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INCREASED INCORPORATION OF ^{3}H-THYMIDINE INTO RAT LIVER DNA AFTER HANDLING OF LIVER LOBES

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SUMMARY.—Handling of part of the rat liver without resection or interference with the vascular supply was associated with an increased rate of incorporation of ^{3}H-thymidine into DNA. The response curve was distinctive and different in shape and in magnitude from that which occurs after partial hepatectomy. Incorporation was greater in the peripheral rather than central parts of the liver, was more striking after i.p. rather than intravenous administration of isotope and was associated with a proliferation of hepatic capsular cells. Radioautographs showed activity in capsular cells, inflammatory cells and also in a few peripherally placed hepatocytes.

The possibility is canvassed that this phenomenon may have led to misinterpretation of results of experiments designed to test for the presence of humoral stimuli or inhibitors in restoration of the liver.

THE search for the mechanism which controls restoration of the liver after damage to or removal of part of the organ has led not only to disagreement about interpretation of results, but in some instances to conflicting findings when apparently similar experiments have been carried out (Adibi, Paschkis and Cantarow, 1959; MacDonald, Rogers and Pechet, 1963; Alston and Thomson, 1963; Grisham, Leong, Albright and Emerson, 1966; Moolton and Bucher, 1967). Several physical and chemical changes are known to occur in the remaining liver cells after partial hepatectomy, but their significance and interrelationships are not yet clearly defined. Some of the observations may relate more closely to the surgical manoeuvres than to the proliferative response but the differentiation is very difficult to establish. The density of negative charge on cell surfaces increases shortly after operation (Ben-Or, Eisenberg and Doljanski, 1960), changes are noted in the flow and pressure of blood to the liver remnant (Benacerraf, Bilbey, Biozzi, Halpern and Stiffel, 1957; Banerjee and Aikat, 1968) and the levels of several enzymes increase (Bucher, 1967). The constituents of the blood are also altered; a foetal α-globulin appears in the plasma of adult rats after partial hepatectomy but this is found also after other injuries (van Gool and Ladiges, 1969). There is also evidence that blood from normal rats differs in biological activity from blood in partially hepatectomised rats.

Exchange transfusions between normal and partially hepatectomised animals are reported to cause stimulation or inhibition of growth in recipients (Moolton and Bucher, 1967; Grisham *et al.*, 1966) and autotransplanted liver tissue is described as proliferating after part of the normally sited main liver is resected (Leong, Grisham, Hole and Albright, 1964).

In most of the published work the experimental procedure involved one or more surgical operations on the liver and subsequent analysis by mitotic counts or assessment, either by biochemical analysis or radioautography, of DNA

synthesis after injection of a radio-active precursor of DNA (^{3}H, ^{14}C or ^{32}P labels). In those instances where partial hepatectomy had been performed and only the posterior 2 lobes of the liver remained the tissues examined probably consisted of the larger portion of the right lobe, but might if not defined, have included fragments of the caudate lobe. In instances where part of the anterior lobes remained as in smaller resections or operations on vessels, it is not always clear which is assayed and the effects of the operative procedures on the handled lobes may also complicate the proliferative response. It seemed important to identify whether the trauma which may be inflicted during surgery could possibly induce changes in the rate of DNA synthesis in the handled tissue, and to define the extent and timing of any such response.

MATERIAL AND METHODS

Animals.—Male albino rats weighing between 150 and 220 g. were used. These were bred in the University of Nottingham, weaned at 3 weeks and subsequently fed on " Research" rat cubes.

Surgical Procedures.—All operations were carried out under ether anaesthesia between 10 a.m. and 1 p.m. Handling of the anterior liver lobes consisted in incising the anterior abdominal wall and delivering the anterior 2 lobes of the liver, replacing them in the abdomen and suturing the abdominal wall in 2 layers. Control animals were subject to ether anaesthesia, incision of the anterior abdominal wall, viewing but not touching the anterior lobes of the liver. Animals were not fasted before operation or before death. The rats were killed by ether anaesthesia, the livers being removed while the animals were still breathing and the portal vein was grasped with a haemostat before the liver was excised.

