Uptake, Distribution and Binding of Beryllium to Organelles of the Rat Liver Cell

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1. Male rats were injected intravenously with amounts ranging from 0.08 to 111.0 μ moles of [^7Be]beryllium sulphate/kg. body wt. The distribution in the rat and the subcellular distribution of beryllium in the liver were determined. 2. Within the entire dose range a higher specific activity of beryllium was present in a mitochondrial fraction containing the lysosomes. Purification of this fraction confirmed that beryllium is taken up by lysosomes. 3. With doses approaching the LD₅₀, beryllium was also found in increasing amounts to be present in the liver cell nuclei. Beryllium also showed affinity towards isolated cell nuclei *in vitro*. Evidence is presented that they have one class of binding sites for beryllium. Mitochondria have less affinity for beryllium. 4. No evidence could be obtained of an affinity of beryllium for DNA or RNA by fractionation of nuclei and dialysis experiments. 5. The presence of beryllium in liver cell nuclei may be relevant to the effects of beryllium on nuclear structure and function.

Beryllium is toxic to many cells (for literature cf. Tepper, Hardy & Chamberlin, 1961; Witschi & Aldridge, 1967). A convenient way of examining this toxicity is to inject soluble beryllium salts into rats. An insoluble beryllium phosphate is produced and this is removed from the circulating blood by the reticuloendothelial system of the liver. This is the reason for the apparent specificity of beryllium in inducing necrosis only in the liver (Aldridge, 1966). In a recent paper (Witschi & Aldridge, 1967) an examination was made of various biochemical activities of the liver cell after the administration of beryllium to the rat. Comparison of the results with the effects of other hepatotoxins indicated no common pathway for the genesis of necrosis.

In order to try to learn more of the mechanism whereby beryllium kills cells, we have used ^7Be to determine its distribution in liver cells. The distribution of beryllium within the rat has been previously examined (Crowley, Hamilton & Scott, 1949; Scott, Neuman & Allen, 1950). From previous work on enzymes (Thomas & Aldridge, 1966; Aldridge & Thomas, 1966) it is probable that beryllium is highly specific and reacts with proteins containing a specific molecular configuration. As a preliminary to finding other macromolecules having an affinity for beryllium we studied the subcellular distribution of beryllium in the liver.

MATERIALS AND METHODS

Radioactive beryllium. Solutions were prepared by adding carrier-free $^7\text{BeCl}_2$ (The Radiochemical Centre, Amersham,

Bucks.) to solutions of known concentrations of $^9\text{BeSO}_4$. Radioactivity of samples was measured in a Packard Autogamma scintillation spectrometer with an overall efficiency for ^7Be of approx. 5%. Appropriate dilutions of the active solutions were always counted together with the sample and served as standards. A sufficient number of counts was always collected to keep the standard error below 2%.

Procedure for the determination of the distribution of beryllium. Male albino rats (180–200 g.) of the Porton strain fed with M.R.C. diet 41B (Bruce & Parkes, 1956) were used. Solutions of [^7Be]BeSO₄ were injected into a tail vein at 9 a.m. After the animals had been killed by a blow on the head or with ether-chloroform, samples of different organs were removed, weighed and their radioactivity was counted.

Preparation of subcellular fractions of liver. Liver was removed from the animal as quickly as possible, rinsed and immersed in ice-cold 0.3 M-sucrose. All further operations were then carried out as near 0° as possible. Homogenates (10%, w/v) were prepared in 0.3 M-sucrose by using the homogenizer previously described (Aldridge, Emery & Street, 1960; Webster & Smith, 1964; difference in diameter of pestle and tube 0.02 in. and revolving at 1100 rev./min.). The homogenate was centrifuged in the 6 × 100 ml. angle rotor of the MSE Highspeed 18 centrifuge for 10 min. at 800g to give the nuclear and debris fraction, and for 15 min. at 5000g and 15 min. at 13000g to yield the heavy and light mitochondrial fractions respectively. Both mitochondrial fractions were washed once with 0.3 M-sucrose. The remaining supernatant was centrifuged either for 1 hr. at 40000g in the MSE Highspeed 18 centrifuge or for 1 hr. at 105000g in Rotor no. 40 of the Spinco model L ultracentrifuge to give microsomal fractions and a supernatant fraction. Each pellet was suspended in 0.3 M-sucrose before the determination of radioactivity and protein.

Preparation of nuclei by using high-density sucrose solution.

Clean and easily suspendable nuclei were obtained by the following modification of the method of Chaveau, Moulé & Rouiller (1956). Liver tissue (7g.) from rats starved for 24 hr. was homogenized as described above at 1900 rev./min. in 15 ml. of solution containing 0.25 M-sucrose, 3 mM-MgCl₂ and 0.1 M- or 0.05 M-tris-HCl buffer, pH 7.4. The homogenate was mixed into 140 ml. of 2.1 M-sucrose containing 3 mM-MgCl₂ and centrifuged for 60 min. at 40000g in the MSE Highspeed 18 centrifuge. The nuclei so obtained were resuspended in the sucrose-MgCl₂-tris medium, centrifuged for 10 min. at 800g in the MSE Mistral 6L centrifuge and finally suspended in the same medium. The recovery of DNA from the original homogenate was 50 ± 16% (19) and the ratio mg. of protein/mg. of DNA P for the nuclear preparation was 38 ± 8. When examined by phase-contrast microscopy the nuclei were well preserved with only a slight contamination with other material. No erythrocytes or unbroken cells were seen.

