DELAYED INCORPORATION OF ^{32}P FROM ORTHOPHOSPHATE INTO DEOXYRIBONUCLEIC ACID OF RAT LIVER AFTER SUBTOTAL HEPATECTOMY

K. WEINBREN AND ELSA WOODWARD

From the Department of Pathology, Postgraduate Medical School, London W.12

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Restoration of the liver after partial removal or damage has been studied in several species and in a variety of circumstances. Most of the published observations have been made on the rat liver remnant after removal of the anterior two lobes, which are equivalent to about 2/3 of the total liver weight.

Cellular proliferation in the remnant has been assessed by measuring changes in weight, in the incidence of mitotic figures and in the incorporation of labelled precursors into DNA. During the early post-operative period a tracer method seems to be most suitable as increases in weight might result from changes in water, fat or blood content, and mitoses are not found in great numbers until the end of the first day, some 9–12 hr. after the first increase in DNA synthesis (Nygaard and Rusch, 1955). The level of mitoses in parenchymal cells begins to increase at 24 hr. and reaches a peak some 4 or 5 hr. later (Cater, Holmes and Mee, 1956). After excision of fractions smaller than the usual 2/3, the response is proportionately smaller and more gradual (Drabkin, 1947 ; Pack and Islami, 1956 ; Glinos, 1958 ; Islami, Pack and Hubbard, 1959 ; Straube and Patt, 1961 ; Bucher and Swaffield, 1962) and no measurable response has been reported when the amount resected is smaller than a minimal threshold level (MacDonald, Rogers and Pechet, 1962). These observations suggested that resections larger than 2/3 might induce a response with a sharp peak of activity which was higher and occurred earlier than that occurring after 66 per cent partial hepatectomy. Such an altered response might be associated with more easily measurable alterations in the tissue or serum levels of possible growth-regulating factors which have been postulated by several authors (Weiss and Kavanau, 1957 ; Glinos, 1958 ; Stich and Florian, 1958 ; Smythe and Moore, 1958), but the existence of which has not been unequivocally demonstrated (MacDonald and Rogers, 1961).

The changes in the remnant after resections larger than 2/3 do not appear to have been reported and it therefore seemed necessary to extend this work by comparing the response in the remaining liver after removal of the 2 anterior lobes (standard 2/3 partial hepatectomy) with that found after removal of a larger proportion of liver (subtotal hepatectomy). In the experiments described here the remnants have been examined at 3–5 hr. intervals during the first 42 hr. and thereafter at 48 and 72 hr.

We chose to use ^{32}P-orthophosphate as the labelled precursor for measuring the synthesis of DNA. Although ^{3}H-thymidine has the advantage of being a much more specific precursor and has been widely used in work of this kind, it has been considered by some to interfere with normal DNA synthesis (Hiatt and Bojarski, 1960, 1961 ; Greulich, Cameron and Thrasher, 1961).

The measurement of the incorporation of ^{32}P-orthophosphate into DNA, however, presents some problems, because this incorporation is accompanied by a higher rate of uptake into other types of molecules in the liver and consequently there is a risk that the DNA fraction may be contaminated by extraneous ^{32}P (Smellie, McIndoe, Logan, Davidson and Dawson, 1953). Davidson, Frazer and Hutchison (1951) reported that the method of Schmidt and Thannhauser (1945) was not suitable for the measurement of ^{32}P incorporation because the specific activity of the DNA fraction obtained by this procedure was higher than that of purified, whole DNA prepared from the same homogenate. In some investigations DNA has been isolated and purified before the specific activity has been measured (Zaki, Barnum and Hoffbauer, 1959 ; Gould, Floyd, Whitehead and Sanders, 1961 ; Feigelson, Gross and Feigelson, 1962), but these procedures are not suitable for the examination of hundreds of small samples derived from individual animals.

With the method described here, which is a simplified modification of the one used by Smellie, Kier and Davidson (1959) when working with ^{3}H-thymidine, it is possible to assay 20 samples simultaneously and in duplicate using 300 mg. for each determination. The results thus obtained have been compared with the specific activity of whole DNA produced from the same homogenate and also with two other methods (Schmidt and Thannhauser, 1945 ; Ogur and Rosen, 1950) which have been used for the measurement of ^{32}P incorporation into DNA (Holmes, 1947 ; Smythe and Moore, 1958).