DNA Synthesis.—This was measured by a method described previously (Weinbren and Woodward, 1964), but modified slightly so that ^{3}H-Thymidine (Radiochemical Centre Amersham, T RK 61-Thymidine-6-T (n), Specific Activity 17·4 Ci/mM), was used in place of ^{32}P-orthophosphate. The ^{3}H-thymidine, 0·5 μCi/g. body wt was injected 1 hr before death either i.p., or i.v. *via* the right femoral vein. The radioactivity was measured in a Packard Tri-Carb scintillation counter, using NE 240 scintillant (Nuclear Enterprises, Edinburgh). Corrections were made for quench using external standardisation and the d.p.m. were calculated from c.p.m.

Radioautography.—These were prepared of median and right lobes of every specimen using AR 10 stripping film, exposing at 4° for 21 days, developing with D 19, and staining with Meyer's haemalum.

Microscopy.—Microscopical preparations were made of the median and right lobes in every instance after formol alcohol fixation. Paraffin sections were stained with haematoxylin and eosin.

Arrangement of experiments

Group I.—The anterior liver lobes were handled in 90 rats which were killed in groups of 6 or more at 6, 12, 15, 21, 24, 29, 36, 48, 72, 96, 120 hr and 10 days after the operation. For each group of 6 rats, 2 controls subjected to laparotomy but no handling of the liver, were killed at the same time.

Group II.—The specific activity of the DNA in the sub-capsular and capsular regions was compared with that in the central regions of right and median liver lobes in 6 animals 24 hr after handling of the liver and in 2 controls.

Group III.—A comparison was made between the effects of i.v. and i.p. injections of the labelled thymidine. This was done by subjecting 11 animals to the handling procedure (with 4 controls) and after 23 hr 6 were anaesthetised and the tritiated thymidine was given by the right femoral vein, the same procedure being applied to 2 controls. The remaining 7 animals were subjected to similar anaesthesia and given ^{3}H-thymidine i.p. All animals were killed 1 hr after injection and 24 hr after the original operation.

Group IV.—A group of 6 rats were subjected to handling for different periods of time, 3 for 60 sec., 3 momentarily, together with 2 non-handled controls. The animals were killed at 24 hr, 1 hr after I.P. ^{3}H-thymidine.

RESULTS

The radioactivity of the DNA extract is expressed as disintegrations per min. per unit of extinction at E_{260}. The reference unit has been previously defined, its P content measured and the extraction procedure has been evaluated and found to be valid (Weinbren and Woodward, 1964). The present results are summarised in Table I. The mean level of radioactivity in anterior and posterior lobes of control animals is approximately 300 d.p.m./unit E_{260} and extracts from anterior handled lobes show an increase from 21 hr postoperatively. At this time the level is 945 $\pm$ 181 (S.E.) d.p.m./unit E_{260} for handled anterior lobes, 194 $\pm$ 20 for corresponding posterior lobes and 284 and 255 for anterior and posterior lobes respectively, of controls. There is a slight increase in the rate of incorporation

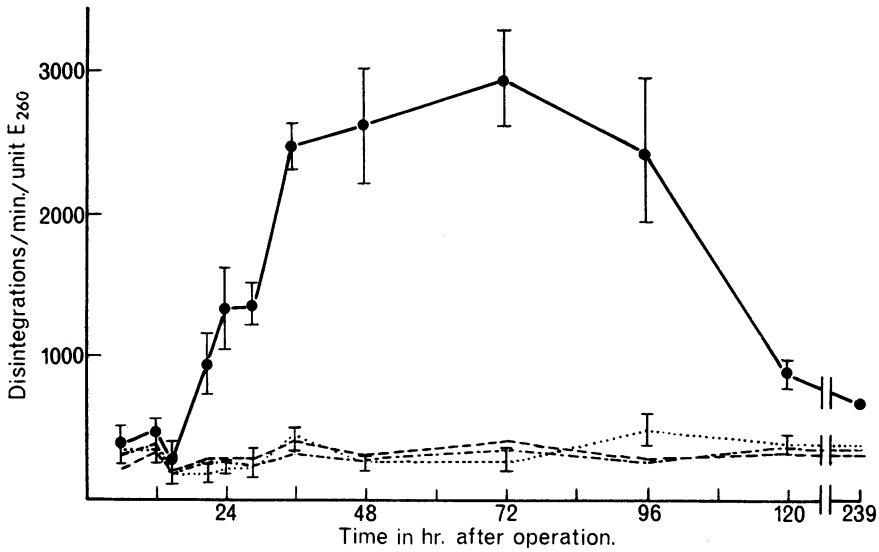


FIG. 1.—The incorporation of ^3H thymidine into DNA of anterior and posterior liver lobes after handling of anterior lobes.

