMIN, EXTRACT OF ALOE HISTOLOGIC AND HISTOCHEMICAL STUDIES ON THE INFLUENCE OF BIOSTYMIN ON REGENERATION OF HEPATIC PARENCHYMA IN WHITE RATS

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EDITORIAL COMMENT: This is the first of 2 articles written by Dr. Fortak on his work with Aloe Vera. This first is a study on healing of injured white rats by a product developed in Poland from Aloe juice, called Biostymin, meaning biogenic stimulator. The particular type of Aloe used by the Poles is *Aloe arborescens*, the same as used by the Russians.

Active biogenic substances released by damaged animal and plant tissues have been described by different authors under various names: Carrell – "trephones," Tusznow – "histolysates," Haberlandt – "wound hormones," Caspari – "necrohormones," Fischer – "desmones," Filatov – "biogenic stimulators," and Menteuffel – "counter substances" (cit. acc. to[1]). Although their chemical structure is unknown, the biologic stimulating action of these substances on repair processes in the body is beyond doubt, as demonstrated by numerous experimental and clinical studies.[3, 4, 6, 10, 11, 21, 24, 28, 29, 33, 36, 48]

However, no mention was encountered in the available literature of studies by histologic and histochemical methods of repair processes after injury in normal parenchymatous organs in animals submitted to tissue therapy by means of "stimulators" of plant origin.

This paper contains a partial report of studies carried out by us in the past several years with the purpose of elucidating the influence of active biogenic substances on the metabolism of liver cells regenerating after partial excision of liver parenchyma. The Polish preparation Biostymin**, a biologically active extract of aloes leaves (*Aloe arborescens*), was used.

Material and Methods

The experiments were carried out with 27 male white rats, 8-12 months old, weighing 250-310 g.

The rats were divided into three groups of 8 animals each. Group I received daily intraperitoneal injections of 1 ml of Biostymin during 3 days (4 rats) or 9 days (4 rats). In group II animals partial excision of liver parenchyma was performed after the method of Higgins and Anderson.[19] Under slight ether anesthesia and strictly aseptic conditions the middle and

left lateral lobes of the liver, constituting 65% of the weight of this organ in rats,[8] were removed. The animals then received Biostymin intraperitoneally, the first dose (1 ml) being instilled into the peritoneal cavity at the end of the operation before closing the abdominal wall, and the subsequent doses were injected intraperitoneally during 2 days (4 rats) or 9 days (4 rats). In the group III animals only partial excision of hepatic parenchyma was performed. In addition, three control rats were given intraperitoneal injections of physiologic saline solution daily after having so-called spurious operation performed, i.e., laparotomy followed by closure of the abdominal wall.

After 3 days one-half of the rats of each experimental group and one rat spuriously operated, and after 10 days the remaining animals (and two spuriously operated), were sacrificed by decapitation under light ether anesthesia.

Sections of liver tissue for histologic study were fixed in formol-calcium fluid and in Bouin's fluid and imbedded in paraffin. Sections 7 μ thick were stained with hematoxylin-eosin and with the trichrome method of Dominici as described by Roskin and Lewinson.[37]

For histochemical studies, liver fragments were collected into cooled Gendre's fluid and fixed 24 hours at +4° (material for studies of ribonucleic acid). Glycogen was studied in the sections by the method of Bauer as amended by Roskin and Lewinson,[37] and neutral mucopolysaccharides (NMPS) by the PAS method as described by Pearse.[34] Control preparations were digested for 3 hours at 37° with 1% solution of diastase. Ribonucleic acid (RNA) was stained by the method of Brachet, modified by Trevan and Sharrock.[40] Controls were digested with ribonuclease, prepared from calf pancreas after the method of Gomori,[17] for 3 hours at 37°.

Glycogen and RNA in normal rat livers were studied histochemically in sections secured during operation, which were fixed and stained together with the preparations from livers of the experimental groups of animals.

In view of the fact that the glycogen content of the liver is subject to diurnal fluctuation, the operations were always performed at the same time of day, between 14 and 15 hours.

