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Effect of Liv.52 on the Liver *In Vitro*

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INTRODUCTION

Tissue culture has proved to be a fruitful methodology, which has not only solved many fundamental problems in biology but also explained many of the unsolved questions related to other disciplines. L. Loeb in 1827 was the first to maintain the blood cells, connective tissue and some other tissue, outside the body in a living condition. This was followed by the development of various new techniques by many workers at different times. (R.G. Harrison, 1907, Carrel and Burrows, 1910, M.R. Lewis, 1911, Ebling, 1913). Strangeways and Fell (1926) have made a real break-through in the field of tissue culture by developing the 'watch glass' technique for organ culture. This was further modified in 1965 when a synthetic medium was employed in place of the natural plasma clot medium.

Similar to other tissues, mammalian, avian and human liver tissue has also been grown by many workers in organo type culture. Heaton (1926) was the first to grow avian liver tissue in a saline medium supplemented with many other tissue and yeast extracts. Similarly, Maximov (1925) has grown rabbits' embryonic liver fragments in hanging drop culture by using rabbits' plasma clot. Human embryonic liver was also grown in organ culture either on chicken plasma clots or agar gel medium. Kauffmann (1924-25) was apparently the first to culture human embryonic liver. The growth of human embryonic liver in roller tube cultures on a collagen substrate and also in organ culture has been further investigated by Hillis and Bang (1962).

Liv.52, an indigenous compound has been claimed as a remedy for various hepatic disorders with some encouraging results. (Sule, 1968, Arora, 1969, Jaffaari and Shyam Raj, 1969, Dayal *et al.* 1970, Mukherjee and Das Gupta, 1971, Patel, 1972, Mitra *et al.* 1974). It has been observed by many workers that Liv.52 stimulates liver function, which is claimed to be due to reduction in intra-hepatic congestion thus relieving cholestasis. In addition to this action Liv.52 also acts as an anabolic with regenerative properties (Damle and Deshpande 1966). Studies on this drug have been carried out both in experimental animals and also in various disorders of livers in human beings.

However, the exact mode of action of Liv.52 on the liver has not yet been clearly understood. Keeping in view the above facts the direct effect of this drug on the liver was studied by using organo type culture *in vitro*.

MATERIALS AND METHODS

The experiment has been carried out in five stages, where the liver tissue of animals (mice and rats), both normal and abnormal, and also of human beings were grown *in vitro* in a control medium as well as the medium containing Liv.52.

Experiment I: In this experiment small (1 mm – 2 mm size) pieces of liver tissue of adult mice were grown *in vitro*, to find out a suitable environmental condition. The explants were grown in two different media i.e. medium Parker 199 and medium B.G.J. All the cultures were cultivated in the atmosphere of 50% O_2, 45% N_2 and 5% CO_2. The total numbers of cultures grown in this experiment were 48. Eight explants were harvested at intervals of 2, 4 and 6 days after culture. After harvesting the explants, they were subjected to histological studies.

Experiment II: After establishing the ideal requirements for the growth of liver tissue *in vitro*, further experiments were carried out. In this experiment similar liver tissue was grown in the B.G.J. medium alone and also in the medium containing Liv.52 (in the dose of 15 μgm/ml). Ninety six explants were cultivated in this series of experiments at different intervals.

Experiment III: In this experiment 20 adult mice were given subcutaneous injections of Carbon tetrachloride (in the dose of 0.1 ml in arachis oil) twice a week up to the end of 60 days. Degenerative changes in the liver were confirmed histologically after sacrificing the animals at different intervals. After establishing the liver damage by carbon tetrachloride, such liver tissue was cut into several small pieces and then grown *in vitro* for 6 days. In this study 112 explants were grown *in vitro*, which were divided equally into two groups i.e. control and treated. The control explants were grown in B.G.J. (modified) medium and others in the medium containing Liv.52.

Experiment IV: In this experiment 20 young albino rats of 100-120 g in weight were given absolute alcohol orally in the dose of 0.5 ml daily for 30 days. During this period all the animals were given a protein free diet. Degenerative changes in the liver were confirmed histologically. after sacrificing the animals at different intervals. At the end of thirty days the animals were sacrificed by decapitation and the liver was dissected out. It was cut into several small pieces and then grown *in vitro* for six days. In this study 60 explants were cultivated. They were equally divided into two groups i.e. control and treated.

Experiment V: In this experiment human liver tissue both normal (collected during operation) and from those suffering from liver disorder (collected by needle biopsy) were cut into small pieces and grown *in vitro* for six days both in the control medium and also in the medium containing Liv.52.

