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Effects of Beryllium on Deoxyribonucleic Acid-Synthesizing Enzymes in Regenerating Rat Liver

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1. The incorporation of thymidine into DNA of regenerating rat liver was measured at various times after partial hepatectomy. A single intravenous injection of 30 μmol of beryllium/kg given immediately after the operation inhibited DNA synthesis 12, 16, 20, 24 and 28h later. 2. The activity of several enzymes critical to DNA synthesis (thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidylate deaminase and DNA polymerase) increased in control rats 20–24h after partial hepatectomy severalfold over the activity found in resting livers. After beryllium treatment this rise in activity was much less and it seemed as if beryllium would partially block the induction of DNA-synthesizing enzymes after partial hepatectomy. 3. Enzymes whose activities do not rise during liver regeneration were not affected by beryllium (aspartate transcarbamoylase, carbamoyl phosphate synthetase, uridine kinase and glucose 6-phosphatase). 4. No evidence was found *in vitro* that beryllium would specifically inhibit thymidine kinase or DNA polymerase. 5. The time–effect relationship between beryllium administration and thymidine kinase activity *in vivo* was examined. Measured 24h after partial hepatectomy, thymidine kinase activity was only affected if beryllium was given within the first 9–12h after partial hepatectomy. Beryllium given later, even in greatly increased doses, failed to have any effect on thymidine kinase. The possibility is discussed that beryllium inhibits enzyme induction at the transcriptional level.

Living tissue exposed to beryllium rapidly becomes badly and generally irreversibly damaged. In humans exposure to airborne beryllium dusts produces a granulomatous chronic lung disease (Tepper, Hardy & Chamberlin, 1961). In rabbits and rats beryllium acutely administered causes liver necrosis (Aldridge, Barnes & Denz, 1949), and long-term exposure is carcinogenic and sarcomagenic (Vorwald, Reeves & Urban, 1966). Beryllium has also been found to interfere with growth of animals and plants, to inhibit limb regeneration in amphibia and to disturb gross differentiation of embryonic tissue (Kimmerle, 1966). Finally beryllium elicits different immunological responses in different strains of guinea pigs (Polak, Barnes & Turk, 1968).

The underlying biochemical mechanism(s) responsible for all these bewilderingly diverse effects are not yet elucidated. It was already known for some time from work with cells in culture that beryllium had a specific affinity for one particular cell organelle, the nucleus (Firket, 1953). Biochemical studies have since fully confirmed these histological observations, and the affinity of beryllium for rat liver cell nuclei has now been put on a quantitative basis (Truhaut, Boudène & Le Talaer, 1965; Witschi & Aldridge, 1968). It was also found that beryllium not only accumulated in the cell nucleus, but was also capable of disturbing nuclear function: beryllium inhibited DNA synthesis in chicken muscle cells and fibroblasts cultivated *in vitro* (Chèvremont & Firket, 1951; Chèvremont, 1961; Bassleer, 1965). Similar observations *in vivo* were made in studying the effects of beryllium on rat liver regeneration (Truhaut *et al.* 1965; Witschi, 1968). The inhibition of DNA synthesis *in vivo* was directly dependent on the amount of beryllium present in the liver, and 30 μmol of beryllium/kg injected immediately after partial hepatectomy prevented the net synthesis of DNA (Witschi, 1968). Despite this very dramatic effect of beryllium on DNA synthesis, RNA synthesis and protein synthesis have not yet been found to be inhibited to any significant extent by beryllium in resting or regenerating livers or dividing tissue culture cells (Witschi & Aldridge, 1967; Witschi, 1968; Chèvremont & Firket, 1951).

The inhibition of DNA synthesis by beryllium in regenerating rat liver was subsequently analysed in greater detail. In an attempt to define more

precisely how beryllium would interfere with DNA replication, the activities of several enzymes critical to DNA synthesis were studied *in vivo* within the first 28h after partial hepatectomy. An attempt was made to correlate the findings with the intracellular distribution of beryllium. The results, presented in this paper, showed that beryllium affects all enzymes involved in DNA synthesis that were examined. However, beryllium had little or no effect on certain enzymes involved in RNA synthesis or on one microsomal enzyme, glucose 6-phosphatase. An analysis of the kinetics of inhibition of DNA-synthesizing enzymes suggested that beryllium most probably did not act directly on these enzymes, but rather prevented their 'induction', normally triggered by partial hepatectomy.

Part of this work has been presented in preliminary form (Witschi, 1970).

MATERIALS AND METHODS

Materials

All radioactive compounds were purchased from New England Nuclear Corp., Waltham, Mass., U.S.A. All nucleosides and nucleoside mono-, di- and triphosphates, DNA (type V), RNA (from yeast), NADP, D(−)-3-phosphoglyceric acid, dithiothreitol, glucose 6-phosphate, carbamoyl phosphate, DL-aspartic acid, snake venom (*Crotalus adamanteus*), (±)-L-tetrahydrofolic acid [(±)-5,6,7,8-tetrahydropteroyl-L-glutamic acid], L-ornithine, 2-mercaptoethanol, 2,3-dimercaptopropan-1-ol and dimedone were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. DEAE-cellulose filter paper (Whatman DE81) was obtained through Reeve-Angel Co., Clifton, N.J., U.S.A. Rats were Wistar-derived and purchased from R. Robidoux, Montreal, Que., Canada.

Methods

General. Male rats (170–190g) were partially hepatectomized under light ether anaesthesia by the technique of Higgins & Anderson (1931). Beryllium was injected into the tail vein as $BeSO_4,4H_2O$ dissolved in water (injection volume: 0.2ml); controls received 0.9% NaCl. After the operation the rats had access to food and water *ad libitum*. They were killed by decapitation at different time-intervals after the operation. Incorporation of thymidine into DNA *in vivo* was measured as previously described (Witschi, 1968). Procedures for measuring the concentration of beryllium within the liver and its distribution in subcellular fractions were as presented in detail by Witschi & Aldridge (1968).