In order to eliminate the effect on glycogen and RNA of the quantity or quality of diet, the operated rats were fasted 24 hours before operation, receiving only drinking water, and after operation received a uniform diet as described by Kovalewsky.[33]

Results

It may be noted first that histologic and histochemical studies of the livers of rats of the control group which received intraperitoneal injections of physiologic saline solution after spurious operation did not show any histologic or histochemical abnormalities, compared with the normal liver.

It may be concluded therefore that the observed histologic alterations and changes in distribution and content of glycogen and RNA in the hepatic parenchyma of rats of the different experimental groups were the direct effect of the action of Biostymin (group I), respectively of excision of liver parenchyma (group II), or both factors jointly (group III).

1. Morphochemical observations on the livers of nonoperated rats receiving Biostymin (group I). A very characteristic feature observed in the livers

both after 3 and after 9 injections of Biostymin consisted in the appearance of numerous amitotic figures in liver cells, as a result of which the number of binucleated cells increased from 20% (in controls) to about 40%. In addition, polynuclear cells (with 3-7 nuclei per cell), not encountered in control preparations, constituted about 7% of the total number of liver cells (mean calculated from 10,000 cells in 10 preparations from each series). The nuclei of these cells are arranged in "circular" or "axial" fashion, resembling isogenic groups in cartilage cells (Fig. 1).

Amitotic figures, or, strictly speaking, direct biphasic division (the nucleus dividing first, followed after an interval by division of the cytoplasm) appeared earlier than mitotic division, which began to predominate first after 10 days, i.e. after 9 intraperitoneal injections of Biostymin.

After 3 injections of Biostymin the glycogen content of the hepatic lobules was greater than in controls, and after 9 injections decidedly so (Fig. 2).

Deposition of glycogen in the liver cells begins at the periphery of the lobule. Cells grouped around the central venule of the lobule contained the least glycogen.

Glycogen appears in the liver cells in the form of fine granules (lumps) filling the cytoplasm completely. In peripheral areas of the lobules "lucid" hepatic cells containing no glycogen were observed sporadically.

The RNA content of the liver cells after 9 injections of Biostymin exceeded that in the control animals and in those which received only 3 injections. Accumulation of RNA in the form of intensely pyroninophilic granules (disappearing completely after digestion with ribonuclease) in the perinuclear cytoplasm and nucleoli is striking (Figs. 3 and 4).

After 3, as well as after 9, injections of Biostymin the Browicz-Kupffer cells lining the sinus capillaries in the lobules showed marked "activation." The numbers of these cells increased, as well as their dimensions, and the cytoplasm and nucleoli showed marked pyroninophilia, indicating accumulation of RNA.

In many of the Browicz-Kupffer cells mitotic figures were observed; some of these cells became detached from the vascular wall, assuming stellate shape and lying in the lumen of the capillaries. Groups of dividing cells were seen in the portal-biliary spaces, among which plasma cells, histiocytes (macrophages) and mast cells were the most numerous. The cytoplasm and nucleoli of the plasma cells and macrophages gave strongly positive RNA reactions, and the cytoplasm of the plasma cells, in addition, contained large numbers of PAS-positive granules which did not disappear after diastase digestion.

The Browicz-Kupffer cells and histiocytes exhibited enhanced phagocytic activity, evidenced by the presence of numerous residual bodies in their cytoplasm. Phagocytosis of similar intensity was not encountered in any of the studied control livers.

2. Morphochemistry of the liver after partial excision of liver parenchyma (group II). The most characteristic histologic alteration of the parenchyma three days after operation consisted in enlargement of the liver cells and of their nuclei and nucleoli, appearance of numerous mitotic figures in liver cells, and areas of transformation of "differentiation" of the

epithelial cells of small bile ducts into young liver cells forming new marginal layers on the periphery of the lobules.

Histochemical observations at this stage disclosed very large amounts of RNA in the liver cells.