CULTURE TECHNIQUE

Liver tissue was cut into several small pieces of approximately 1 mm – 2 mm in size. They were kept immersed in tyrodes solution during the dissection. After washing with a similar fresh solution, two equal size of explants were kept separately on the stainless steel grid small table lying in the culture vessel, one for the control and another for the treated. the culture vessels were similar to those described by Fell and Weiss (1965). These culture dishes were then impregnated into a McIntosh Jar and gassed with mixture of Oxygen, Nitrogen and CO_2. This McIntosh Jar was then incubated in a water jacket incubator at 37.5^{o}C.

Culture Medium: In this initial stage of the experiment the explants were grown in the following chemically defined synthetic medium:

1. Parker 199
2. B.G.J. (Plain)
3. B.G.J. (modified by Prasad and Reynolds, 1968) 1.5 ml of these media were used each time after changing, on every alternate day. At the time of changing the medium the explants were turned over. After establishing the appropriate culture medium, the water soluble fraction of Liv.52 powder was added (in those dose of 15 µgm/ml) into the medium.

History: The explants harvested at the end of 2, 4 and 6 days after culture, were fixed in Bouins fixative solution, dehydrated in alcohol, embedded in paraffin and sections were cut at the thickness of six microns. Paraffin section were stained with Haematoxylin and Eosin and P.A.S. stain.

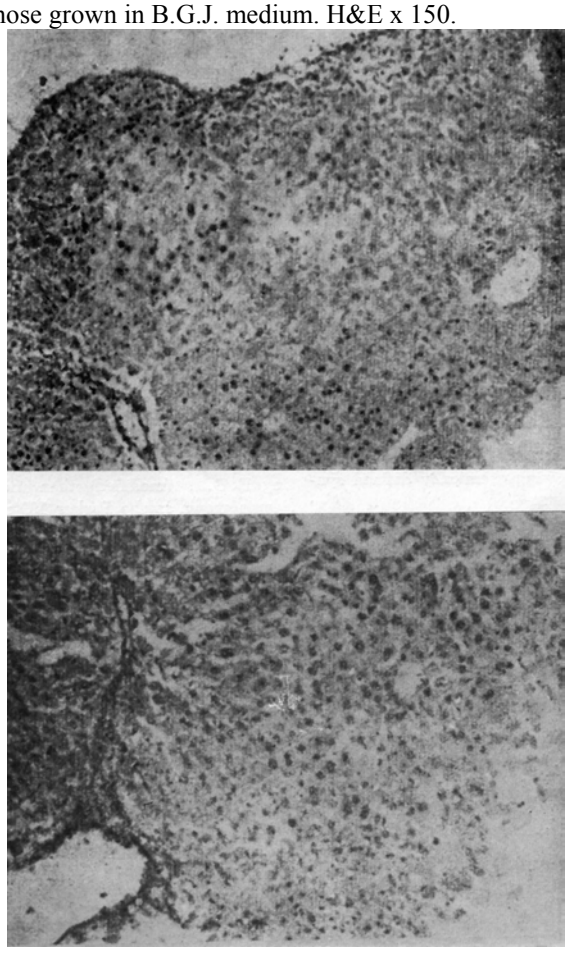
Biochemical: Used media at different intervals after cultivation of the tissue were subjected to the estimation of glucose, alkaline phosphatase, bilirubin, S.G.O.T. and S.G.P.T. by the following methods:

Bilirubin and Alkaline Phosphatase – by the method of Wootton (1964).

Glucose – by the method of Folin and Weiss (1920).

S.G.O.T. and S.G.P.T. – by kit method supplied by Bharat Lab., Bombay-7.

Fig. 1: Histological picture of adult mice liver tissue grown *in vitro* in Parker 199 medium (A) and in B.G.J. modified medium (B). Showing fairly healthy cells in those grown in B.G.J. medium. H&E x 150.



A

B

RESULTS

Selection of Media: The result of this experiment showed that the explants were more healthy till the 6th day after culture when cultivated in the medium containing B.G.J. (modified) than those explants grown in the Parker 199 medium alone. Histologically also necrotic changes were more marked in the explants grown in Parker 199 medium (Fig. 1A) whereas the parenchymatous cells with haemopoietic factors were alive and healthier in those explants cultivated in B.G.J. modified medium till the end of the 6th day of culture *in vitro* (Fig. 1B).