Preparation of nuclei by using Triton X-100. In a few experiments nuclei were prepared exactly as described by Hymer & Kuff (1964) by centrifugation at low speeds and washing with the non-ionic detergent Triton X-100.

Purification of lysosomes by differential centrifuging. After injection with [⁷Be]BeSO₄, rats were starved for 6 or 24 hr. and their livers homogenized in 0.25 M-sucrose. The homogenate of each liver was fractionated individually by the centrifugation scheme of Sawant, Shibko, Kumta & Tappel (1964). At each stage of their purification procedure (FI to FIV) one or several pellets were taken as representative samples; if not enough material was available in the pellet from one liver, two pellets were pooled before the assays. All pellets were made up to volume with deionized water, and samples for the determination of protein, ⁷Be and acid phosphatase were removed, the last being frozen immediately in liquid N₂.

Purification of lysosomes by density-gradient centrifugation. From a 10% (w/v) rat liver homogenate in 0.25 M-sucrose, nuclei and some mitochondria were removed by centrifugation for 10 min. at 1000g. A mitochondrial fraction was then isolated by centrifuging the supernatant for 15 min. at 16500g. After the pellet had been suspended in 25 ml. of 0.25 M-sucrose, four 5 ml. portions were layered in four 50 ml. centrifuge tubes on top of a discontinuous sucrose gradient as described by Rahman & Lindenbaum (1964). The tubes were centrifuged for 120 min. at 1350g in the 12 × 50 ml. swing-out head of the MSE Mistral 6L centrifuge. The centrifuge was allowed to stop without braking, and eight 5 ml. fractions were collected from each tube with the aid of a syringe and needle, proceeding from top to bottom (fraction 1=lowest-density sucrose, fraction 8=highest-density sucrose). The corresponding fractions from four tubes were pooled, centrifuged for 10 min. at 30000g, the supernatants discarded and the pellets suspended in deionized water. Samples were taken for the determination of acid phosphatase, ⁷Be and protein. Recoveries were 118 ± 6% for acid phosphatase, 98 ± 2% for beryllium and 99 ± 4% for protein (means ± S.E.M., 14 experiments).

Subfractionation of nuclei. Nuclei from 20–25 g. of liver were prepared by using high-density sucrose solution (Chaveau *et al.* 1956), pooled and fractionated exactly as described by Rendi (1960) except that the deoxycholate-soluble material was not further fractionated.

Analytical methods. Protein was determined by the biuret method (Robinson & Hogden, 1940, as modified by Aldridge,

Table 1. *Distribution of beryllium in organs of rat at various times after injection*

Male rats (200g.) were injected intravenously with 83 μmoles of [⁷Be]BeSO₄/kg. body wt. At 24 hr. the heart contained 0.020, lung 0.075, brain 0.001, duodenum 0.079 and muscle 0.004 μmole of beryllium/g. wet wt. of tissue.

Time after injection (hr.)	Beryllium (μmole/g. wet wt. of tissue)			
	Blood	Liver	Spleen	Kidney
Immediately	1.0	0.14	0.04	0.12
1	0.79	0.15	0.13	0.096
2	0.79	0.20	0.15	0.085
4	0.61	0.32	0.21	0.12
6	0.43	0.49	0.22	0.096
8	0.29	0.52	0.37	0.096
12	0.25	0.70	0.32	0.091
24	0.045	1.02	0.33	0.067

1957), crystalline bovine serum albumin being used as reference substance. DNA was determined by the method of Burton (1956), highly polymerized DNA from calf thymus (British Drug Houses Ltd.) being used as standard. The phosphorus content of the standard DNA was determined by the method of Fiske & Subbarow (1925). Total acid phosphatase was determined in the following way. The frozen samples were thawed and incubated for 30 min. at 0° in the presence of Triton X-100 (final concn. 0.1%). Incubation of the enzyme was then carried out at 37° in a medium containing 0.25 M-sucrose, 0.1 M-sodium citrate-citric acid buffer, pH 5.8, and 0.05 M-β-glycerophosphate. Inorganic phosphate was determined by the method of Berenblum & Chain (1938), after no and 10 min. incubation.