●——● anterior lobes after handling.
..... posterior lobes after handling of anterior lobes.
– – – – anterior lobes from controls.
—·—·—·posterior lobes from controls.
The vertical lines represent $\pm$ S.E.

at 24 and 29 hr (1334 and 1336 d.p.m./unit E_{260}) and the high rate is maintained from 36–96 hr. It is difficult to define a single peak during this period and the plateau at the most active level is apparently sustained for some 60 hr, presuming there are no serious changes in rate of incorporation during the 12 hr periods between the times at which assays were done (Fig. 1). The rate of incorporation is still slightly increased at 865$\pm$65 d.p.m./unit E_{260} at 120 hr and approaches a normal level between then and the 10th day when the level is approximately 516 $\pm$ 42 d.p.m./unit E_{260}. At all the times studied the posterior lobes of experimental animals showed no significantly increased rate of synthesis as compared with control animals. The difference between results derived from anterior handled lobes and those derived from posterior (non-handled) lobes of the same

experimental series is emphasised by expressing the values as a ratio (Table I, Fig. 2). In control animals the ratio is very much lower (between 0·91–1·28) than that derived from animals in which the highest levels of incorporation are found (up to 11·25 at 72 hr).

Radioautography confirms this general finding and there are abundant grains over handled lobes and few over non-handled lobes, but activity appears to be confined mainly to the capsular and sub-capsular regions. Activity of the nuclei

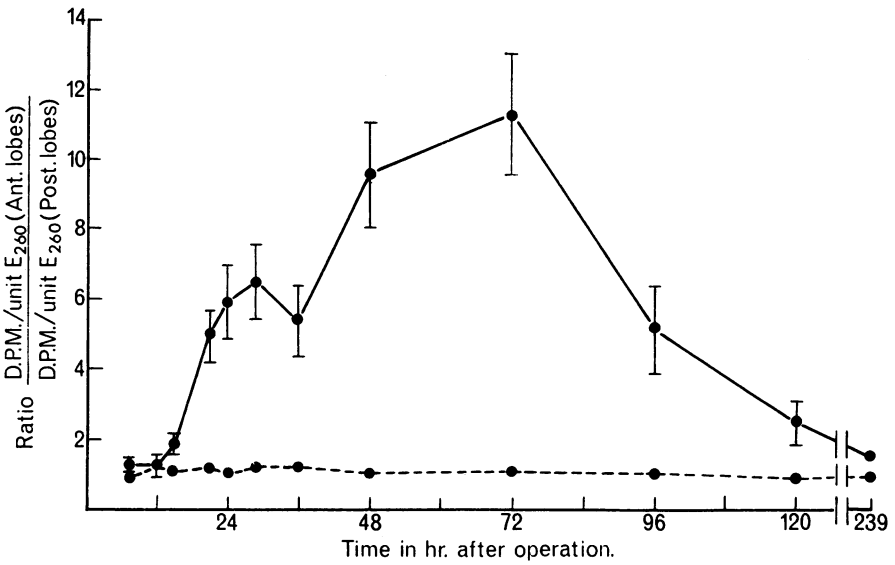


FIG. 2.—Ratio of incorporation of ^{3}H thymidine into DNA of anterior lobes to that of posterior lobes after handling of anterior lobes and controls.

●———● ratio after handling.
●– – –● ratio in control non-handled rats.

TABLE I.—*Incorporation of ^{3}H-Thymidine into Liver DNA after Handling the Anterior Lobes*

Time after operation (hr)	Handled livers: No. of rats	Handled livers: D.P.M./Unit E_{260} in DNA extract: Ant. Mean	Ant. SE	Post. Mean	Post. SE	Ratio A/P	Non-handled livers: No. of rats	Non-handled livers: D.P.M./Unit E_{260} in DNA extract: Ant. Mean	Post. Mean	Ratio A/P
6	6	385	138	317	102	1·349	2	212	343	0·910
12	6	479	94	325	31	1·349	2	308	360	1·252
15	6	282	48	154	28	1·873	2	188	184	1·045
21	11	945	181	194	20	4·819	4	284	255	1·200
24	17	1334	215	225	28	5·908	7	290	276	1·053
29	8	1336	143	222	33	6·508	2	290	239	1·211
36	6	2483	116	453	65	5·417	2	408	329	1·230
48	6	2605	427	268	30	9·604	2	309	289	1·078
72	6	2936	267	267	39	11·250	2	402	355	1·150
96	6	2423	506	497	73	5·128	2	286	273	1·038
120	6	865	65	394	38	2·426	2	325	368	0·911
239	6	516	42	354	31	1·458	2	268	283	0·947

in the substance of the liver tissue, away from the capsule does not appear to differ significantly between the control and experimental animals.