MATERIAL AND METHODS

Rats.—Male white rats bred in our own laboratory and weighing between 150 and 180 g. were used. The rats were weaned at 3 weeks and thereafter maintained on " Research " rat cubes. They were not fasted before operation or killing.

Chemicals.—^{32}P-orthophosphate in isotonic phosphate buffer pH 7 and containing 1 mg. phosphorus/ml. was obtained from the Radiochemical Centre, Amersham, Bucks. This was diluted from approximately 1 mc./ml. to 140 μc./ml. with 0·9 per cent saline. Deoxyribonuclease 1 (crystalline) and venom phosphodiesterase were obtained from the Worthington Biochemical Corporation.

Surgical procedures.—Ether anaesthesia was used. Standard 2/3 partial hepatectomy was carried out by the method of Higgins and Anderson (1931). Subtotal hepatectomy involved the removal of the 2 anterior lobes, then the 2 subdivisions of the caudate lobe and finally the inferior division of the right lobe (for nomenclature see Greene, 1935) (Fig. 1–4). Each of these regions was ligated at the hilum and then severed distal to the ligature with a sharp knife.

Measurement of ^{32}P incorporation.—Each animal received an intraperitoneal injection of 70 μc. ^{32}P 3 hr. before being killed. The livers were removed under ether anaesthesia and transferred to ice-cold containers. Samples of 600 $\pm$ 20 mg. were homogenised in 10 ml. 0·9 per cent saline in a Potter-Elvehjem type homogeniser and the coarse suspension divided approximately equally between two 8 ml. centrifuge tubes. After adding 0·2 ml. 10 N-$HClO_4$ the suspension was immediately stirred and centrifuged. The radioactivity was measured in the supernatant from one of each pair of duplicates. The precipitate was extracted successively at room temperature with 5 ml. alcohol-ether (3 : 1, v/v), alcohol-chloroform (3 : 1, v/v) and ether and the residue digested with 5 ml. 0·3 N-KOH at 37° overnight in a stoppered tube. The digest was cooled in the deep freeze for 30 min., acidified with 0·2 ml. 10 N-$HClO_4$, stirred and spun. After washing twice with 5 ml. water, the residue was extracted with 6·5 ml. 0·5 N-$HClO_4$ at 70° for 20 min., cooled in the deep freeze for 40 min. and then centrifuged.

A portion (1 ml.) of the supernatant was diluted to 5 ml. with water and the extinction at 260 mμ measured against a similar dilution of 0·5 N-$HClO_4$. A further 5 ml. of supernatant was made up to 10 ml. with water and the radioactivity was measured in a liquid dipping counter.

In many experiments the extinction provided an adequate measure of the amount of DNA in the extract and the results could conveniently be expressed as counts/min./unit of extinction. For this purpose 1 unit of extinction was taken as 1 ml. of extract having an extinction value of 1. In other experiments the P content of the extract was estimated, by heating in a Kjeldahl flask with 10 N-$HClO_4$ and assaying the phosphate by the method of Allen (1940).

Preparation of rat liver DNA labelled with ^{32}P.—Standard 2/3 partial hepatectomy was performed on 3 male rats weighing 400 g. Intraperitoneal injections of 120 μc. ^{32}P were given 20 hr. after operation and the rats were killed 4 hr. later.

A coarse liver homogenate containing 18 g. liver in 100 ml. 0·9 per cent saline, was acidified to pH 2 with 6 N-HCl and centrifuged. After extraction with 50 ml. of lipid solvents as used in the assay procedure, the nucleic acids were extracted with 10 per cent NaCl by the method of Hecht and Potter (1956*a*). The material precipitated by alcohol was collected after 15 min. at 4° and digested with 0·1 N-KOH at 37° overnight. After cooling, the DNA was precipitated with 2 N-HCl and washed with water. Reprecipitation and washing were repeated a further 4 times to remove extraneous ^{32}P. In order to be sure that the ^{32}P was DNA-P, this material was converted quantitatively to the mononucleotides which were then separated by column chromatography (Hecht and Potter, 1956*b*). The DNA was digested with 0·5 mg. deoxyribonuclease at pH 7·5 and 37° for 1½ hr. in a volume of 3·6 ml., followed by 0·3 mg. of diesterase at pH 9 and 37° for 1 hr. in a final volume of 4·3 ml. The digest (2 ml.) was chromatographed on a Dowex 1 × 10—formate column (5 × 1 cm.) and eluted with 50 ml. water, 35 ml. 0·05 N-formic acid (deoxycytidine 5′-monophosphate), 30 ml. N-formic acid (deoxyadenosine 5′-monophosphate), 40 ml. 1·8 N-formic acid (deoxyguanosine 5′-monophosphate) and 60 ml. 2·5 N-formic acid (thymidine 5′-monophosphate) (Hecht and Potter, 1956*b*). The volume of each fraction was noted and the extinction measured at 260 mμ and 280 mμ. The amount of each nucleotide was calculated using the molar extinction co-efficient (shown as " Found " in Table I).