RESULTS

Distribution of beryllium in rats in vivo

After a single intravenous injection of 83 μmoles of beryllium sulphate/kg., the liver and the spleen contained at 24 hr. most beryllium/g. of tissue. In the liver and spleen the concentration of beryllium increased continuously over the first 8 and 24 hr. after administration respectively (Table 1). At 4 hr. and 24 hr. after injection 20% and 50% respectively of the injected beryllium was in the liver (no beryllium could be removed from the liver by perfusion of the whole animal). Blood, kidney, heart, lung, brain, muscle and duodenum contained less beryllium. The uptake of beryllium by the liver and the spleen is in agreement with the view of Cheng (1956) that a colloidal compound of beryllium forms in the blood and is removed by the reticuloendothelial system of these organs. Below a certain dose administered (7 μmoles/kg.) the percentage beryllium uptake by the liver decreased sharply (Scott *et al.* 1950; Table 2). This may indicate that when low doses are administered more of the beryllium might exist in the blood as a non-

particulate, soluble and diffusible form (Feldman, Havill & Neuman, 1953) that would not be trapped by the reticuloendothelial system.

No further studies on the distribution of beryllium in the whole animal were carried out later than 24 hr. after administration, since this information is available in the literature (Crowley *et al.* 1949; Scott *et al.* 1950).

Subcellular distribution of beryllium

Fractionation of liver after injection of beryllium. At 24 hr. after a single intravenous dose of 83 μ moles of beryllium sulphate/kg., rat livers were fractionated by differential centrifugation. Since 63% of the beryllium in the liver was found in the nuclear and debris fraction, purer preparations of nuclei were isolated (Table 3). Pure nuclei whether isolated in a high-density sucrose solution or with the aid of a detergent had always a higher specific activity (m μ moles of beryllium/mg. of protein) than that of the homogenate from which they were isolated

(Table 4). The specific activity of the light mitochondrial fraction was also higher than that of the homogenate or the heavy mitochondrial fraction. The heavy mitochondrial fraction is probably contaminated with some nuclei. This increases the significance of the higher specific activity of the light mitochondrial fraction. It seemed likely that some component other than mitochondria must be considered; lysosomes were clearly a possibility. The microsomal and supernatant fractions had a lower specific activity than the homogenate.

Fractionation of liver homogenates to which beryllium had been added. At 24 hr. after the injection of 83 μ moles of beryllium sulphate/kg. to rats, approx. 8–10 μ moles were present in the liver. A similar amount (8.4 μ moles) was added to a 10% homogenate of normal liver. After fractionation the distribution of beryllium in the fractions was different to that found after administration *in vivo* (Table 3). The microsomal and supernatant fractions contained a much larger proportion of the beryllium. The nuclear and debris fraction contained 34% of the beryllium added and, when nuclei were isolated from similar homogenates by the method of Chaveau *et al.* (1956), their specific activity was 6.07 ± 1.05 times that of the homogenate. The different pattern of distribution found after administration *in vivo* and *in vitro* indicates that it is unlikely that there is a major redistribution of the beryllium during homogenization and fractionation after administration *in vivo*.

Fractionation of liver after injection of various doses of beryllium. The intracellular distribution of beryllium 24 hr. after injection of doses in the range 0.083–110 μ moles/kg. is shown in Table 5. The specific activity of the light mitochondrial fraction was at all doses uniformly greater than that of the corresponding homogenate. The specific activity of nuclei was less than that of the homogenate at low doses and considerably more at the highest dose.

Table 2. *Proportion of administered doses of beryllium found in rat liver*

Male rats (200 g.) were injected intravenously with [7 Be]BeSO₄ and killed 24 hr. later. The numbers of animals are given in parentheses.

Dose of BeSO ₄ (μ moles/kg.)	Beryllium in liver at 24 hr. (% of dose)
111	25–33 (3)
83	32–50 (12)
49	40–52 (7)
28	29–30 (3)
7	24–32 (3)
1.74	5–6 (3)
0.84	2.5–10 (9)
0.084	2–3 (5)

Table 3. *Distribution of protein and beryllium in subcellular fractions from rat liver*

For the experiments *in vivo* 83 μ moles of [7 Be]BeSO₄/kg. body wt. were injected intravenously and the rats killed 24 hr. later. For the experiments *in vitro* 8.4 μ moles of [7 Be]BeSO₄ were added to 80 ml. of a 10% (w/v) homogenate of a liver from an untreated rat and incubated for 15 min. at 0°. The results are the means $\pm$ S.E.M. of six experiments. The recoveries of protein for the experiments *in vivo* and *in vitro* were 95.8 ± 4.0 and $94.0 \pm 4.0\%$ respectively; for beryllium they were 94.4 ± 5.0 and $98.3 \pm 5.0\%$ respectively.