Enzyme assays. Except for glucose 6-phosphatase and carbamoyl phosphate synthetase, enzyme activities were measured in the following way: rapidly excised livers were homogenized with ten strokes with a motor-driven (1000rev./min) Teflon–glass homogenizer (tissue grinder; A. H. Thomas Co., Philadelphia, Pa., U.S.A.) in the appropriate medium. The homogenates were immediately centrifuged for 70min at $102000g_{av.}$ in a Spinco model L-3 centrifuge at 0–2°C. Supernatant was carefully aspirated, avoiding the top layer containing fat, and served as enzyme source. All assays were run at 37°C in duplicate immediately after centrifugation. Conditions were throughout chosen to ensure linear enzyme activites within the incubation period adopted. In each assay background samples were prepared by stopping the reaction as soon as all components were mixed together, and these zero-time values were then subtracted from the values obtained at the end of the incubation period. In each assay a standard sample containing a known amount of radioactive substrate was prepared. These standards showed the same degree of quenching as did the actual samples. Control of the quenching was assessed with external standardization in a Packard Tri-Carb model 3375 three-channel counter. A total number of counts were collected generally within a counting error of 1% and converted with the help of the standards into nmol of substrate. Results were then calculated as nmol of substrate transformed/min per mg of protein in the assay. Protein was determined by a modified biuret method (Aldridge, 1957).

(*a*) DNA polymerase (EC 2.7.7.7) (De Recondo & Fichot, 1969). The liver was homogenized in 2.5vol. of 0.25M-sucrose–2.5mM-tris–HCl buffer (pH8.0)–6mM-KCl. The reaction mixture contained in a final volume of 0.5ml of 0.16M-tris–HCl buffer, pH7.2: 0.03μmol each of dATP, dCTP and dGTP and [Me-^{3}H]dTTP (0.5μCi); 1.5μmol of $MgCl_2$; 1.2μmol of KCl; 0.2μmol of 2-mercaptoethanol; 50μg of freshly heat-denaturated (10min at 100°C) DNA as primer; 0.2ml of enzyme. After 60min 150μg of carrier DNA followed by 0.8ml of ice-cold 0.5M-$HClO_4$ were added. The precipitate was washed twice with 0.2M-$HClO_4$, dissolved in 3ml of 0.3M-KOH, incubated for 60min at 37°C, reprecipitated with ice-cold $HClO_4$ (final concn. 0.2M) and washed once more. The final pellet was dissolved in 1M-NaOH, suspended in Bray's solution containing 4% Cab-O-Sil and after being cooled its radioactivity was counted (Bray, 1960).

(*b*) Thymidine kinase (EC 2.7.1.21) (Breitman, 1963). Livers were homogenized in 5vol. of 0.2M-tris–HCl buffer, pH8. The reaction mixture contained in a final volume of 0.5ml of 0.12M-tris–HCl buffer, pH8.0: 2.5μmol of ATP; 3μmol of 3-phosphoglyceric acid; 2.5μmol of $MgCl_2$; 0.2ml of enzyme. After preincubation for 3min the reaction was started with the addition of 0.025μmol of [2-^{14}C]thymidine (0.025μCi). After 10min the reaction was stopped by immersing the tubes in a boiling-water bath for 1min. After cooling and centrifugation, 0.1ml of the protein-free supernatant was spotted on to a piece (3cm×3cm) of DEAE-cellulose filter paper and dropped into 1mM-ammonium formate (25ml/paper). The papers were washed once in ammonium formate, once in water, again in ammonium formate and water and finally in ethanol. They were dried at 75°C and immersed in a scintillation vial in 15ml of toluene containing 2,5-diphenyloxazole (PPO) (0.4%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (0.0095%). In each experiment the effectiveness of the washing procedure in removing unphosphorylated thymidine from the paper was checked: consistently more than 99% was eluted.

(*c*) Thymidylate kinase (EC 2.7.4.4) (Labow, Maley & Maley, 1969). Livers were homogenized in 3vol. of 0.15M-KCl. The reaction mixture contained in a final volume of 0.5ml of 50mM-K_2HPO_4–KH_2PO_4 buffer, pH7.5: 3μmol of ATP; 3μmol of $MgCl_2$; 5μmol of

dithiothreitol; 2.5 μmol of [2-^{14}C]dTMP (0.05 μCi); 0.2 ml of enzyme. After 30 min 0.02 ml of the reaction mixture was spotted directly on to a strip (1.5 cm × 10 cm) of DEAE-cellulose filter paper. Ascending chromatography was carried out in open beakers with 4 M-formic acid–0.1 M-ammonium formate as solvent. Under these conditions dTTP remained essentially at the origin, dTDP had R_F 0.3 and was well separated from dTMP, which had R_F about 0.7. In each assay a test strip was developed on which dTMP was spotted and after the run detected under short-wave u.v. light. The strips were cut 0.5 cm below the inferior margin of the dTMP spot and the radioactivity of the portion containing the dTTP and dTDP was counted in toluene scintillation liquid.

(*d*) Thymidylate synthetase (EC 2.1.1.*b*) (Lorenson, Maley & Maley, 1967). Livers were homogenized in 3 vol. of 0.15 M-KCl. The reaction mixture contained in a final volume of 0.36 ml of 0.13 M-KH_2PO_4–K_2HPO_4 buffer, pH 6.5: 0.11 μmol of [^{14}C]formaldehyde (1 μCi), 0.1 ml of a mixture made up from 0.8 ml of 20 mM-2,3-dimercaptopropan-1-ol, 0.6 ml of 15 mM-(±)-L-tetrahydrofolic acid (freshly dissolved in 0.1% 2-mercaptoethanol) and 0.6 ml of water; 1.5 μmol of dUMP; 0.1 ml of enzyme. A similar mixture but without dUMP was incubated as control. After 15 min the reaction was stopped by immersing the tubes into a boiling-water bath. Then 1 ml of 0.1 M-tris–HCl (pH 8.5)–30 mM-$MgCl_2$ containing 0.5 mg of snake venom (*Crotalus adamanteus*) was added. After a second incubation at 37°C for 30 min 0.2 ml of 0.1 M-formaldehyde was added, and, after mixing well, 0.4 ml of freshly prepared 0.4 M-dimedone dissolved in 50% (v/v) ethanol. The mixture was heated for 5 min at 100°C with occasional stirring, cooled and centrifuged for 15 min at 1200 *g*. The supernatant was poured on to a column (3 cm × 1 cm) of Dowex 50 (H^+ form; 200–400 mesh) layered on top of a column (3 cm × 1 cm) of Dowex 1 (formate form; 200–400 mesh). The column was washed with water until a total volume of 15 ml was collected and the amount of dTMP formed was determined from the radioactivity that passed through this column.