Glycogen content of the parenchyma cells 3 days after partial excision was somewhat less than in controls; after 10 days, however, the content of glycogen was distinctly greater than in the normal, intact liver.

Ten days after operation the histologic and histochemical picture of the regenerating parenchyma already approached that of the control livers. Cytologically, it was distinguished by the appearance of polyploidal nuclei in liver cells, not observed (or very rarely) in control livers.

3. *Morphochemical observations of livers after partial excision of the parenchyma in rats receiving Biostymin (group III).* A highly characteristic feature in the regenerating livers after 3 and 9 injections of Biostymin was the appearance of large numbers of liver cells with polyploidal nuclei, which were 10 to 20 times more numerous in this than in the remaining experimental groups.

In consideration of the dimensions of the nuclei and accumulation of granules of heterochromatin, many times greater than in normal cells, it was established that these were mainly tetra- and octoploids (Figs. 5 and 6).

Some of these were formed as the result of incomplete endomitosis, that is by doubling of the number of chromosomes without possibility of migration to the poles and loss of ability of the cytoplasm to divide.

The observations described above pertain to mononuclear polyploids. Cells with two polyploid nuclei, also numerous in this group, were formed either by direct division (endoamitosis) from polyploid nuclei of higher order (e.g. two tetraploidal nuclei from one octoploid), or by complete endomitosis also from a polyploid of the same or higher order. In this case very characteristic mitotic figures in metaphase were produced, in which two superimposed asters composed of double numbers of chromosomes formed arrangements resembling spectacles. Hence, at this stage direct biphasic division resulted in "normalization" of the cellular and nuclear picture of the parenchyma, whereas incomplete mitosis led to chaso in polyploidization, i.e. in the structure and dimensions of the nuclei of hepatic cells.

Another very characteristic histologic feature of the hepatic parenchyma three days after operation and after three injections of Biostymin was the very marked vacuolization of the liver cells and appearance in the lobules of small foci of necrosis of the parenchyma. In some lobules the hepatic cells also showed signs of atrophy, leading to distinct thinning of the hepatic trabeculae.

As a rule, these alterations were not observed in cells with polyploidal nuclei.

Apart from its negative influence on the morphologic state of the parenchyma and especially in producing disorders in the normal cell divisions, Biostymin in the early stage (i.e. during the first three days after operation) also exerted an unfavorable influence on the ability of the parenchyma to synthesize and store glycogen. At this stage the liver cells contained no glycogen.

RNA was present in small amounts only in the nucleoli and perinuclear cytoplasm, and very little elsewhere, in the form of weakly pyroninophilic granules scattered irregularly in the cytoplasm. Visual evaluation of the intensity of the reactions indicated that the RNA content was much lower than, for example, three days after partial excision of liver parenchyma.

On the 10th day of regeneration, i.e. after 9 injections of Biostymin, very distinct improvement – if the expression is permissible – was observed. Only traces of vacuolization remain, and the size of the cells and thickness (width) of the trabeculae differ little from controls. Direct and indirect divisions are numerous, and many cells with two polyploidal cells are present (Fig. 7). The necrotic foci have disappeared. The small bile ducts proliferate at this time and their epithelium is transformed into young liver cells forming new marginal trabeculae in the lobules.

"Activation" of the reticuloendothelial cells, previously described, if found also in this group.

Although the liver cells contain more glycogen than after 3 days, its amount does not achieve the levels observed in the parenchyma after administration of Biostymin alone or after excision of liver parenchyma.

The most striking histochemical change observed after 10 days of regeneration in rats which had received 9 injections of Biostymin was a marked increase in RNA content of the liver cells. Unusually intense reactions of RNA were obsèrved both in the nucleoli and in the perinuclear cytoplasm and in the remaining aréas (Fig. 8). The intensity of the RNA reactions in this group was even greater than that seen on the third day of regeneration after partial excision of liver parenchyma (group II), which was the peak accumulation of RNA throughout the whole course of regeneration of the liver after excision.