Fraction	Distribution (% of that in homogenate)			
	<i>in vivo</i>		<i>in vitro</i>	
	Protein	Beryllium	Protein	Beryllium
Nuclear + debris	43 ± 1	63 ± 2	50 ± 1	34 ± 1
Heavy mitochondrial	10 ± 1	17 ± 1	7 ± 1	3 ± 1
Light mitochondrial	3 ± 1	5 ± 1	3 ± 1	3 ± 1
Microsomal	7 ± 1	5 ± 1	8 ± 1	11 ± 1
Supernatant	32 ± 1	7 ± 1	29 ± 1	44 ± 1

The specific activity of the heavy mitochondrial fraction tended to increase with increasing dose though to a smaller extent than that of nuclei, whereas that of the microsomal and supernatant fractions decreased; that of the supernatant fraction decreased to a greater extent than that of the microsomes. If the beryllium in the supernatant is predominantly beryllium in solution this is expected.

Beryllium and lysosomes in liver

The higher specific activity ($m\mu$ moles of beryllium/mg. of protein) in the light mitochondrial compared with the heavy mitochondrial fraction indicated the possibility that at least some of the

beryllium was located in the lysosomes. There is evidence that lysosomes were damaged because 24 hr. after rats were dosed with 83μ moles of beryllium sulphate/kg. the percentage of free acid phosphatase in the liver was increased (Witschi & Aldridge, 1967). To minimize damage to lysosomes a small dose (7μ moles of beryllium sulphate/kg.) and a short time-interval (5 hr.) were used.

Beryllium and lysosomes 5 hr. after the injection of 7μ moles of beryllium sulphate/kg. A mitochondrial fraction from liver was fractionated by the density-gradient centrifugation method of Rahman & Lindenbaum (1964). The specific activities of beryllium and acid phosphatase of the eight fractions from each of four experiments were compared (Fig. 1), and it is clear that there is a constant

Table 4. *Beryllium in various subcellular fractions from rat liver*

Rats were injected intravenously with 83μ moles of $[^7\text{Be}]\text{BeSO}_4/\text{kg. body wt.}$ and killed 24 hr. later. The results are given as means $\pm$ s.e.m. (with the numbers of experiments in parentheses) of the specific activity of each fraction as a percentage of the corresponding homogenate. The specific activity of the homogenates was 4.8 ± 0.4 ($10 m\mu$ moles of beryllium/mg. of protein).

Fraction	Specific activity of beryllium (% of that of homogenate)
Nuclear (prepared by method of Chaveau <i>et al.</i> 1956)	350 ± 52 (8)
Nuclear (prepared by method of Hymer & Kuff, 1964)	320 ± 10 (4)
Heavy mitochondrial (M1)	174 ± 8 (6)*
Light mitochondrial (M2)	204 ± 15 (6)*
Microsomal ($100\,000g$ for 1 hr.)	65 ± 5 (5)
Supernatant ($100\,000g$ for 1 hr.)	18 ± 1 (5)

* M2/M1 specific activity ratio 1.17 ± 0.06 (6).

Fig. 1. Relation between the specific activities of beryllium and acid phosphatase in subfractions of a mitochondrial fraction from rat liver. The experiments are those described in Table 6. The equation to the regression line through the points is $y = 0.0011 + 0.008624x$, where y and x are the specific activities of beryllium and acid phosphatase respectively. The different symbols represent different experiments.

Table 5. *Beryllium in subcellular fractions from rat liver after various doses*

Rats were injected with various amounts of $[^7\text{Be}]\text{BeSO}_4$ from 0.083 to 110μ moles/kg. body wt. and killed 24 hr. later. Specific activity is expressed as $m\mu$ moles of beryllium/mg. of protein. Nuclei were isolated in different experiments by using the high-density sucrose method.

Dose of BeSO_4 (μ moles/kg.)	Fraction ...	Specific activity of beryllium (% of that of homogenate)				
		Nuclear	Heavy mitochondrial	Light mitochondrial	Microsomal	Supernatant
0.083		44	110	260	70	160
0.83		141	98	295	93	125
1.8		98	—	—	—	—
8.3		98	—	315	—	—
28		280	200	310	57	35
83		340	175	204	63	—
110		410	—	—	—	20

Table 6. *Beryllium and acid phosphatase in subfractions of a mitochondrial fraction from rat liver*

The mitochondrial fraction and the subfractions were prepared as described in the Materials and Methods section by the method of Rahman & Lindenbaum (1964). Results are given as means $\pm$ S.E.M. (with the numbers of experiments in parentheses). The specific activities of fraction 4 at 5 and 24 hr. after dosing were for beryllium 2.03 ± 0.25 (4) and 1.80 ± 0.24 (5) $\mu\text{moles/mg.}$ of protein and for acid phosphatase 233 ± 61 (4) and 194 ± 18 (5) $\mu\text{moles of P}_i/\text{min./mg.}$ of protein respectively.