(*e*) Deoxycytidylate deaminase (EC no. not assigned). (Labow *et al.* 1969). The livers were homogenized in 4 vol. of 0.2 M-tris–HCl buffer (pH 8.0)–0.1 M-dithiothreitol–0.75 mM-dCTP (Sneider, Potter & Morris, 1969). The reaction mixture contained in a final volume of 0.5 ml of 0.18 M-tris–HCl buffer, pH 8.0: 15 μmol of NaF; 0.5 μmol of [G-^{3}H]dCMP (1 μCi); 0.2 ml of enzyme. The reaction was stopped after 10 min by heating the tubes briefly in a boiling-water bath. To each tube was added 0.5 mg of snake venom in 1 ml of 0.1 M-tris–HCl buffer, pH 8.5, and the tubes were reincubated for 30 min. The samples were then cooled, centrifuged and poured on top of a column (5 cm × 1 cm) of Dowex 50 (H^+ form; 200–400 mesh). The column was washed with water until a total of 10 ml was collected and dexoycytidylate deaminase activity was determined from the amount of radioactivity that passed through the column (Maley & Maley, 1960).

(*f*) Aspartate transcarbamoylase (EC 2.1.3.2) (Bresnick, 1965). After homogenization of the livers in 7 vol. of 0.25 M-sucrose, each incubation vessel contained in a final volume of 1.5 ml of 0.13 M-tris–HCl buffer, pH 9.2: 1 μmol of DL-[4-^{14}C]aspartic acid (0.1 μCi); 5 μmol of carbamoyl phosphate and 0.4 ml of enzyme. After 10 min 0.2 ml of 4 M-$HClO_4$ was added and the tubes were centrifuged. The deproteinized extract was passed through a column (5 cm × 1 cm) of Dowex 50 (H^+ form; 100–200 mesh) and the column was washed with 1 ml of water. Enzyme activity was determined from the radioactivity in the column eluate, representing carbamoylaspartic acid (Bresnick, 1965).

(*g*) Carbamoyl phosphate synthetase (EC 2.7.2.5) (Bresnick, 1965). Livers were homogenized in 10 vol. of 0.1% cetyltrimethylammonium bromide and the homogenates were spun for 15 min at 4000 $g_{av.}$. In a total volume of 0.9 ml of 25 mM-glycylglycine–NaOH buffer, pH 8.0, were incubated: 0.2 ml of enzyme; 5 μmol of NH_4Cl; 10 μmol of $MgSO_4$; 5 μmol of L-ornithine; 5 μmol of ATP and 10 μmol of $NaH^{14}CO_3$ (0.25 μCi). No ornithine carbamoyltransferase was added to the incubation mixture; according to Bresnick (1965) livers from partially hepatectomized rats contain sufficient enzyme to effect the carbamoylation of ornithine. After 15 min the reaction was stopped with 0.5 ml of 10% (w/v) trichloroacetic acid followed by 0.2 ml of 6 M-HCl (Kerson & Appel, 1968). The reaction mixture was then heated in a boiling-water bath in a ventilated fume-cupboard for 30 min. Enzyme activity was measured from the amount of acid-stable ^{14}C-labelled material in an incubation mixture containing the cofactor *N*-acetyl-L-glutamate (5 μmol/assay mixture) minus the amount of acid-stable ^{14}C-labelled material in a similar mixture but without cofactor.

(*h*) Uridine kinase (EC 2.7.1.48) (Bresnick, 1965). Livers were homogenized in 5 vol. of 0.15 M-KCl. In a final volume of 0.5 ml of 50 mM-tris–HCl buffer, pH 7.5, were incubated: 0.2 ml of enzyme; 25 μmol of ATP; 7 μmol of 3-phosphoglyceric acid; 7 μmol of $MgCl_2$; 0.5 μmol of [2-^{14}C]uridine (0.05 μCi). After 15 min the reaction was stopped by boiling. After cooling and centrifugation 0.1 ml of protein-free supernatant was spotted on to DEAE-cellulose paper squares (3 cm × 3 cm). The papers were then processed as for the measurement of thymidine kinase.

(*i*) Glucose 6-phosphatase (EC 3.1.3.9). A 2% (w/v) liver homogenate was prepared in 0.1 M-tris–maleate buffer, pH 6.2. The reaction mixture contained in a final volume of 1.1 ml: 20 μmol of tris–maleate buffer; 13.4 μmol of glucose 6-phosphate; 0.2 ml of liver homogenate. The reaction was stopped after 20 min by the addition of 5 ml of 10% (w/v) trichloroacetic acid. P_i was determined in the protein-free supernatant by the method of Fiske & Subbarow (1925) as modified by Gomori (1942).

Presentation of results. Unless otherwise stated, all results in the tables are given as mean values ± S.E.M. with the numbers of animals used given in parentheses. Significance levels were assessed according to Student's *t* test. Degrees of freedom (d.f.) were estimated in the following way: if $n_1 = n_2$, d.f. $= n-1$; if $n_1 > n_2$, d.f. $= n_2 - 1$ (Snedecor, 1953). *P* values of 0.05 or less were considered to be significant.

RESULTS

In a previous study (Witschi, 1968) on the inhibition of DNA synthesis in regenerating rat liver by beryllium all animals were killed exactly 28 h after partial hepatectomy and the beryllium had been injected at various times before death. It was

Table 1. *Incorporation of thymidine into rat liver DNA after partial hepatectomy*

Rats were partially hepatectomized and injected intravenously with 30 μmol of beryllium/kg; controls were given 0.9% NaCl. At 1 h before the rats were killed 10 μCi of [Me-^{3}H]thymidine was injected intraperitoneally. The rats were killed at the times indicated, DNA was extracted from liver homogenates with hot $HClO_4$ and its specific radioactivity was determined as described previously (Witschi, 1968).