The specific activity of each nucleotide was determined by digesting approximately 0·5 μ-mole with 1 ml. 10 N-$HClO_4$ and assaying for phosphorus. The colorimetric solution was preserved quantitatively for the measurement of ^{32}P.

In order to test for contamination by ribonucleotides 0·3 μmole of the dCMP fraction was examined by descending paper chromatography using the borate solvent of Reichard (1958), but with versene omitted. After u.v. lamp inspection, the chromatogram was used to prepare an autoradiograph. No contamination was observed.

After preliminary assessment of the biochemical assay procedure as outlined above, the experiments were arranged in 2 main groups :

Determination of relative amounts of tissue removed by the operative procedures.—Livers from 14 control rats were removed immediately after death, the lobes dissected and weighed separately. The right lobe was further subdivided into anterior and posterior parts, corresponding to the dissection usually effected at the subtotal hepatectomy operation.

Comparison of ^{32}P *incorporation into the DNA of the remnants after each of the* 2 *surgical operations.*—The rats were subjected to the operation between 10 a.m. and 12 noon. Groups of 6 or more were given intraperitoneal injections of 70 μc. ^{32}P at different times after the operations and killed 3 hr. later. Animals examined in this way were injected at the following post-operative times, 3, 6, 9, 12, 15, 18, 21, 26, 31, 36, 39, 45 and 69 hr. and the livers were assayed for specific activity of DNA as described above. A total of 251 animals was used after rejection of 15 in which there was evidence of poor absorption of ^{32}P, as indicated by the

EXPLANATION OF PLATE

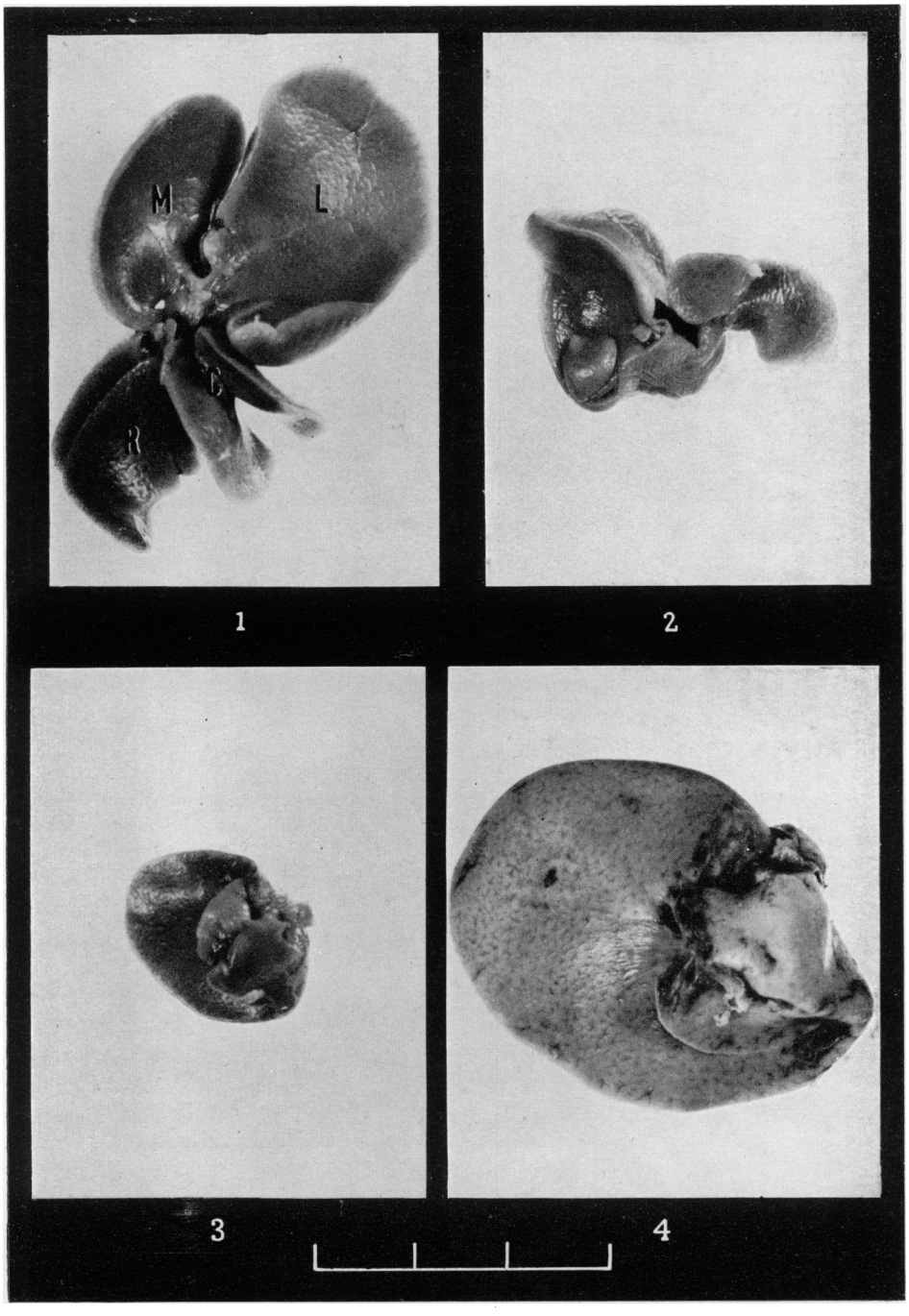
FIG. 1.—Ventral view of normal rat liver to show the 4 lobes. M—median lobe, L—left lateral lobe, R—right lobe, C—caudate lobe.