Time after 7 μmoles of BeSO ₄ /kg. (hr.)	Fraction no.	Specific activity (% of that of fraction 4)		P
		Beryllium	Acid phosphatase	
5	1	14.1 $\pm$ 1.1	18.1 $\pm$ 3.5	0.35
	2	36.5 $\pm$ 6.2	47.5 $\pm$ 7.5	0.35
	3	82.0 $\pm$ 7.6	88.0 $\pm$ 11.5	0.7
	4	100	100	—
	5	55.7 $\pm$ 4.5	53.8 $\pm$ 5.4	0.8
	6	24.1 $\pm$ 3.4	25.1 $\pm$ 5.6	0.9
	7	12.3 $\pm$ 5.3	13.4 $\pm$ 3.7	0.85
	8	10.1 $\pm$ 0.9	8.6 $\pm$ 1.1	0.3
24	1	16.2 $\pm$ 2.5	31.3 $\pm$ 3.1	< 0.01
	2	23.2 $\pm$ 2.4	53.6 $\pm$ 4.0	< 0.01
	3	49.6 $\pm$ 5.0	92.8 $\pm$ 6.0	< 0.01
	4	100	100	—
	5	87.3 $\pm$ 7.8	53.6 $\pm$ 5.3	< 0.01
	6	43.8 $\pm$ 9.5	22.9 $\pm$ 4.8	0.09
	7	23.2 $\pm$ 5.9	11.9 $\pm$ 2.5	0.09
	8	17.6 $\pm$ 4.0	7.5 $\pm$ 1.3	0.05

ratio between these variables, the regression line passing through the origin. Livers were also fractionated by the differential centrifugation method of Sawant *et al.* (1964). In this case there was also a linear relation between the specific activities of beryllium and of acid phosphatase of the various fractions. However, the straight line did not pass through the origin. We have no explanation for this, but consider it may be associated with the complex fractionation procedure leading to damage to the lysosomes and consequent loss of acid phosphatase. In Table 6 the distribution of beryllium and acid phosphatase among fractions is shown, the fraction with the highest specific activity (fraction 4) being taken as 100. These results indicate that beryllium concentration and acid phosphatase specific activity are correlated, and therefore it may be inferred that after this dose and time-interval beryllium is in the lysosomes of the liver. It is also clear, since the regression line passes through the origin (Fig. 1), that most of the beryllium in this fraction must be so located.

Beryllium and lysosomes 24 hr. after the injection of 7 μmoles of beryllium sulphate/kg. When a mitochondrial fraction from liver 24 hr. after a dose of 7 μmoles of beryllium sulphate/kg. was further fractionated by the density-gradient technique no simple correlation between beryllium content/mg. of protein and acid phosphatase activity/mg. of protein was found. The results of five experiments are shown in Table 6. The distribution of beryllium among the fractions is different to that of the acid

Fig. 2. Relation between the specific activities of beryllium and acid phosphatase in subfractions of a mitochondrial fraction from rat liver. Rats were injected intravenously with 83 μmoles of $[^7\text{Be}]\text{BeSO}_4/\text{kg.}$ body wt. and killed 5 and 24 hr. later. The mitochondrial fraction and the subfractions were prepared as described in the Materials and Methods section by the method of Rahman & Lindenbaum (1964). The equations to the regression line through the results obtained at 5 and 24 hr. were $y = 0.143x - 0.73$ and $y = 0.978x - 8.3$ respectively, where y and x are the specific activities of beryllium and acid phosphatase respectively. The different symbols represent different experiments.

phosphatase. This result may or may not indicate that beryllium is located in particles other than lysosomes.

Beryllium and lysosomes 5 and 24 hr. after the injection of 83 μ moles of beryllium sulphate/kg. Similar experiments with a greatly increased dose of beryllium sulphate indicated that there was a direct correlation between specific activities of beryllium and of acid phosphatase both at 5 hr. and at 25 hr. (Fig. 2). The relation between these variables was not, however, the same at each time, the beryllium/acid phosphatase ratio being 7.7 at 5 hr. and 1.9 at 24 hr.

Binding studies in vitro

It has been shown (Table 4) that, after injection of 83 μ moles of beryllium sulphate/kg. into the rat, liver nuclei isolated and purified by two different methods had a higher specific activity than that of the corresponding homogenate. With lower doses a relatively lower specific activity was found. On the other hand, after the addition of beryllium sulphate even in amounts in the range 0.2–97 μ moles/ml. of 10% homogenate of rat liver, followed by separation of nuclei by the high-density sucrose method, the specific activity of the nuclei was always higher than that of the homogenate. This range of doses is equivalent to the administration of 2–83 μ moles of beryllium sulphate/kg. body wt. It was therefore decided to try to determine the affinity of beryllium for nuclei by studies *in vitro*. However, when nuclei were suspended in sucrose buffered to pH 7.4, incubated for 1 hr. at 40° and the nuclei separated by centrifugation, it was found that there was a linear relation between the amount of beryllium bound and the amount available in the system over a range of concentrations from 0.14 μ M to 1.4 mM. However, mitochondria behaved in an identical fashion. The results obtained by fractionation of liver after the animals had been dosed with beryllium or those obtained by adding beryllium sulphate to homogenates of control livers both indicated a higher affinity of beryllium for nuclei than for mitochondria. It seemed possible that in these binding studies *in vitro* the beryllium was present at pH 7 as a charged colloid of beryllium hydroxide; such a colloid would be readily adsorbed to most biological material. We therefore attempted to maintain the beryllium in solution as a soluble complex of stability low enough to allow binding to nuclei to take place. Such an approach has also been used for studying the binding of mercury to biological material, penicillamine being the competitive complex-forming agent used (Clarkson & Magos, 1966). For beryllium Feldman *et al.* (1953) have shown that citrate may be used for this purpose, though not all of the beryllium is freely diffusible and equilibrium is not reached within 16 hr. (Feldman, Neuman, Danley & Havill, 1951). After preliminary experiments 1 mM-citrate was