Time after partial hepatectomy (h)	Radioactivity incorporated (c.p.m./mg of DNA)		
	Controls	Beryllium-treated	P
0 (basal value)	92 ± 30 (3)	—	—
12	406 ± 43 (4)	187 ± 45 (4)	<0.05
16	499 ± 106 (6)	230 ± 57 (6)	<0.10
20	1405 ± 277 (7)	619 ± 99 (6)	<0.05
24	5163 ± 1004 (6)	1843 ± 390 (7)	<0.05
28	5366 ± 1269 (14)	1401 ± 233 (12)	<0.02

therefore necessary to determine the rate of DNA synthesis when beryllium was administered immediately after the operation and the animals then killed at various stages of liver regeneration. The experimental period was limited to the first 28h after partial hepatectomy. Up to this time beryllium-treated rats appeared to be in good health regardless of the dose administered. No gross histopathological alterations were seen in slices obtained from livers during this period; the only change was a conspicuous decrease in the number of mitoses in livers of beryllium-treated rats (P. Goldblatt, M. Lieberman & H. P. Witschi, unpublished work). All biochemical measurements described below were thus done on livers that by the usual histopathological criteria were essentially normal. Animals allowed to survive longer, however, developed fully necrotic livers 36–48h after partial hepatectomy, even if given as little as 7.5 μmol of beryllium/kg, and eventually died. In these animals the livers looked histologically similar to those previously described (Aldridge *et al.* 1949; Cheng, 1956). The action of beryllium on regenerating liver therefore seemed to be irreversible.

The incorporation of thymidine into DNA is presented in Table 1. In control animals incorporation was already slightly increased over basal values (measured in non-regenerating livers) 12h after the operation and a sharp increase became evident between 20 and 24h and at 28h. At this time thymidine incorporation was about 60 times the basal values. In beryllium-treated animals incorporation rates at any given time except 16h were significantly lower than in control animals. There was no difference in the amount of acid-soluble radioactivity per unit weight of liver, suggesting that in both control and treated rats thymidine uptake into the liver was the same. As in earlier observations (Witschi, 1968), it was confirmed that 28h after partial hepatectomy the DNA concentration, measured chemically with diphenylamine (Burton, 1956), was significantly lower in beryllium-treated animals [μg of DNA/g of liver in treated rats: 1.73 ± 0.07 (12); in control rats: 2.00 ± 0.08 (14) ($P<0.05$)]. Therefore beryllium interfered not only with the incorporation of labelled thymidine into DNA, but also with net synthesis of DNA. The differences in incorporation rates of labelled thymidine presented in Table 1 are therefore indicative of an inhibitory effect of beryllium on DNA synthesis.

Uptake of beryllium in the liver and its subcellular distribution was followed over the same time-period. Contrary to an earlier observation in which a maximal beryllium concentration in normal liver was found only 24h after administration of beryllium (Witschi & Aldridge, 1968), a maximal concentration seemed to be reached in regenerating liver 12h after injection. From there on up to 24h no more beryllium was taken up in the liver. However, a redistribution of at least some of the beryllium seemed to occur during this time; at 24h more beryllium was present in the nuclear fraction than at 12h, although the protein content of this fraction did not change (Table 2). On the other hand beryllium and to a smaller extent protein were lost from the light-mitochondrial fraction between 12 and 24h. Calculation of the specific activities (nmol of beryllium/mg of protein) showed that isolated and purified nuclei practically doubled their beryllium concentration between 12 and 24h. The specific activities rose slightly in the microsomes but remained essentially unchanged in the light mitochondria, heavy mitochondria and the supernatant (Table 2).

Whether the inhibition of DNA synthesis *in vivo* could be explained by an inhibition of enzymes involved in DNA synthesis was examined. During experimental liver regeneration several enzymes directly engaged in the metabolism of DNA precursor molecules and DNA synthesis display markedly increased activity (Bucher, 1967*a*,*b*).

Table 2. *Subcellular distribution of beryllium in regenerating rat liver*

Rats were injected intravenously immediately after partial hepatectomy with 30 μmol of beryllium/kg. At 12 and 24h later eight rats each were killed and the livers were fractionated as described by Witschi & Aldridge (1968). Distribution of beryllium and protein in the different fractions was calculated as percentages of the total amounts of beryllium and protein recovered during the fractionation procedure. Recoveries as percentages of the original homogenate were at 12h: 88±1% for beryllium, 88±2% for protein; at 24h: 95±2% for beryllium, 95±2% for protein.

	Distribution of beryllium (% of amount of total homogenate)			
	12h after partial hepatectomy		24h after partial hepatectomy	
Fraction	Protein	Beryllium	Protein	Beryllium
Nuclei + debris	42±1	43±1*	43±2	50±2*
Heavy mitochondria	15±1	34±2	15±1	34±2
Light mitochondria	7±1†	9±1*	4±1†	5±1*
Microsomes	10±1	4±1	10±1	5±1
Supernatant	26±1†	8±1	30±1†	7±1

* Percentage of beryllium significantly different between 12 and 24h.
† Percentage of protein significantly different between 12 and 24h.

	Beryllium content (nmol/mg of protein)	
Fraction	12h after partial hepatectomy	24h after partial hepatectomy
Homogenate	2.51±0.16	2.95±0.14
Purified nuclei	3.73±0.11	7.29±1.06
Heavy mitochondria	6.52±0.89	6.54±0.60
Light mitochondria	3.94±0.71	3.73±0.51
Microsomes	1.07±0.09	1.48±0.12
Supernatant	0.79±0.05	0.70±0.06

Table 3. *Activities of enzymes involved in DNA synthesis in* 24h *regenerating liver*

Rats were partially hepatectomized and injected intravenously with 30 μmol of beryllium/kg; controls received 0.9% NaCl. At 24h the animals were killed and enzyme activities were measured as described in the Materials and Methods section. Basal enzyme activities were evaluated in non-regenerating livers.