DISCUSSION

1. *Remarks concerning methods used in the experiments.* The histochemical methods of demonstrating glycogen, neutral mucopolysaccharides and ribonucleic acid cover only a small section of the cellular metabolism. None the less, the study of changes in the metabolism of carbohydrates and proteins in the liver (in conjunction with the cytologic observations) appear to provide adequate grounds for the evaluation of two very important functions of liver and, to a certain extent, of the general functional state of that organ.

The stages of the process of regeneration which were selected for observation, i.e. the 3rd and 10th days, were dictated by the fact that the "model of the regenerating liver" on the third day after operation is characterized by the greatest mitotic activity of the cells and highest RNA content, increasing content of glycogen, and highest activity of a number of enzymes participating in the process of regeneration.

This stage represents the peak of the metabolic activity of the liver parenchyma.[13,14,38,41,46]

The tenth day, on the other hand, represents that stage in the regeneration of the liver, when processes of repair have reached an end, and the morphologic pattern of the hepatic parenchyma and various metabolites return to normal levels. It was therefore expected that a comparison of the effects of Biostymin at these two stages in the different

experimental groups would furnish the most conclusive evidence of its influence.

2. *Discussion of the results in different experimental groups.* The morphochemical observations of livers of group 1 rats show that intraperitoneal injections of Biostymin exert a very distinct effect on the normal, intact liver parenchyma, and, moreover, that this effect is favorable.

This is evidenced by the numerous cell divisions, both direct (amitotic) and indirect (mitotic), increasing content of glycogen in the parenchyma, increasing RNA content, and functional stimulation of the reticuloendothelial system with selective and very marked increase in the numbers of plasma cells.

Amitotic cell division, or more strictly speaking direct biphasic division, is the fundamental mechanism by which the normal liver restores its physiologic cell losses. The increased numbers of amitotic divisions, which as a rule precede appearance of mitotic divisions in case of marked loss of parenchyma, are a highly characteristic phenomenon in the liver during periods of intensified proliferation.[14]

As is known, mitotic division of parenchymatous cells in the normal liver of mammals is an extremely rare phenomenon. Holmgren[10] found only one mitotic figure in each of two cells out of 176 rat livers. According to Brues and Marble,[8] in the livers of white rats one mitotic figure occurs in 10,000-20,000 liver cells.

In our material examination of several dozens of lobes from control cases revealed mitotic figures in only two instance, which is in agreement with the findings of the authors cited above, besides confirming the observations of Blomquist[5] and our own previous results.[14]

It may be concluded that Biostymin is a very active "stimulator" of cell divisions in the liver parenchyma. This action of Biostymin may be of practical importance for liquidating losses of hepatic cells arising as the results of various types of liver damage.

However, the property of Biostymin of stimulating division of hepatic cells, especially mitotic division, is not a specific one. Homogenates of kidney or liver tissue injected intraperitoneally exert a similar influence.[5,39,40] This effect is probably due to similar, although unknown, biogenic substances present in extracts of aloes and in homogenates of different organs endowed with the property of "stimulating" cell division. Marshak and Walker[25] found that the mitotic activity of hepatic cells is stimulated by the chromatin fraction of organ homogenates containing large amounts of deoxyribonucleic acid (DNA). According to Weiler,[42] on the other hand, it is the microsomal fraction of homogenates containing large amounts of ribonucleic acid (RNA) and organ-specific antigens which exerts "mitogenic" action on the liver parenchyma.

These observations are worth mentioning because in the literature on tissue therapy dealing with the physicochemical properties of plant and animal stimulators, many authors express the opinion that nucleic acids may also act as biostimulators.[35] The histochemical study on the behavior of glycogen suggests that the effect of Biostymin on carbohydrate metabolism in the normal liver may consist in enhancing the ability of the liver cells to synthesize and store glycogen.

This appears to be confirmed by the biochemical studies of Filatov and Vachon and Prunieras (cit. acc. to[33]), who found lowered blood sugar levels during tissue therapy with biostimulators. The mechanism of hypoglycemia is probably connected with the increased storage of glucose in the form of glycogen in the liver. This observation may have some clinical value, since it is known that high glycogen content of the liver parenchyma renders it more resistant to various exo- and endogenous destructive factors.[33]

Our own observations relative to the behavior of RNA in the intact liver indicate that prolonged administration of Biostymin markedly enhances protein synthesis in the liver parenchyma.