Fig. 3. Binding of beryllium to nuclei from rat liver. Rat liver nuclei (7–8 mg. of protein) were suspended in 10 ml. of a solution containing sucrose (0.3 M), tris-HCl buffer, pH 7.4 (0.05 M), sodium citrate (1 mM) and $[^7\text{Be}]\text{BeSO}_4$ ranging from 5.5 μ M to 1.1 mM. This solution was incubated for 1 hr. at 4°. After centrifuging at 2000g for 15 min. the beryllium in the pellet and supernatant was determined. The results from five separate experiments (each with a different symbol) are plotted and the line is the regression line through the points excluding the first six from the left.

chosen as that concentration which gave a large discrimination between the binding of beryllium to nuclei and heavy mitochondria, light mitochondria or microsomes. The results of experiments with a large range of concentrations of beryllium in sucrose containing 1 mM-citrate, pH 7, are shown in Fig. 3. The concentration range examined was 5.5 μ M to 0.9 mM. The method of plotting is that of Scatchard (1949), where the ratio of bound to free form is plotted against bound form. If a straight line is obtained crossing each ordinate then a single class of groups is involved, and the concentration of such groups and the association constant may be evaluated from the intercept on the axis for the bound form and the slope respectively. This was found to be the case, the concentration of sites involved being 25.5 m μ moles of beryllium/mg. of nuclear protein and the association constant being 1.8×10^4 . This constant cannot, however, be converted into absolute values for the affinity of Be^{2+} ions for nuclei because of the complexity of the affinity constant of the beryllium-citrate system above pH 7 (Feldman, Toribara, Havill & Neuman, 1955). The six points on the left obtained from very

Table 7. *Distribution of beryllium in fractions of nuclei from rat liver*

Nuclei from the liver of each of four rats were isolated by the high-density sucrose method 24 hr. after the intravenous injection of 83 μ moles of $[^7\text{Be}]\text{BeSO}_4/\text{kg. body wt.}$ The four preparations were mixed and fractionated by the method of Rendi (1960) and the fractions are named as in his paper. The results are the mean of two experiments. Recoveries of protein, DNA and beryllium in the fraction were 94, 96 and 100% respectively.

Fraction	Specific activity of beryllium (m μ moles/mg. of protein)	Recovery in each fraction (% of that in original nuclei)		
		Protein	DNA	Beryllium
Original nuclear	8.9	100	100	100
Deoxycholate-insoluble material				
Soluble in water	3.1	10	11	3
Soluble in M-NaCl	13.2	40	78	59
Residue	23.2	8	0.7	22
Deoxycholate-soluble material	4.1	36	6.3	16

Table 8. *Equilibrium dialysis of various biological materials after the addition of beryllium*

To 5 ml. of a solution containing sucrose (0.3 M), tris-HCl buffer, pH 7.4 (0.05 M), and sodium citrate (1 mM) was added 0.2 μ mole of $[^7\text{Be}]\text{BeSO}_4$. This mixture either alone or after the addition of rat liver nuclei, mitochondrial fraction, DNA or RNA (prepared from rat liver by method of Kirby, 1964) was dialysed against 5 ml. of medium without beryllium for 16 hr. at 4°. DNA and RNA equivalent to 22 mg. of nuclear protein were added. The results are the means of two experiments except for the control dialysis against medium, where the result is the mean $\pm$ S.E.M. for six experiments.

Addition to medium inside dialysis sac	Concn. of beryllium outside dialysis sac (% of equilibrium concn.)
Nuclei (28.6 mg. of protein)	18
Nuclei (22.0 mg. of protein)	32
Nuclei (9.4 mg. of protein)	47
Mitochondrial fraction (16 mg. of protein)	72
DNA (7.25 mg. dry wt.)	70
RNA (1.15 mg. dry wt.)	66
No addition	72 $\pm$ 3.1 (6)

low concentrations of beryllium (2.2–11.0 μM) are probably due to the diffusion of a small amount of a beryllium-binding component from the nuclei into the supernatant. With similar experiments with mitochondria a line was obtained horizontal to the axis for the bound form and crossing the axis for the bound form/free form ratio at 15. Such a result indicates a constant distribution coefficient rather than a saturatable class of binding site.