	Specific activity (nmol or pmol of substrate transformed/min per mg of protein)			
Enzyme activity	Basal value	Controls (24h)	Beryllium-treated	*P* (controls versus beryllium-treated)
Thymidine kinase (nmol of thymidine phosphorylated)	0.028±0.006 (7)	0.326±0.039 (23)	0.021±0.006 (8)	<0.001
Thymidylate kinase (pmol of dTMP phosphorylated)	0.67 ±0.28 (6)	5.06 ±0.73 (6)	1.37 ±0.36 (6)	<0.01
Deoxycytidylate deaminase (nmol of dCMP deaminated)	0.24 ±0.04 (9)	0.71 ±0.10 (8)	0.30 ±0.05 (8)	<0.01
Thymidylate synthetase (pmol of formaldehyde transformed)	Not detectable	18.5 ±2.2 (9)	8.6 ±1.6 (9)	<0.01
DNA polymerase (pmol of dTTP incorporated)	5.07 ±0.70 (4)	37.95 ±2.95 (8)	13.65 ±1.87 (7)	<0.001

The following enzymes were assayed: thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidylate deaminase and DNA polymerase. At 24h after partial hepatectomy the activities of all these enzymes were throughout higher than in non-regenerating resting livers (Table 3). But, where activities in control animals rose between seven- and 12-fold (except that of deoxycytidylate

deaminase, which rose only threefold), the corresponding increase was much less in beryllium-treated animals, ranging from two- to about threefold only. In all cases the activities in beryllium-treated livers were significantly lower than in the controls. In one experiment measuring the incorporation of labelled dTTP into DNA the three complementary non-radioactive triphosphates were omitted from the incubation mixture. Under these circumstances incorporation of radioactivity into an acid-insoluble product was about 40% of that of the complete system. This incorporation could reflect activity of the terminal nucleotidyltransferase (De Recondo & Fichot, 1969) and was in beryllium-treated animals decreased proportionally to the overall decreased activity in the complete system.

It was now important to test whether this inhibition was due to a direct interaction of beryllium with the enzymes examined. Beryllium has been found to inhibit a few selected enzymes only. The inhibition is, however, very specific and under appropriate experimental conditions is observed at concentrations as low as 1 μM (Thomas & Aldridge, 1966; Aldridge & Thomas, 1966). It was possible to measure the actual beryllium concentrations in our enzyme sources with ^{7}Be. At 24h after partial hepatectomy, when enzymes were maximally inhibited, beryllium concentrations in the enzyme sources were between 2 and 6 μM. Preincubation of enzyme from control livers with the same beryllium concentrations *in vitro* for 10min at 37°C failed to inhibit thymidine kinase and affected DNA polymerase only little (Table 4). Applying the criteria of Thomas & Aldridge (1966) for judging inhibitory effects of beryllium on enzymes, these experiments demonstrate that most probably beryllium has no specific inhibitory effect on thymidine kinase or DNA polymerase.

It did not seem attractive to follow further studies *in vitro*, and another explanation for the enzyme inhibition observed *in vivo* was sought. The kinetics of inhibition *in vivo* were now examined for thymidine kinase, thymidylate kinase and DNA polymerase (Table 5). The patterns that emerged were remarkably similar to the pattern of DNA synthesis and its inhibition by beryllium. In control livers enzyme activities rose sharply and severalfold between 16 and 24h after partial hepatectomy, whereas in the livers exposed to beryllium enzyme activities rose only slightly. Although at 28h they were definitely higher than at 12h or those found in non-regenerating livers, the activities were considerably lower than in the controls. Beryllium therefore prevented the rise in enzyme activity within the first 28h after partial hepatectomy.

These results could be interpreted as if beryllium would inhibit enzyme induction in regenerating liver. If this was so, then it might be expected that beryllium would not produce any effect on such enzymes that seem to work in normal liver at a diminished rate, but whose activities become enhanced during regeneration in some fashion that does not immediately involve an increase in enzyme synthesis. Such enzymes are, among others, uridine kinase, aspartate transcarbamoylase and carbamoyl phosphate synthetase (Bresnick, 1965). The effect of beryllium on these enzymes is shown in Table 6. Activity of uridine kinase did not rise over a 24h period of regeneration. Beryllium somewhat depressed its activity at 24h, but the difference was not significant. Aspartate transcarbamoylase activity rose in control animals to about the same extent as reported by Bresnick (1965), but it rose also in the beryllium-treated rats and the differences observed were not significant. Carbamoyl phosphate synthetase activity was not depressed by the beryllium 24h after partial hepatectomy.

A further enzyme measured was glucose 6-phosphatase, which was also not affected by beryllium at all over a 24h period (Table 7). This enzyme has been found to decrease in activity in some forms of liver injury (Feuer, Golberg, & Le Pelley, 1965). The results show that beryllium

Table 4. *Activities of thymidine kinase and DNA polymerase* in vitro *after preincubation with beryllium*

High-speed supernatant (60min at 102000$g_{av.}$) from normal 24h regenerating liver was incubated for 10min at 37°C in the presence of various concentrations of $BeSO_4$. Enzyme assays were then performed as described in the Materials and Methods section.

Concn. of beryllium during preincubation (mM)	Activity measured (% of activity of untreated control enzyme)	
	Thymidine kinase	DNA polymerase
1.0	105 (1)	28.5 (1)
0.1	112±1 (3)	88±3 (5)
0.05	113±4 (2)	86±7 (3)
0.01	102±3 (3)	92±1 (4)
0.005	101±4 (2)	97 (1)
0.001	93 (1)	99±2 (3)

Table 5. *Activities of thymidine kinase, thymidylate kinase and DNA polymerase in regenerating rat liver at various times after partial hepatectomy*

Partially hepatectomized rats were injected intravenously with 30 μmol of beryllium/kg; controls were given 0.9% NaCl. The animals were killed at the times indicated after the operation and enzyme activities were measured as described in the Materials and Methods section.