FIG. 2.—The remnant immediately after standard partial hepatectomy, removal of the 2 anterior lobes leaving the right and caudate lobes.

FIG. 3.—The remnant immediately after subtotal (82 per cent) hepatectomy, leaving only the superior sub-division of the right lobe.

FIG. 4.—The corresponding sub-division of the right lobe 9 months after subtotal hepatectomy.

FIG. 1, 2 and 3 illustrate specimens taken from rats of comparable weight (160 g.). Fig. 4 is from a rat 9 months older and weighing 430 g.



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radioactivity in the first supernatant. Of this total 109 rats were subjected to 67 per cent partial hepatectomy and 142 to 82 per cent subtotal hepatectomy.

RESULTS

The findings relating to the validity of the procedure used for measuring the incorporation of ^{32}P are summarised in Tables I, II and III. Table I shows that

TABLE I.—*Analytical Results Obtained on DNA Labelled with ^{32}P and Prepared from Rat Liver Homogenate as Described*

Nucleotide	Ratio $\frac{E_{280}}{E_{260}}$ in acid		$\varepsilon_{260} \cdot 10^{-3}$ in acid		μ moles found	Specific activity counts/min./μg. P
	*Quoted	Found	*Quoted	Found		
dCMP	2·12	2·08	6·5	5·96	4·55	25·2
dAMP	0·23	0·214	14·1	14·5	5·95	36·1
dGMP	0·70	0·697	10·6	11·5	4·24	24·7
TMP	0·73	0·727	8·4	8·4	5·92	32·2

Specific activity of whole DNA (calculated from the two right-hand columns)	30·3 counts/min./μg./P.
Specific activity of DNA extracts (prepared from the same homogenate by the assay procedure described in the text)	28·1, 29·8, 29·2, 28·7, 27·4, 28·8 Mean = 28·7 counts/min./μg. P.

* " Properties of the Nucleic Acid Derivatives. 4th Revision, April, 1961 " in *Biochemical Data and Price List* (April, 1962). published by the California Corporation for Biochemical Research.

the specific activity of whole DNA, calculated from the molar proportions and specific activities of the constituent nucleotides, is 30·3 counts/min./μg. P and that the mean value derived by the assay procedure is 28·7.

Extracts of DNA prepared by this method were found to have much lower specific activities than values obtained on the same liver material by the procedures of Schmidt and Thannhauser, and Ogur and Rosen (Table II).