Effect of Liv.52 on Normal Liver (Exp. II)

The histological examination showed little difference between the control and treated explant throughout the culture. The biochemical analysis of the used medium revealed that alkaline phosphatase had slightly increased from 3.79 K.A.% on the 2nd day to 4.8 K.A.% on the 4th day followed by decrease on the 6th day i.e. to 3.29 K.A.% in the control medium. Those media used for treated explants showed more excretion of alkaline phosphatase on 2nd day after culture i.e. 5.02 K.A.% to reach maximum on 4th day after culture i.e. 7.14 K.A.%. This was followed by a similar fall as observed in control explants. The bilirubin was almost negative in the control medium, whereas the treated medium showed a little bilirubin throughout the culture period. The estimation of S.G.O.T. and S.G.P.T. revealed a gradual decline in the content from 2nd day to 6th day of the culture, whereas, a higher level was maintained in the control medium (Table I).

Table 1

Estimation	2nd day		4th day		6th day	
	C	T	C	T	C	T
Alkaline phosphatase (K.A. %)	3.79	5.02	4.80	7.14	3.29	4.01
Bilirubin (mg%)	0.00	0.02	0.00	0.02	0.00	0.04
S.G.O.T. (Unit %)	6.00	2.25	4.00	1.20	4.00	0.50
S.G.P.T. (Unit %)	6.00	1.50	5.00	0.75	1.00	0.05