As is known, the function of RNA is directly connected with the synthesis of proteins in cells (including the cells' own proteins and those of general use to the body), the rate of which is proportional to the amount of RNA accumulated in the cell.[12,14,22] Confirmation of this was found in this study in the observed increase in RNA content of the nucleoli and perinuclear cytoplasm during administration of Biostymin, cellular protein synthesis being concentrated in these areas of the cell.[7,9,16]

The observed markedly increased numbers of Browicz-Kupffer cells containing large amounts of RNA, grouping of macrophages in the portal-biliary spaces, and enhanced phagocytosis by these cells under the influence of Biostymin indicate activation of the reticuloendothelial system.

The appearance of large numbers of plasma cells, with markedly increased content of RNA and NMPS and very intense Brachet and PAS reactions, denotes increased production of glycoproteids by these cells.[17,34,37] In addition, it was found that the intensity of the PAS reaction is proportional to the globulin content of cells and tissues;[26] the synthesis of these proteins is supposed to depend directly on the nucleolar and cytoplasmic RNA.[47] Plasmocytes being regarded as the main producers of γ-globulin antibodies in general,[1,30,31,43] the above phenomenon may be associated with increased synthesis of nonspecific immune bodies in these cells under the influence of Biostymin; biochemical studies have demonstrated that these immune bodies of the nature of glycoproteids (cit. acc. to[27]). This may be an important link in the still obscure chain of the mechanism of the action of Biostymin in the body.

Histologic and histochemical study of the liver after partial excision of liver parenchyma in rats receiving Biostymin revealed a number of specific alterations probably representing direct effects of the action of Biostymin on the intracellular metabolism in regenerating parenchyma.

In the first place this pertains to distinct disorders in the normal mitotic divisions with subsequent polyploidization of the nuclei of hepatic cells. This phenomenon is particularly characteristic on the third day of regeneration in the liver after three doses of Biostymin and after partial excision of the hepatic parenchyma. Isolated polyploids occurred also in the livers of rats after only excision of hepatic parenchyma, but much later, first after 10 days; earlier, i.e. after 3 days, at the peak of mitotic activity normal indirect cell divisions did not give rise to polyploids. On the basis of the observations of Wilson et al.[45] and our own findings[14] we expressed the opinion in a previous paper that occurrence of polyploids in the late stage of hepatic regeneration (10th day) is probably associated with the

phenomenon of gradual extinction of "mitogenic stimuli," leading to incomplete endomitotic divisions and temporary polyploidization of the parenchyma.

This accords with the view held by a number of investigators (cit. acc. to[16]) that polyploidia is the expression of the general biologic phenomenon of "transition" of a tissue or organ from the stage of growth and proliferation to that of functional differentiation. During embryonic development as well as in later stages of regeneration it may therefore be regarded as physiological. On the other hand, early differentiation of newly formed liver cells in the presence of marked quantitative deficiency can hardly be considered favorable or normal. Functional differentiation markedly restricts their biologic power of division.

The findings presented above indicate that Biostymin, being a potent "mitogenic factor," does not induce polyploidia in the intact liver; during the early stages of regeneration, however, it affects cellular metabolism specifically, in a sense distorting the normal course of posttraumatic regeneration of the parenchyma. At this stage Biostymin disturbs the normal course of mitotic division, inhibits regeneration of the parenchyma, and induces premature differentiation (polyploidization) of the quantitatively still insufficiently developed hepatic parenchyma.

When assessing the influence of this phenomenon on the function of the liver at this stage, it is difficult to conclude whether even numerous liver cells with one or two polyploidal nuclei are able to perform double or triple metabolic work, especially since the cytoplasmic increase in these cells is disproportionate to the very marked increase in nuclear mass. However, it may be recalled that at this stage no pathologic alterations (vacuolization, atrophy) were observed only in the polyploids, whereas the remaining cells were more or less affected by them. It may be concluded that polyploids are more resistant to various destructive factors.