TABLE II.—*Comparison of Results Obtained by Different Assay Procedures on the Same Rat Liver Homogenate*

Assay procedure	Specific activity of ^{32}P in DNA counts/min./μg. P.
Schmidt and Thannhauser (1945)	17, 14, 31, 41
Ogur and Rosen (1950)	37, 306, 55, 80
Smellie *al al.* (1953)	4·5, 3·9, 3·7, 3·1

In Table III are presented the values of E_{260}/μg. P which were obtained on 48 DNA extracts taken at random over several months. Although more than half the results lie within 5 per cent of the mean, the total variation is up to 20 per cent of the mean.

The mean percentage of total liver of the two anterior liver lobes, those which would be resected by the standard 2/3 partial hepatectomy procedure, is 66·6

TABLE III.—*Variation of Relationship between Extinction and P Content of 0·5N-$HClO_4$ Extracts of DNA*

E_{260} of extracts containing 1 μg. P/ml.	Number of results	
0·35 –0·360	10	
0·361–0·370	6	
0·371–0·380	14	= 52 per cent of all results
0·381–0·390	11	
0·391–0·400	4	
0·401–0·410	3	
Total :	48	Mean value = 0·375

± 1·4 (S.D.) and that of the lobes which would be resected by the subtotal hepatectomy procedure is 81·5 ± 1·5 (S.D.).

Fig. 5 and Table IV show the clear differences between the patterns of response after partial (67 per cent) and subtotal (82 per cent) hepatectomy procedures respectively. The latent period after subtotal hepatectomy is almost twice as long as that after partial hepatectomy.

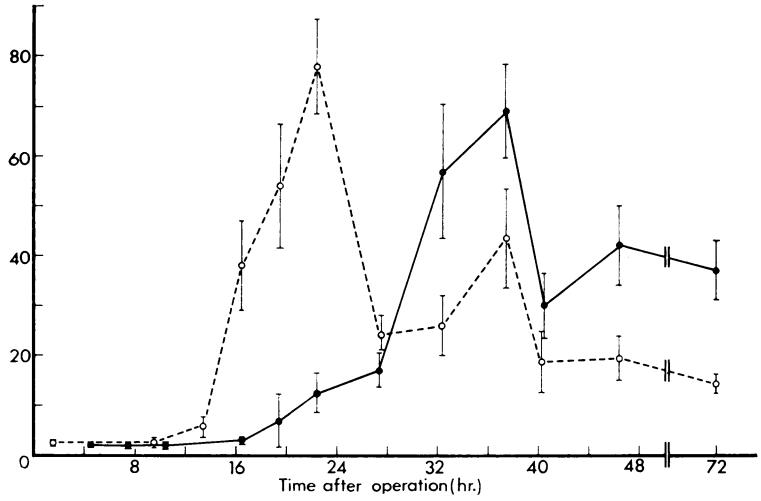

FIG. 5.—The incorporation of ^{32}P into DNA after partial hepatectomy and subtotal hepatectomy. - - - - ○ - - - - Remnant after partial hepatectomy. ——— ● ——— Remnant after subtotal hepatectomy.

DISCUSSION

The close similarity between the specific activity of the whole DNA and the DNA extracts prepared from the same homogenate indicates that the assay method may be considered satisfactory. However, the use of the extinction measurement to assay the DNA content must be applied with some reserve. All the extinction values in Table III are higher than the figure of 0·274 given by Ceriotti (1955) for a solution of calf-thymus DNA in N-perchloric acid, and also higher than the theoretical value, even if one assumes the DNA to have been hydrolysed as far as the pyrimidine mononucleotides and the free purine bases. Of more importance

TABLE IV.—*Incorporation of ^{32}P-Orthophosphate into Rat Liver DNA. Intraperitoneal Injection of 70 μc. in 0·5 ml. given 3 hr. Before Killing*