Fractionation of nuclei. Some information was sought about the intranuclear distribution of beryllium. When nuclei isolated from rats given 83 μ moles of beryllium sulphate/kg. 24 hr. previously were fractionated by the procedure of Rendi (1960)

the residual protein fraction had a high specific activity with respect to beryllium but contained virtually no DNA (Table 7). Beryllium sulphate was added *in vitro* to rat liver nuclei and mitochondria and to DNA and RNA prepared from rat liver by the method of Kirby (1964). The suspensions were dialysed against the same volume of suspending medium consisting of sucrose (0.3 M), tris-hydrochloric acid buffer (0.05 M) and citrate (1 mM). In this medium alone beryllium was only 72% freely diffusible on dialysis within 16 hr. Whole nuclei prevented the diffusion of some of the beryllium and the extent of this effect was dependent on the amount of nuclei present (Table 8). With an amount of DNA equivalent to nuclei containing 22 mg. of protein beryllium diffused to the same extent as in the control medium. Similarly RNA had no effect. Finally, in agreement with the previous experiment, mitochondria in the presence of 1 mM-citrate had no affinity for beryllium.

DISCUSSION

Evidence has been presented that *in vivo* beryllium concentrates in two cell organelles, the lysosomes and the nucleus. The work of Cheng (1956) and Reeves & Vorwald (1961) suggested that soluble beryllium salts once in the blood form and are transported as colloidal particles of beryllium phosphate and hydroxide, possibly loosely associated with some serum globulins (Vacher & Stoner, 1968). Colloidal metal salts have, by histology or electron microscopy or both, been shown to be present in lysosomes [colloidal iron (Kent, Minick, Volini, Orfei & de la Huerga, 1963; Theron, Hawtrey, Liebenberg & Schirren, 1963); colloidal mercuric sulphide (Oudea, 1963; Hampton, 1958); thorium oxide (Hampton, 1958); copper (Goldfisher, 1965); colloidal gold (Shnitka, 1965); tellurium (Blinzinger & Hager, 1965)]. In one instance biochemical techniques have been used (colloidal

Table 9. *Distribution of beryllium, acid phosphatase and protein in subfractions of a mitochondrial fraction from rat liver*

The results in this Table are from experiments described in Table 6, but expressed as means $\pm$ S.E.M. of the percentage distribution of the total recovery in all eight fractions. The total recoveries 5 and 24 hr. after dosing with 7 μ moles of [^7Be]BeSO₄/kg. body wt. were for acid phosphatase 4.6 ± 0.5 and 4.2 ± 0.2 m-moles of P_i/min., for beryllium 40.4 ± 2.3 and 40.6 ± 4.8 m μ moles of beryllium, and for protein 72.3 ± 4.8 and 73.9 ± 4.3 mg. of protein. The total amount of beryllium in the livers from which these fractions were derived 5 and 24 hr. after dosing were 407 ± 25 and 411 ± 21 m μ moles respectively.

Fraction no.	Acid phosphatase			Beryllium			Protein		
	Distribution (% of total recovery)			Distribution (% of total recovery)			Distribution (% of total recovery)		
	5 hr.	24 hr.	P	5 hr.	24 hr.	P	5 hr.	24 hr.	P
1	11.0 $\pm$ 1.4	12.6 $\pm$ 0.5	0.35	9.8 $\pm$ 0.5	6.6 $\pm$ 0.5	< 0.02	20.0 $\pm$ 0.7	13.4 $\pm$ 1.3	< 0.05
2	17.7 $\pm$ 1.7	17.0 $\pm$ 1.1	0.75	14.5 $\pm$ 0.7	6.8 $\pm$ 0.5	< 0.01	12.3 $\pm$ 1.5	9.8 $\pm$ 1.1	0.35
3	19.2 $\pm$ 1.1	20.6 $\pm$ 0.5	0.35	18.5 $\pm$ 1.5	10.2 $\pm$ 1.2	< 0.02	6.3 $\pm$ 0.5	6.6 $\pm$ 0.3	0.65
4	17.5 $\pm$ 1.9	16.2 $\pm$ 0.6	0.55	18.7 $\pm$ 0.7	15.8 $\pm$ 1.4	0.15	5.0 $\pm$ 0.4	5.2 $\pm$ 0.4	0.75
5	13.2 $\pm$ 1.1	12.4 $\pm$ 0.3	0.55	15.0 $\pm$ 0.4	19.4 $\pm$ 0.8	< 0.02	7.5 $\pm$ 0.3	7.2 $\pm$ 0.7	0.75
6	8.3 $\pm$ 1.3	8.4 $\pm$ 0.8	0.95	7.5 $\pm$ 1.7	15.4 $\pm$ 1.1	< 0.05	9.5 $\pm$ 1.3	12.0 $\pm$ 1.3	0.35
7	5.0 $\pm$ 1.4	5.0 $\pm$ 0.5	—	5.5 $\pm$ 1.2	9.0 $\pm$ 1.1	0.15	11.0 $\pm$ 0.7	14.0 $\pm$ 2.0	0.35
8	7.8 $\pm$ 0.7	8.0 $\pm$ 1.0	0.85	10.2 $\pm$ 1.0	16.8 $\pm$ 1.5	< 0.05	27.8 $\pm$ 2.6	32.2 $\pm$ 1.7	0.35