Although the facts presented above throw some light on the role of polyploids, this problem is far from its solution, which will require further detailed studies.

In view of the occurrence (after 3 days of regeneration and 3 doses of Biostymin) of pathologic changes in the parenchyma such as vacuolization, partial atrophy of cells, and even small necrotic foci on the first day after operation,[2,13,14,46] the influence of Biostymin on the morphologic state of the liver in the early stage of regeneration must be considered unfavorable. Biostymin upholds and increases the pathologic alterations which appear temporarily in the course of normal regeneration, and thus delays regeneration at this stage.

Biostymin exerts an unfavorable influence also on the functional state of the liver at this time, as evidenced by complete lack of glycogen and only scanty amounts of RNA in the liver cells.

The present and previous histochemical studies on the liver after partial excision of parenchyma show that the third day after operation is a period of intensive synthesis of proteins and glycogen and of peak activity of various cellular enzymes taking part in these processes.[14] The conclusion that Biostymin exerts a negative influence in the early stage of hepatic regeneration is based on the fact that it impairs the synthesis and storage of glycogen in the cells and inhibits protein synthesis by impairing production and storage of RNA.

A radical change in the situation occurs after 10 days of regeneration and 9 doses of Biostymin; the histologic and histochemical observations at this time point to a favorable influence of Biostymin on the process of posttraumatic regeneration of the hepatic parenchyma. This is evidenced by the disappearance of the pathologic alterations of the parenchyma, numerous cell divisions, both mitotic and amitotic, greatly exceeding those in the regenerating livers of rats not receiving Biostymin. On the other hand, the numbers of polyploids are only slightly higher compared with that group.

Another characteristic feature is the stimulation of proliferation and rapid differentiation of the epithelium of the small bile ducts into young hepatic cells which form new marginal trabeculae, thus distinctly increasing the volume of the lobules.

The most important indication of improvement in the function of the hepatic parenchyma at this stage, however, was the increased glycogen content of the cells and the high content of RNA with characteristic stimulation of the zones of protein synthesis in the cells.

The different effects of Biostymin on parenchymatous regeneration in the early and late stages of regeneration of the liver require explanation. The difference may be due to the fact that in the early stage the action of two potent biologic stimuli, i.e. trauma (partial excision of parenchyma) and release of active autogenic substances from the tissue is added to that of Biostymin, which is also a powerful tissue stimulator as shown by the observations on intact liver. The summation of these two stimuli at this stage of regeneration presumably led to a sort of biologic and metabolic exhaustion of the parenchyma. The stimuli evoking the response exceeded the limits of the adaptive, reproductive and functional reactivity of the cells.

Gradual expiration of the action of the active biogenic substances released from the tissues while stimulation by Biostymin continues, restores the normal reactivity of the cells. The pathologic changes in the parenchyma then quickly recede, and proliferation and function are normalized.

The data cited above point to the practical conclusion that in the early stages of trauma of the parenchymatous organs or tissues, tissue therapy by means of Biostymin is not indicated. On the other hand, its effect in late stages of reparative-regenerative processes is favorable.

In conclusion, it should be mentioned, as already stated, that the action of Biostymin on the liver is not, strictly speaking, a direct one. The mechanism of the action of Biostymin on intact as well as regenerating hepatic parenchyma is probably complex, involving a number of systemic phenomena of an immunologic and hormonal nature. This study represents one step in an attempt to elucidate some of these problems.

CONCLUSIONS

1. In the intact liver Biostymin increases storage of glycogen and protein synthesis in the liver cells. The action of Biostymin as a tissue stimulator is manifested by marked stimulation of the mitotic activity of the liver cells.
2. Biostymin causes marked "activation" of the reticuloendothelial system and selectively increases the numbers of plasma cells.
3. In the early stage of regeneration of the liver parenchyma Biostymin