Time after partial hepatectomy (h)	Specific activity of thymidine kinase (nmol of thymidine phosphorylated/min per mg of protein)		
	Controls	Beryllium-treated	*P*
12	0.025 ± 0.005 (8)	0.009 ± 0.003 (8)	<0.05
16	0.016 ± 0.004 (8)	0.004 ± 0.001 (8)	<0.05
20	0.147 ± 0.028 (8)	0.009 ± 0.002 (7)	<0.01
24	0.326 ± 0.039 (23)	0.021 ± 0.006 (8)	<0.001
28	0.311 ± 0.082 (4)	0.028 ± 0.003 (4)	<0.05
	Specific activity of thymidylate kinase (pmol of dTMP phosphorylated/min per mg of protein)		
	Controls	Beryllium-treated	*P*
16	0.852 ± 0.144 (4)	0.362 ± 0.077 (4)	<0.10
20	1.730 ± 0.258 (5)	0.735 ± 0.118 (6)	<0.05
24	5.063 ± 0.734 (6)	1.365 ± 0.360 (6)	<0.01
28	3.226 ± 0.474 (8)	0.861 ± 0.140 (7)	<0.01
	Specific activity of DNA polymerase (pmol of dTTP incorporated/min per mg of protein)		
	Controls	Beryllium-treated	*P*
12	5.84 ± 1.56 (4)	4.80 ± 0.56 (4)	<0.10
16	10.26 ± 1.14 (4)	6.58 ± 1.12 (4)	<0.20
20	22.71 ± 2.60 (4)	8.84 ± 1.13 (4)	<0.02
24	37.95 ± 2.94 (8)	13.65 ± 1.87 (7)	<0.001
28	51.71 ± 5.60 (4)	12.07 ± 3.23 (4)	<0.01

in the dose used in the present experiments did not have a general toxic effect on the liver.

It was decided to examine whether and how the time of beryllium administration affected enzyme induction. Actinomycin D has been shown to be capable of inhibiting steroid-induced enzyme synthesis only within a comparatively short period after stimulation. This led to the conclusion that actinomycin D interfered with the enzyme induction at the transcriptional level rather than having an effect on translation, i.e. synthesis of enzyme molecules as such (Tomkins, Gelehrter, Granner, Peterkofsky & Thompson, 1969). Some experiments were now done to test the relationship of time of beryllium administration to the extent of inhibition of thymidine kinase. Thymidine kinase activity was measured exactly 24h after partial hepatectomy and beryllium was injected either at the time of the operation or 6, 9, 12, 20 or 22h later. Fig. 1 demonstrates that beryllium is effective in inhibiting thymidine kinase activity only if it is administered within the first 9–12h after partial hepatectomy. If the administration of beryllium is delayed for 12h or more then thymidine kinase activity, measured at 24h, is no longer significantly lower than in controls.

It was of course possible that beryllium injected 12h or later after partial hepatectomy would not reach a high enough concentration in the liver to affect thymidine kinase. This possibility was dismissed by studying dose–response effects: beryllium administered immediately after partial hepatectomy decreased thymidine kinase activity proportionally to the beryllium concentration in the liver (Fig. 2). As little as 0.061 μmol of beryllium/g of liver depressed enzyme activity significantly. Beryllium injected 20h after hepatectomy failed to produce a dose–response effect, despite the fact that much more beryllium was administered, and 2.9 μmol of beryllium/g of liver failed to affect thymidine kinase activity. The time of beryllium administration was thus clearly more important than the final beryllium concentration in the target organ.

Table 6. *Activities of enzymes involved in pyrimide synthesis in regenerating rat liver*

Partially hepatectomized rats were injected intravenously with 30 μmol of beryllium/kg; controls were given 0.9% NaCl. At the times indicated after the operation the animals were killed and enzyme activities were measured as described in the Materials and Methods section. In no instance was the difference between treated and control animals significant (P values throughout >0.05).

Time after partial hepatectomy (h)	Specific activity of uridine kinase (nmol of uridine phosphorylated/min per mg of protein)	
	Controls	Beryllium-treated
0 (basal value)	1.46±0.07 (12)	—
12	2.01±0.19 (4)	2.14±0.17 (4)
16	2.05±0.10 (6)	2.06±0.10 (6)
24	2.11±0.12 (8)	1.77±0.13 (8)
	Specific activity of aspartate transcarbamoylase (nmol of aspartic acid carbamoylated/min per mg of protein)	
	Controls	Beryllium-treated
0 (basal value)	0.94±0.16 (11)	—
16	1.08±0.15 (8)	0.79±0.08 (8)
20	1.57±0.30 (8)	1.05±0.13 (8)
24	2.22±0.42 (8)	1.41±0.30 (8)
28	1.10±0.31 (4)	0.97±0.20 (4)
	Specific activity of carbamoyl phosphate synthetase (nmol of $NaH^{14}CO_3$ converted/min per mg of protein)	
	Controls	Beryllium-treated
0 (basal value)	23.28±6.28 (6)	—
24	21.18±3.06 (8)	20.26±3.18 (8)

Table 7. *Activity of glucose 6-phosphatase in regenerating rat liver*

Partially hepatectomized rats were injected intravenously with 30 μmol of beryllium/kg; controls were given 0.9% NaCl. The animals were killed at various times after partial hepatectomy and the activity of glucose 6-phosphatase was measured in liver homogenates as described in the Materials and Methods section. In no case was there a statistically significant difference between treated animals and controls.

Time after partial hepatectomy (h)	Specific activity of glucose 6-phosphatase (nmol of P_i liberated/min per mg of protein)	
	Controls	Beryllium-treated
0 (basal value)	67.8±2.4 (6)	—
8	80.5±8.6 (6)	76.3±2.2 (6)
16	70.0±5.8 (6)	76.5±7.6 (6)
24	67.0±3.5 (5)	65.8±2.01 (6)

DISCUSSION

The presented results show that the inhibition of DNA synthesis by beryllium in regenerating rat liver (Witschi, 1968) is most probably the consequence of a decreased activity *in vivo* of several key enzymes: thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidylate deaminase and DNA polymerase. All these enzymes showed greatly diminished activities in 24h regenerating livers exposed to beryllium. It was found for three of them that the kinetics of inhibition were remarkably parallel to the inhibition of DNA synthesis itself; both the latter and the activity of DNA polymerase, thymidine kinase and thymidylate kinase sharply increased in control animals between 16 and 20h after partial hepatectomy. In beryllium-treated rats, little or no