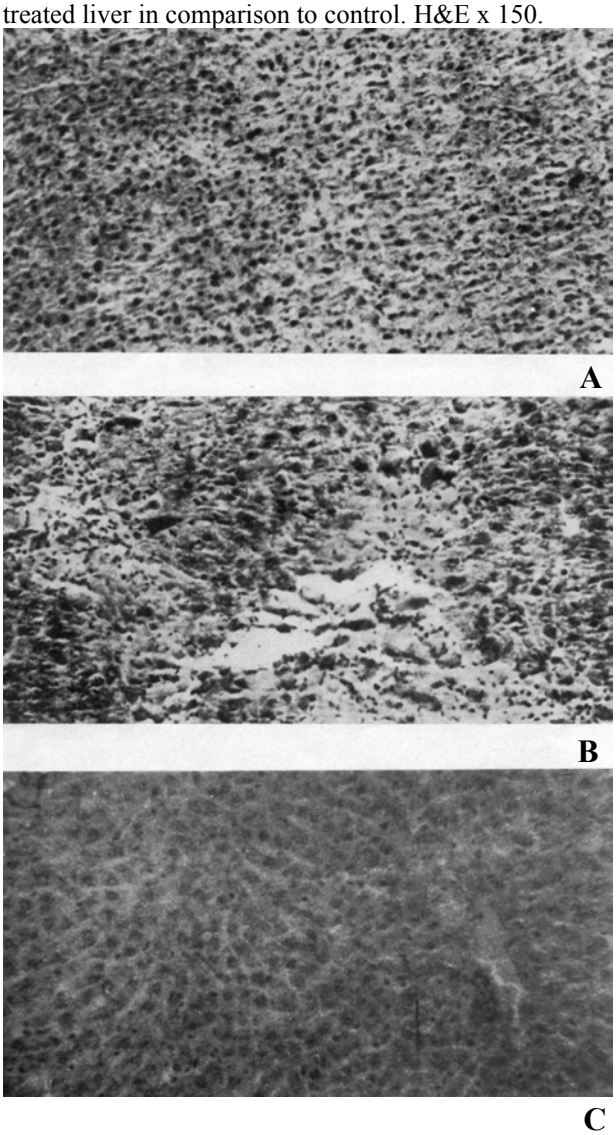


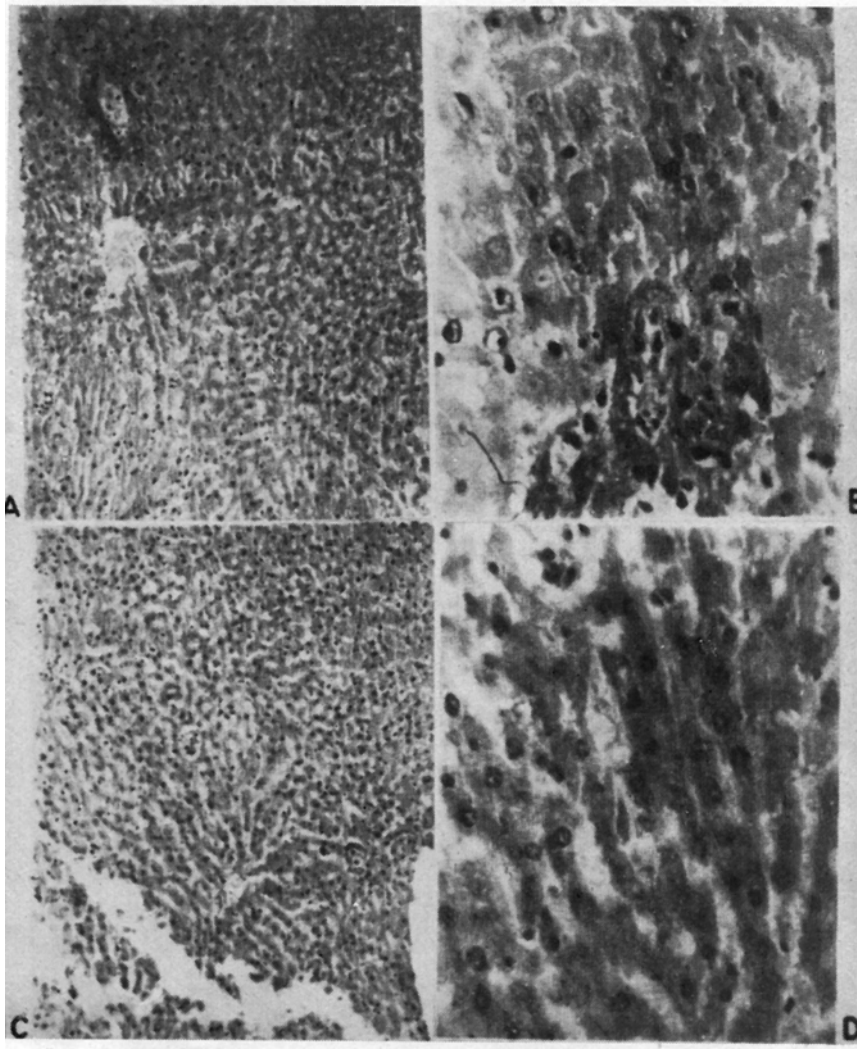
Fig. 2: Histological picture of rats' liver tissue normal. (A) and those treated with carbon tetrachloride (B) and alcohol (C). Showing the degenerative changes in the treated liver in comparison to control. H&E x 150.

Effect of Liv.52 on Carbon Tetrachloride Treated Liver (Exp. III)

The results of this experimental study showed nearly 40% mortality when treated with CCl_4. The liver in these animals lost its normal colour and showed necrotic changes histologically (Fig. 2B). When such liver tissue was grown *in vitro*, it did not change its colour; rather it became slightly brittle by the end of the 6th day of culture in the control medium. Whereas those explants grown in the medium containing Liv.52 revealed a little firmness in the tissue. Histological examination of those explants which were cultured in the control medium showed degenerative changes of the liver cells with accumulation of some fatty materials (Figs. 3A and 3B) whereas those explants grown in the medium containing Liv.52 showed gradual

replacement by normal cells with fair amount of P.A.S. positive material and haemopoietic factors (Figs. 3C and 3D).

Fig. 3: Histological picture of carbon tetrachloride treated liver grown *in vitro* in the control medium (A–x 150, B–x 600) and also in the medium containing Liv.52 (C–x 150, D–x 600). Showing regeneration of the parenchymatous cells in the treated explant in comparison to the control.



Biochemical examination of the used media revealed gradual increase in the utilisation of the glucose throughout the culture, both, in the control and treated groups. However, those explants treated with Liv.52 utilised more glucose in comparison to the control explants. The level of alkaline phosphatase was almost the same throughout the period in the control medium, whereas in the treated medium a higher level was seen initially, but, a gradual decline was observed in the later phase. No bilirubin could be traced in the control medium, but from the 4th day onwards traces were observed in the treated medium. The estimation of S.G.O.T. and S.G.P.T. showed higher units in the control medium in comparison with the treated one (Table II).