In experimental series II, extracts derived from the central part of the anterior handled lobes provided 805 ± 222 d.p.m./unit E_{260} and those from the periphery 2234 ± 579 d.p.m./unit E_{260}. This difference is not seen when values from peripheral and central parts of posterior lobes are compared, although the values in this series are unusually high (659 ± 176 for central and 711 ± 180 for peripheral parts of posterior lobes). These differences were similarly not found in extracts taken from peripheral and central parts of control livers (Table II). The results

TABLE II.—*Incorporation of ^{3}H-Thymidine into DNA from Central and Peripheral Parts of Rat Liver Lobes 24 hr after Handling*

		D.P.M./Unit E_{260}							
		Anterior				Posterior			
		Central		Peripheral		Central		Peripheral	
	No. of rats	Mean	SE	Mean	SE	Mean	SE	Mean	SE
After handling	6	805	222	2234	579	659	176	711	180
Controls	2	419	—	488	—	437	—	471	—

of the experiment (III) designed to test the effect of different routes of administration of the labelled DNA precursor indicate that a greater rate of incorporation is associated with i.p. injection than with i.v. administration. Extracts derived from anterior lobes 24 hr after handling and 1 hr after i.p. injection of the radioactive thymidine gave a mean value of 1414 ± 343 d.p.m./unit E_{260} while the comparable value for handled anterior lobe extracts after intravenous injection of labelled thymidine was 379 ± 37 d.p.m./unit E_{260}. Posterior lobe extracts from both series and all samples from relevant non-handled controls, given i.v. or i.p. injections were uniformly low between 210 and 261 d.p.m./unit E_{260} (Table III).

TABLE III.—*Incorporation of ^{3}H-Thymidine into Rat Liver DNA 24 hr after Handling of Liver and Intravenous or Intraperitoneal Administration of Compound*

	D.P.M./Unit E_{260} of extract Intraperitoneal Injection				D.P.M./Unit E_{260} of extract Intravenous Injection				
	No. of rats	Ant.	SE	Post.	SE	No. of rats	Ant.	SE	Post.	SE
After handling Liver	5	1414	343	205	54	6	379	37	261	11
Controls	2	244	—	210	—	2	244	—	240	—