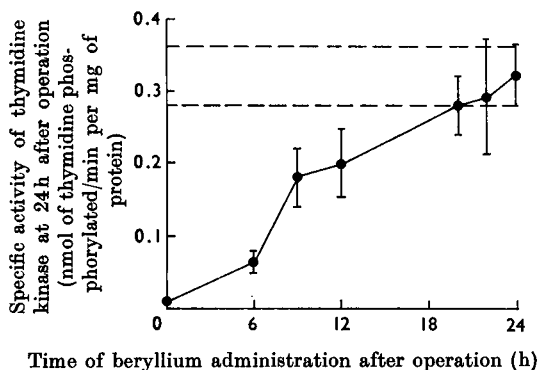

Fig. 1. Relation between time of beryllium administration and activity of thymidine kinase in 24h regenerating rat liver. Rats were partially hepatectomized, killed exactly 24h later and thymidine kinase activity was measured as described in the Materials and Methods section. Beryllium (30μmol/kg) was injected intravenously at the times after operation indicated. Results are plotted as means ± S.E.M.; each point represents six to eight animals. The area between the two broken lines represents the values for untreated controls [0.326 ± 0.039 nmol of thymidine phosphorylated/min per mg of protein (23 animals)].

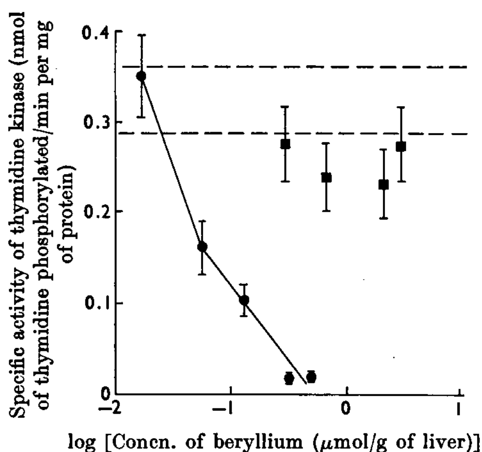

Fig. 2. Dose–response curve for the activity of thymidine kinase in regenerating rat liver 24h after partial hepatectomy and 24h (●) or 4h (■) after intravenous injection of beryllium. The amounts of beryllium given to the animals immediately after partial hepatectomy (●) were, from left to right, 3, 7, 15, 20 and 30μmol/kg. Animals injected with beryllium 20h after partial hepatectomy only (■) received 30, 60, 120 or 240μmol/kg. All animals were killed exactly 24h after partial hepatectomy. Thymidine kinase activity and beryllium concentrations in the livers were measured as described in the Materials and Methods section. The area between the broken lines represents the normal enzyme activity for untreated controls and is the same as in Fig. 1.

increase was observed within the same time-period. Impaired DNA synthesis after beryllium might therefore be attributable to a diminished activity of critical enzymes.

Although beryllium is a very specific inhibitor for a few selected enzymes (Thomas & Aldridge, 1966) it does not seem likely that the decreased enzyme activities found in the present experiments are due to a direct interaction of beryllium with these enzymes. Thomas & Aldridge (1966) reported that beryllium-susceptible enzymes are inhibited at about 1μM; inhibition *in vitro* of enzymes at concentrations of 1mM or more has to be considered an unspecific artifact. No large effect of beryllium on thymidine kinase or DNA polymerase was found if the enzyme sources were preincubated for 10min in the presence of beryllium concentrations up to 0.1mM, and at 1mM only DNA polymerase was inhibited (Table 4). Since the beryllium concentrations measured in the enzyme sources after beryllium administration *in vivo* never exceeded 10μM, the absence of any effect of much higher beryllium concentrations in the system *in vitro* makes a direct interaction of beryllium with these two enzymes rather unlikely. This conclusion is supported by the observation that beryllium given at a time (20h after partial hepatectomy) when thymidine kinase activity is almost maximal failed to have any inhibitory effect even when very high beryllium concentrations in the liver were obtained (Fig. 2). DNA polymerase and thymidine kinase also have different locations within the cell. The polymerase is located within the nucleus (Behki & Schneider, 1963), whereas thymidine kinase seems to be an enzyme of the cell sap (Baugnet-Mahieu, Goutier & Semal, 1968). If beryllium affected these enzymes directly it might be expected that the DNA polymerase would suffer more since 24h after partial hepatectomy nuclei contain ten times as much beryllium as does the cell sap (Table 2). The results in Table 3 demonstrate that at this time thymidine kinase is more inhibited relative to controls than is DNA polymerase. And, finally, it has been observed that beryllium more probably affects the events leading to DNA synthesis than DNA synthesis itself (Witschi, 1968).

All the enzymes whose activities were affected by beryllium (Table 3) show very low activities in normal non-regenerating livers. In control rats, however, activities increased considerably during the first 20–28h after partial hepatectomy. These enzymes are all critical to DNA synthesis, an event practically non-existent in resting liver (Bucher, 1967*a*,*b*). There are on the other hand enzymes present in more than adequate amounts in normal liver but that do not, for one reason or another, operate at full capacity and that also do not show much increased activity during regeneration

(Bresnick, 1965). These enzymes are those synthesizing pyrimidine precursors of RNA. It was found that beryllium had no effect on those few tested: uridine kinase, aspartate transcarbamoylase and carbamoyl phosphate synthetase. These results might explain why the incorporation of orotic acid into RNA in regenerating rat liver was not inhibited by beryllium, whereas the incorporation of the same precursor into DNA was (Witschi, 1968). Finally it was necessary to establish that the inhibition of some enzymes by beryllium was not simply a manifestation of toxic liver damage. Gross histopathological changes were absent throughout in livers examined 28h after partial hepatectomy (P. Goldblatt, M. Lieberman & H. P. Witschi, unpublished work). Glucose 6-phosphatase activity, an enzyme thought to be an index of toxic liver damage even in the absence of gross histopathological changes (Feuer *et al.* 1965), was also not affected by beryllium. In regenerating rat liver DNA synthesis thus seems to be inhibited because enzymes involved in forming the necessary precursors for DNA fail to increase in activity. Beryllium is, however, without effect on such enzymes already present in normal liver and that do not rise in activity during partial hepatectomy. This makes the effect of beryllium a rather specific one.