Post-operative time killed (Hr.)	Partial (67 per cent) hepatectomy			Sub-total (82 per cent) hepatectomy		
	No. of rats in group	Counts/min./unit E_{260} in DNA extract		No. of rats in group	Counts/min./unit E_{260} in DNA extract	
		Mean	S.E.		Mean	S.E.
3	11	2·7	0·47	—	—	—
6	17	2·2	0·34	7	2·2	0·43
9	—	—	—	8	1·85	0·61
12	3	2·3	1·0	7	2·0	0·58
15	5	6·1	1·74	—	—	—
18	6	37·7	9·26	8	2·9	0·52
21	6	54·2	12·3	5	7·1	5·62
24	12	78·3	9·6	18	12·7	3·88
29	16	24·4	3·42	24	16·9	3·61
34	7	25·9	5·94	15	56·9	13·5
39	6	43–6	10·0	14	69·2	9·74
42	3	19·0	6·4	10	29·6	6·5
48	5	19·3	4·59	17	42·2	8·1
72	12	14·4	1·73	9	36·8	5·9

when using the extinction as a measure of DNA content is the range of variation shown in the table. While it is not clear why this variation is present, one possibility is that there may be slight variable losses of adenine and guanine, since the purines, which make a major contribution to the extinction at 260 mμ, are released comparatively easily by mild acid treatment (Tamm, Hodes and Chargaff, 1952). In the present assay procedure the method of Smellie *et al.* (1959) has been modified to reduce the exposure to acid to a minimum. This may have particular relevance in the circumstances of this experiment, as when large numbers of samples are processed together, it is difficult to ensure that all are acidified for the same period. Nonetheless, since differences of ^{32}P uptake between the experimental groups varied from 50–5000 per cent and the variation of the phosphorus/extinction ratio appeared to be limited to less than 20 per cent of the mean value, it seemed reasonable to use the extinction procedure in those experiments yielding large numbers of results in each experimental group.

Our findings confirm the view of Davidson *et al.* (1951) that significant contamination of the DNA fraction with extraneous ^{32}P may occur if the tissue is processed by the Schmidt and Thannhauser method. We have found also that the extract prepared by the Ogur and Rosen (1950) procedure appeared to possess an even higher degree of contamination.

The operation for subtotal hepatectomy involved an arbitrary section near the hilum of the inferior division of the right lobe of the liver, but after the technique had been developed, it was found to be reproducible. The operative mortality was never more than 10 per cent in the series used for this project and, when required, long-term survivors were kept for 9 months.

The main finding of this work is the delayed incorporation of ^{32}P into liver DNA after the subtotal (82 per cent) resection as compared with that occurring after partial (67 per cent) hepatectomy. Although the latent period before DNA synthesis increases is prolonged, the rate of incorporation into DNA is eventually greater than that found after the smaller resection.

So far we have not found published reports of a similar delay in restorative response after very large resections in other tissues and the mechanisms are difficult to elucidate.

It is possible that the subtotal resection is so traumatic from a surgical point of view, that the affected animal is in some way unable to respond. This may be related to excessive secretion of adrenal hormones which may play some part in depressing DNA synthesis. Both adrenaline and cortisone have been reported as having anti-mitotic effects the former particularly in the mouse epidermis (Bullough and Laurence, 1961) and the latter in the liver in certain circumstances (Hemingway, 1961), but we have no clear evidence that this is relevant to the present experiment. It is perhaps noteworthy that almost all the animals survive the operative procedure and there is in fact an increased response after the delay period is over.

It may be that the composition of the remnant is altered in such a way that DNA synthesis is inhibited, but if this were so, any unusual constituent may be the result and not the cause of the delay. Yet another possibility is the effect of gross haemodynamic changes associated with a sudden reduction in the portal vascular bed, but this might be considered to increase the rate of the reaction according to one current theory based on experimental observations (Glinos, 1960).

Whether the situation imposed by these circumstances is unique or whether this delay might have relevance to the overall problem of the control and initiation of restorative growth, cannot be assessed from the present data. If there is an active inhibition for a time of the usually anticipated proliferation in the remnant after partial extirpation, then the mechanism might be related to what usually controls restoration of the liver.

SUMMARY

The incorporation of ^{32}P into DNA of the liver remnant after standard partial hepatectomy (67 per cent resection) has been compared with that found after subtotal hepatectomy (82 per cent resection).

After standard 67 per cent resection the usual latent period of 12–15 hr. before increased ^{32}P incorporation into DNA was confirmed, but after 82 per cent resection this latent period was much increased.

A simplified method requiring 300 mg. liver, is described for measuring the incorporation of ^{32}P into DNA, and the results have been compared with the specific activity of purified whole DNA prepared from the same homogenate. The method has also been compared with and found to be superior to two other assay procedures.

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