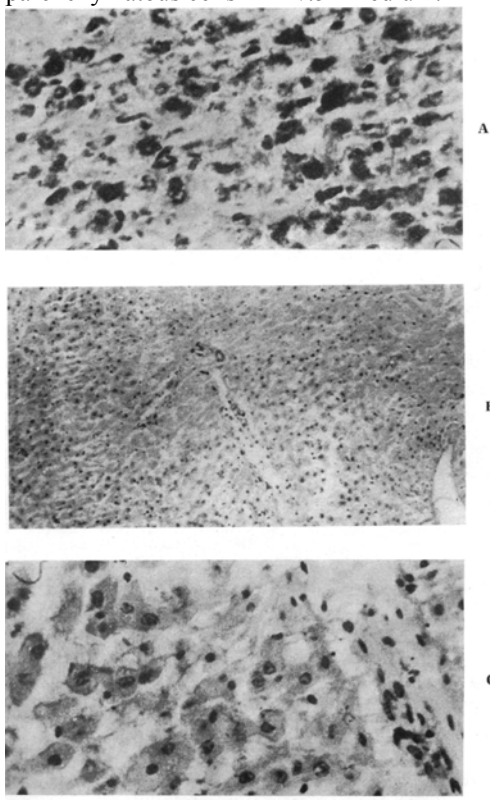
Table II

Estimation	2nd day		4th day		6th day		
	C	T	C	T	C	T	
Glucose utilised (mg%)	1.00	1.54	2.72	3.70	2.91	4.00	Increased utilisation
Alkaline phosphatase (K.A.%)	2.35	3.27	2.10	3.20	2.11	2.95	Gradual decline
Bilirubin (mg%)	Nil	Nil	Nil	0.04	Nil	0.02	Traces observed
S.G.O.T. (Unit %)	16.0	10.0	8.0	6.0	10.0	Nil	Higher in control
S.G.P.T. (Unit %)	70.0	26.0	67.0	40.0	50.0	30.0	

Effect of Liv.52 on the Alcoholic Liver (Exp. IV)

Results of this experiment showed some patchy discoloration of the liver, which was slightly enlarged compared to the normal. Histologically, destruction of the cell as well as diminution in the size of the cell and nucleus with some fibrotic changes could be seen (Fig. 2C). Such liver explants when grown *in vitro* in the control medium for six days showed some improvement in the cellular pattern with slight amount of P.A.S. positive (glycogen) material (Fig. 4A) whereas those explants cultivated in the medium containing Liv.52 revealed regeneration of the liver cells along with the growth of some fibroblastic cells and haemopoietic factor. There was a fair amount of P.A.S. positive material (Figs. 4B and 4C).

Fig. 4: Histological picture of alcoholic liver tissue when grown in the Control medium (A–x 600) and also those cultivated in the Liv.52 medium (B–x 150 and C–x 600). Showing degenerative changes in the Control medium, whereas normalization of parenchymatous cells in Liv.52 medium.



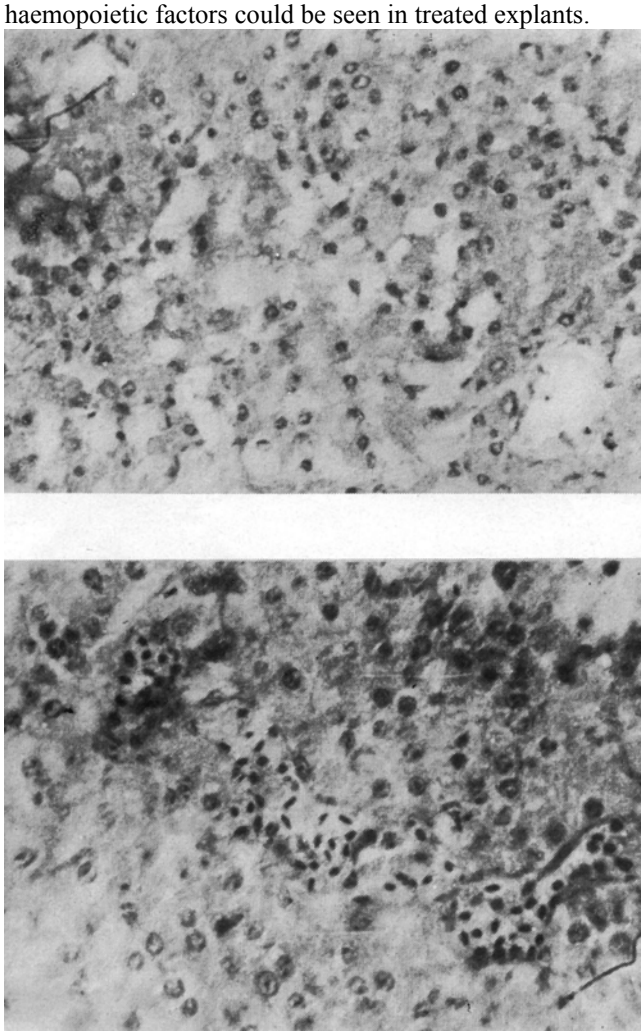
The biochemical studies showed up to 2-3 times more utilisation of glucose by the explants cultivated in the medium containing Liv.52 in comparison with those explants cultured in the control medium. Similarly the excretion of bilirubin was more in the treated group than those of the control. The alkaline phosphatase level showed a gradual decline as the culture period advanced both in the control and treated media. However, the level was higher in the treated medium than in the controls. S.G.O.T. and S.G.P.T. estimation revealed a gradual lowering in the content on 2, 4 and 6 days after culture in both groups. The S.G.O.T. was almost nil in the treated medium by the end of six days after culture, whereas 9.3 unit % was still observed in the control medium.