The rise in activity of some enzymes after partial hepatectomy can be considered to represent a special case of enzyme induction. Operative removal of two-thirds of the liver would then be the trigger or stimulus for inducing enzymes critical to DNA synthesis. It is assumed for the following discussion that the increase in enzyme activity observed after partial hepatectomy reflects actually increased synthesis of new enzyme molecules, an assumption for which there is at least some circumstantial evidence (Bresnick, Williams & Mossé, 1967). In experiments similar to the ones reported several substances have been shown to inhibit a rise in enzyme activity after partial hepatectomy: ethionine, actinomycin D, *p*-fluorophenylalanine and puromycin (Schneider, Cassier & Chordikian, 1960; Maley, Lorenson & Maley, 1965; Gottlieb, Fausto & Van Lancker, 1964; Bresnick *et al.* 1967); cycloheximide and methotrexate (Labow *et al.* 1969); chlortetracycline (Hurwitz & Carter, 1969). Most of these inhibitors act maximally when given immediately after partial hepatectomy (actinomycin D: Bresnick *et al.* 1967; vinblastine: Luyckx & Van Lancker, 1966; chlortetracycline: Hurwitz & Carter, 1969; asparaginase: Becker, Baserga & Broome, 1970). Others are still effective when given up to and shortly before the maximal rise in DNA synthesis (cycloheximide: Verbin, Sullivan & Farber, 1969; cortisol: Raab & Webb, 1969). The damage produced by some of these agents is temporary only (asparaginase: Becker *et al.* 1970), whereas with others it is irreversible (actinomycin D: Schwartz, Sodergren, Garofalo & Sternberg, 1965; methotrexate: Labow *et al.* 1969). Beryllium now has to be added to this list. It falls under the class of agents that are active only if given within the very first hours after partial hepatectomy, but that inflict a lasting and irreversible damage.

The mechanism(s) whereby some of the abovementioned substances prevent enzyme induction on regenerating livers are known. Actinomycin D binds to DNA, inhibits DNA-dependent RNA synthesis and thus might prevent transcription of new mRNA needed for stepped-up enzyme synthesis. One possible piece of evidence for this hypothesis is the observation during a study of the induction of tyrosine aminotransferase by steroids (Tomkins *et al.* 1969). Actinoymcin D only prevents enzyme induction if given with or soon after the stimulus. If administration is delayed beyond a critical time-lapse, mRNA is supposedly fully transcribed and the increased production of enzyme can no longer be blocked by the drug. Studies on the inhibition of thymidine kinase in regenerating liver lead essentially to the same conclusions (Bresnick *et al.* 1967). Puromycin, *p*-fluorophenylalanine and possibly also ethionine might conceivably inhibit enzyme induction by interfering with protein synthesis, blocking translation rather than transcription.

From the results presented in Fig. 1 it seems that beryllium resembles actinomycin D: beryllium disturbs the process of adapting thymidine kinase synthesis to the new rates required for regeneration, but it is no longer capable of interfering with enzyme already formed, nor does it apparently disturb enzyme production once an increased rate of synthesis has been programmed. However, an important difference exists: so far beryllium has not been found to inhibit RNA synthesis (Chèvremont & Firket, 1951; Witschi, 1968), one of the most immediate effects of actinomycin D. There is also conflicting evidence on whether beryllium binds to DNA (Witschi & Aldridge, 1968; Reiner, 1970; Truhaut, Festy & Le Talaer, 1968; Festy, Le Pecq, Paoletti & Truhaut, 1965). Beryllium also does not interfere with incorporation of leucine into liver protein *in vivo* (Witschi & Aldridge, 1967), although it has been found that beryllium causes breakdown of liver polyribosomes proportionally to the dose of beryllium administered (H. P. Witschi, unpublished work). No metabolic alteration has therefore yet been defined that might give a rational basis for the observation of the inhibited enzyme induction.

It is of course tempting to attribute the effect of beryllium on enzyme induction at the transcriptional level directly to the presence of beryllium in the cell nucleus. Work by Reiner (1970) suggests

that beryllium binds within the nucleus rather specifically to a special protein fraction, not yet defined. The affinity of this fraction towards beryllium is modified in the presence of DNA. The work of Pogo, Pogo, Allfrey & Mirsky (1968) has shown that chemical alterations of nucleoproteins such as histones play an important role in influencing template activity of the chromatin for RNA synthesis. It might well be that the binding of beryllium to one or several nucleoproteins in the end might be the cause for the present findings. This hypothesis seems especially likely since beryllium has any effect only if present very early after hepatectomy within the liver; acetylation of histones is also a very early phenomenon after partial hepatectomy (Pogo *et al.* 1968). More work on both the intracellular and intranuclear distribution of beryllium and on nuclear protein and RNA metabolism within the very first hours after partial hepatectomy hopefully will clarify whether and how beryllium interferes with those early metabolic changes during liver regeneration. The following point has, however, to be borne in mind: if the effects of beryllium on enzyme induction are due only to the beryllium that accumulates within the nucleus within the first few hours after partial hepatectomy, then the doubling of the nuclear beryllium concentration between 12 and 24h (Table 2) would be irrelevant to this aspect of the toxic action. It remains to be seen whether beryllium is responsible for other metabolic effects or whether nuclei also serve simply as some kind of 'storage place' for intracellular beryllium.

The present results allow us to understand perhaps somewhat better some earlier observations of beryllium toxicology. Witschi & Aldridge (1967) pointed out that in normal rat liver beryllium did not depress several biochemical functions, but rather seemed to prevent normal livers from adapting to a 24h starvation period; mitochondrial and microsomal enzymes in livers exposed to beryllium failed to display an increase in activity, which, however, was very pronounced in control rats. This could mean that beryllium blocks 'enzyme induction' due to starvation. Ethionine, an agent known to interfere with enzyme induction (Farber, 1963), has also been reported to produce an altered pattern of adaptation to starvation (Tani & Hanson, 1969). It is also conceivable that one well-known feature of human beryllium disease might be explained by the fact that beryllium prevents enzyme induction. Clinically more-or-less stable and stationary beryllium diseases suddenly flare up during periods of stress such as operations or pregnancies (Tepper *et al.* 1961). It could well be that such conditions of stress are counteracted by a defensive response in different tissues. The presence of beryllium effectively might block this adaptive response, depriving the affected tissue of means to deal successfully with altered demands.

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