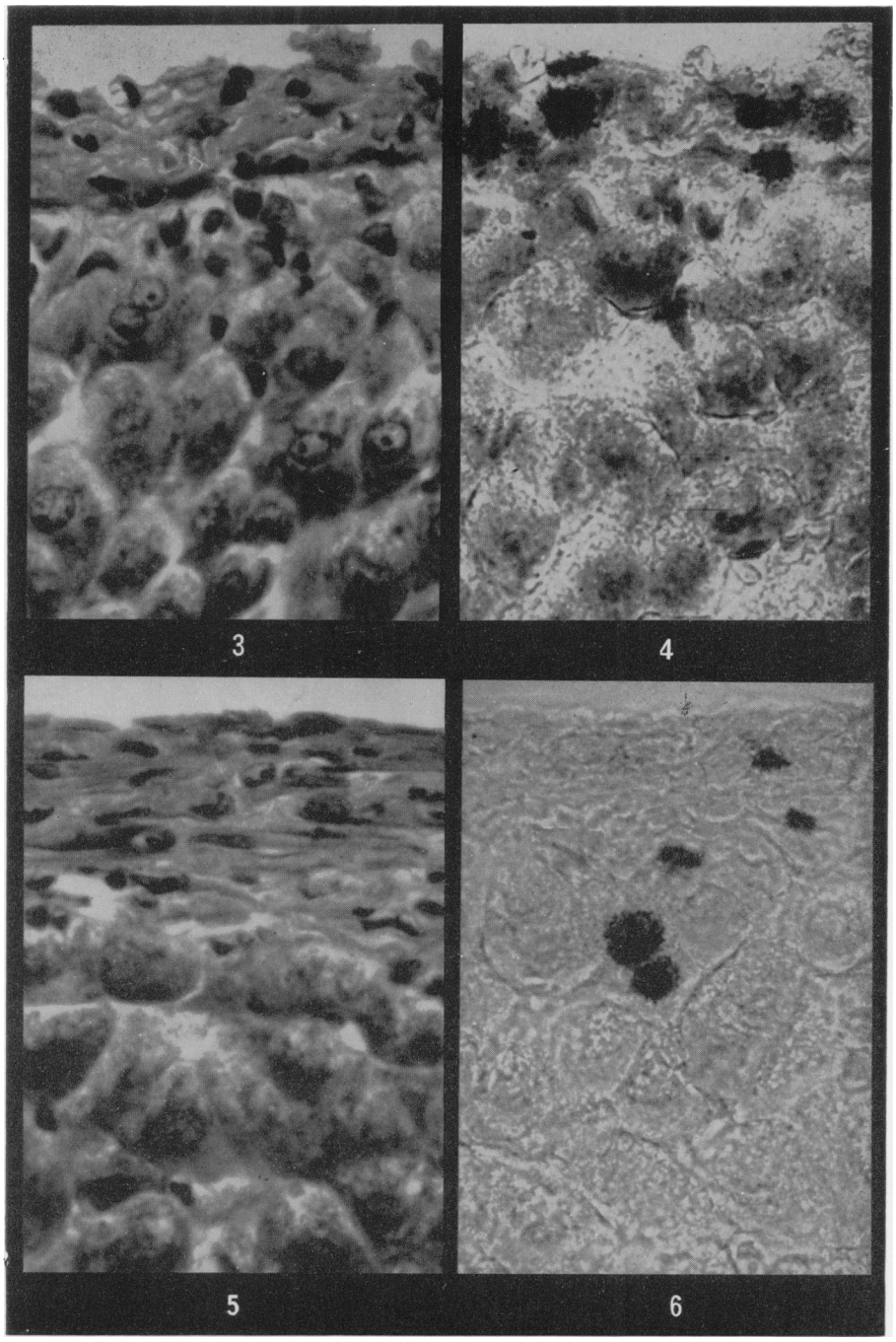
EXPLANATION OF PLATE

FIG. 3.—A mild inflammatory reaction and capsular cell proliferation in the anterior liver lobes 24 hr after handling. H. and E. × 800.

FIG. 4.—Radioautograph of the same anterior liver lobes as in Fig. 3 showing labelling of cell nuclei in the capsular tissue and inflammatory exudate. × 800.

FIG. 5.—An inflammatory reaction with organisation of the exudate and capsular cell proliferation in the anterior liver lobes 72 hr after handling. H. and E. × 800.

FIG. 6.—Radioautograph of the same anterior liver lobes as in Fig. 5 showing labelled nuclei of subcapsular hepatocytes and of cells in the capsule 72 hr after handling. × 800.



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After prolonged handling (for 60 sec.) of the anterior lobe the incorporation of labelled thymidine was not greater at 24 hr than that which occurred after the brief handling involved in delivery of the liver lobes (Series IV). DNA extracts after prolonged handling gave a value of 1418 d.p.m./unit E_{260} as compared with 1409 d.p.m./unit E_{260} after brief handling. Posterior lobe extracts after prolonged and brief handling were similar to control non-handled tissue (278, 256 and 293 d.p.m./unit E_{260} respectively, Table IV).

TABLE IV.—*Effect of Prolonged Handling on Incorporation of ^{3}H-Thymidine into Liver DNA. Animals Killed after 24 hr*

Handling	No. of rats	D.P.M./Unit E_{260} Extract	
		Ant. lobes	Post. lobes
Handling for 60 sec.	3	1418 (range 1039–1847)	278 (190–336)
Brief handling	3	1409 (range 1159–1738)	256 (225–315)
Control (no handling)	2	316 (range 300–332)	293 (248–338)