Effect of Liv.52 on Human Liver (Exp. V)

In this experiment diseased (cirrhotic) liver (obtained by liver biopsy) and normal liver (obtained during operation) were grown *in vitro* in both media i.e. control and with Liv.52. Those explants obtained from normal patients and grown *in vitro* showed not much difference, microscopically between control and treated groups. When cirrhotic liver tissue was cultivated *in vitro* in the medium containing Liv.52 it showed a slight decrease in the firmness in comparison to that grown in the control medium. Histological examination of such explants when cultivated in the control medium showed patchy degeneration of the liver cells with an accumulation of fatty materials (Fig. 5A). Whereas those explants grown in the medium containing Liv.52 showed presence of haemopoietic factor in blood vessels, a decrease in fatty deposition along with regeneration of liver cells. The fibrotic bands, which were observed in the control explants had decreased when grown in Liv.52 (Fig. 5B). The

biochemical analysis of the used medium revealed similar observation as seen in other previous experiments. The utilisation of glucose was more by those explants, which were grown in the medium containing Liv.52 in comparison to the control explants. Similarly the level of bilirubin was higher in Liv.52, whereas in control medium it was absent. The alkaline phosphatase was found more in the treated medium than in the control medium throughout the culture period.

Fig. 5: Shows the histological picture of the human liver (cirrhotic) tissue grown *in vitro* in Control medium (A–x 600) and those grown in the medium containing Liv.52 (B–x 600) for a similar period. The Control explant shows the presence of degenerative and some fibrotic changes, whereas normalization of liver cells along with haemopoietic factors could be seen in treated explants.



DISCUSSION

Liver tissue has been grown *in vitro* by many workers in different parts of the world. Most of them have cultivated it in monolayer culture, and observed that *in vitro* it is mainly the fibroblasts, which grow, rather than the liver cells, whereas the result of our study has shown that liver tissue could be grown *in vitro* by using organo type culture in the synthetic medium. Further, the results of our studies, where diseased liver was grown *in vitro* in the medium containing Liv.52 has revealed increased utilisation of glucose. This suggests that Liv.52 probably stimulates the liver cells to convert more glucose into glycogen. This could be further correlated with increased P.A.S. positive materials on histochemical studies. This also correlates with the almost similar observation made by Mukerjee and Dasgupta (1970). Khetarpal and Kumar (1974) and Kulkarni *et al.* (1972). The higher values of bilirubin in the treated medium indicate that the addition of Liv.52 in the medium may also stimulate the liver cells directly to excrete the bilirubin into the culture medium. Clinically such action has also been reported by Dayal *et al.* (1971). At the same time the decrease in the S.G.O.T. and S.G.P.T. in the treated medium is indicative that Liv.52 probably prevents further necrotic changes *in vitro* produced by carbon tetrachloride or alcohol. Moreover the activity of the liver cells, and the regenerative process may be confirmed by the observation of higher levels of alkaline phosphatase. These observations, however, suggest that the water-soluble fraction of Liv.52 probably also acts directly on the liver and stimulates it to increase the function and normalise the cells.

SUMMARY

(i) Liver tissue was grown *in vitro* in organo type culture for six days in chemically defined synthetic medium and also in the medium containing water-soluble fraction of Liv.52.

(ii) Liver damage was produced by carbon tetrachloride in mice and by alcohol in rats. Thereafter such liver was cultivated *in vitro*, both in control and Liv.52 containing media for 6 days. It has been observed that when such liver tissue was grown in the medium containing Liv.52 it showed regeneration of cells and increase in the function. The glucose utilisation was found more in the treated explants in comparison to control.

(iii) In addition to the experimental liver tissue, some human liver (normal and cirrhotic) was also grown *in vitro* in the similar media upto six days. The effect of Liv.52 on such explants was studied and a directly stimulating response was obtained *in vitro*.

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