plutonium; Rahman & Lindenbaum, 1964). All these observations indicate that the uptake of beryllium into lysosomes in liver is not specific but is common to many colloidal metal salts. From the point of view of cell death it is important whether beryllium damages the lysosomal membrane. There is evidence that the cytotoxicity of silica for macrophages depends on the release of enzymes, presumably from the damaged lysosomes (Allison, Harington & Birbeck, 1966; Allison, Magnus & Young, 1966), but this is not generally true for hepatotoxins (de Reuck & Cameron, 1963). Though liver lysosomal enzymes are released *in vivo* by beryllium (Witschi & Aldridge, 1967) this is a late phenomenon and we do not know if the toxic action of beryllium on liver cells is due to damage to lysosomes. Beryllium also binds to the nuclei of the liver cell, but there is no reliable reason to link this with liver necrosis. There are, however, a growing number of hepatotoxins that affect nuclear function and are located within the nucleus or react with components of it [dimethylnitrosamine (Magee, 1966); aflatoxin (Clifford & Rees, 1967); α -amanitin (Stirpe & Fiume, 1967); beryllium (this paper)].

In the mitochondrial fractions from the livers from rats treated with a low dose (7 μ moles/kg.) of beryllium sulphate the distributions of acid phosphatase and beryllium were not the same 5 and 24 hr. after dosing (Table 6). In these experiments identical proportions of acid phosphatase, beryllium and protein were separated into the eight fractions (Table 9). The distribution of acid phosphatase and

protein among the fractions was identical 5 and 24 hr. after dosing (except for protein in fraction 1; cf. Table 9). However, at 5 hr. most of the beryllium (62%) was found in fractions 1–4, whereas at 24 hr. it was in fractions 5–8 (61%). This beryllium apparently moved from the less to the more dense fractions without any accompanying changes in protein or acid phosphatase. No explanation for these changes can be made that does not involve many as yet untestable assumptions. Perhaps the simplest is that at 5 hr. lysosomes either containing or without beryllium have the same density in sucrose. At 24 hr. changes have occurred in those containing beryllium such that they become more dense in sucrose, and also have lost acid phosphatase that is exactly balanced by the synthesis of new enzymes in new lysosomes.

After the injection of 83 μ moles of beryllium sulphate/kg. beryllium is present in lysosomes but the beryllium/acid phosphatase ratio varies at 5 and 24 hr. (Fig. 2). With this dose the lysosomes become damaged within 24 hr. and the concentration of beryllium in the liver doubles between 6 and 24 hr. (Table 1). Unlike the previous experiments with a low dose of beryllium no steady state in the concentration of beryllium or acid phosphatase exists, and in addition the livers so exposed will be necrotic within 24–48 hr. The interpretation of the findings is thus impossible at the present time.

Isolated rat liver nuclei have a high affinity for beryllium *in vitro*. Beryllium accumulated in the nuclei *in vivo* as the injected dose approached the LD₅₀. Others have provided histochemical evidence

for the presence of beryllium in nuclei of various cells [fibroblasts (Firket, 1953); phagocytic cells in subcutaneous beryllium granulomata (Gusek & Mestwerdt, 1963); nuclei from lung (Reeves, 1959); nuclei from normal and regenerating rat liver (Truhaut, Boudène & LeTalaer, 1965)]. Effects have also been found on mitosis and enzymes in nuclei of various cells (Chevremont & Firket, 1951a,b; Jacobson & Webb, 1952; Hirokawa, 1953; Stirpe & Aldridge, 1961; Monoyer, 1963; Bassleer, 1965).

The nature of the component in nuclei that binds beryllium is clearly of interest. Festy, LePecq, Paoletti & Truhaut (1965) have shown that concentrations of 0.1–1 mM-beryllium sulphate have effects on the physical properties of DNA, but some of the effects were shown to be prevented or reversed by citrate. Such a binding would not be strong enough to account for our results. Other evidence is provided against binding to DNA. Though we do not know to what beryllium binds in nuclei, treatment of various cells with beryllium does impair nuclear activity. Mitosis is prevented and in addition Bassleer (1965), using histochemical techniques, claimed an inhibition of DNA synthesis and of nucleolar protein synthesis but with no effects on RNA metabolism. In regenerating rat liver, injection of soluble beryllium salts inhibits the incorporation of thymidine and orotic acid into DNA without preventing the incorporation of orotic acid into RNA or of leucine into protein (H. P. Witschi & H. Petering, unpublished work). This rather specific action of beryllium on DNA metabolism, its general toxicity to cells and the growing evidence that under certain circumstances it is a carcinogen (Barnes, Denz & Sissons, 1950; Reeves, Deitch & Vorwald, 1967) make the nature of the binding site for beryllium in the nucleus of great interest.

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