Microscopical examination of the sections and radioautographs for the most part show a good correlation with the radioactivity of the corresponding DNA extracts. Thus, at 6 hr after operation, there was either no, or minimal, capsular cell proliferation, no inflammatory exudate and no labelled capsular or liver cells in any of the sections studied. In the 15 hr group, sections of 4 out of 6 handled lobes contained a few (1–4) labelled capsular cells and a very mild inflammatory exudate. The other 2 appeared normal. Posterior lobes and controls showed no inflammation, capsular proliferation or labelling. In sections prepared from most anterior lobes, 21 or more hr after handling, an inflammatory reaction was evident and the presence of proliferated capsular cells, polymorphonuclear leucocytes and mononuclear cells correlated well with high radioactivity. At 24 hr 1 of 6 cases showed no inflammatory or capsular reaction and high radioactivity was found in the DNA extract, but the microscopical features in the remaining 5 cases correlated well with the level of radioactivity in the extracts. At 29 hr, there was good correlation in 5 specimens, at 36 hr, all specimens correlated well with the radioactivity assay. At 48 hr in 1 instance an inflammatory exudate was found, but no labelled cells were seen and the radioactivity of the extract was low. The remaining 7 specimens contained labelled cells when the extracts were highly radioactive. The microscopical findings at 72 hr agreed with assay results for the most part, but it was difficult to correlate exactly the presence of labelled parenchymal cells with radioactivity measurements. The types of microscopical findings in handled anterior lobes varied from normal to severe proliferative reactions but in most instances they were sufficiently distinctive for the anterior lobe to be easily identified (Figs. 3–6).

DISCUSSION

The finding of increased radioactivity in the DNA of liver tissue subjected to handling provides yet another situation in which DNA synthesis takes place in the liver after surgical stimuli. The shape of the precursor incorporation curve (Fig. 1) is distinctive and differs from that which occurs in the liver after other surgical

procedures. The curve after removal, or destruction of, or induction of atrophy to, part of the liver shows a steep ascending limb after increased DNA synthesis begins at about 15 hr postoperatively, achieves a sharply peaked synthesis rate at about 6 hr later and then declines (Weinbren and Woodward, 1964; Bucher, 1963, 1967) while the present curve shows a much more gradual ascent with a plateau from approximately 36–96 hr. The proliferative response after handling thus differs in several respects from that which occurs after partial resection. It begins later, achieves a peak much later and maintains its highest rate of synthesis for longer. The height of the response at its peak is lower than that usually associated with partial resection (approximately a 6–10 fold increase in rate of incorporation as compared with up to 50 fold increase after partial hepatectomy in young adult rats (Bucher, Swaffield and DiTroia, 1964)).

The mechanism is not clear but certain observations can be made. The capsular cells which are associated with an inflammatory reaction are stimulated to proliferate and undergo DNA synthesis and a similar capsular proliferation has been described adjacent to tangential hepatic wounds (Cameron, Hassan and De, 1957). The proliferation is clearly associated with the surface inflammatory reaction, although the precise growth stimulus in these circumstances is not defined. The reason for the increased radioactivity of some peripheral hepatocytes (Fig. 6) is uncertain and parenchymal mitoses were noted in similar cells by Cameron *et al.* (1957). It is possible that handling may result in lysosomal activation which has been described as occurring even after minor trauma (Bitensky, 1963). Such a sequence could occur in the present circumstances and it has been suggested that activation of lysosomal enzymes including DNase is associated with DNA synthesis (Allison and Mallucci, 1964). The precise mechanism involved in this sequence is not clear but Bollum (1960) has proposed that DNase may activate DNA as a primer for DNA synthesis. It is known that DNase accumulates shortly before the wave of increased DNA synthesis after the usual standard partial hepatectomy (Brody and Balis, 1959).

The incorporation of radioactive thymidine into the handled liver lobes was much lower after i.v. injection than after i.p. administration. This implies that the capsular elements when stimulated were able to absorb the compound directly. This is known to take place in newt liver cells and a relationship has been found between i.p. dose and capsular incorporation (Grillo, Urso and O'Brian, 1965). It is possible however that inflamed areas within liver lobes are likely to incorporate DNA precursors even when administered i.v. and inflamed foci of necrosis after vascular interference might well incorporate DNA precursors in certain experimental situations. Such incorporation, although leading to increased specific activity of DNA, does not represent a process of parenchymal growth.

It is possible also that the response after handling may have led to serious misinterpretation of the results derived from attempts at growth stimulation or inhibition during restoration experiments. Similarly in some experiments autotransplants may have been handled when normally-sited liver was resected and in such cases the response measured in the transplant could reflect an effect of handling. The phenomenon may play a part also in experiments describing a stimulatory effect in livers whose vessels have been subjected to temporary ligation (Lieberman and Short, 1965). Other proliferative reactions, formerly thought to be due to a humoral stimulus might conceivably be related to the effects of handling.

From a practical point of view, whatever the mechanism, it is clear that for accurate measurements when the various complex phenomena are to be dissociated, it is important not to handle the liver lobes and also not to introduce the radioactive precursor intraperitoneally unless these 2 factors are controlled.

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