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COVALENT LINKAGE OF CARBOXYPEPTIDASE G_2 TO SOLUBLE DEXTRANS—II

IN VIVO DISTRIBUTION AND FATE OF CONJUGATES

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Abstract—The *in vivo* fate of the therapeutic enzyme, carboxypeptidase G_2 (CPG_2) in native form and covalently-linked to soluble dextrans was studied in the mouse using radiolabelled compounds. Clearance, from the blood, of all compounds tested was found to be as intact, active material, whilst excreted radiolabel was associated in all cases with low molecular weight substances. The clearance and excretion rates of native CPG_2 were found to balance, but this was not so for dextran–CPG_2 conjugate or CNBr-activated dextran. Tissue distribution studies demonstrated that there was little or no tissue uptake of native CPG_2, whereas dextran–CPG_2 conjugate, and CNBr-activated dextran were retained in the liver. Within the liver, the CPG_2 component of dextran–CPG_2 conjugate was degraded more rapidly than the dextran moiety. Blockade of reticulo-endothelial system (RES) led to increased half-lives of dextran CPG_2 conjugate and CNBr-activated dextran, demonstrating the involvement of the RES in the clearance of these compounds. Impairment of RES activity did not affect the clearance rate of native CPG_2.

These results are discussed in relation to the potential use of dextran–CPG_2 conjugates in cancer chemotherapy.

A number of workers have demonstrated that the covalent attachment of proteins to soluble dextrans can greatly increase the half life of the protein in blood circulation [1–3] and that the increase can be controlled by variation of the molecular weight of the dextran carrier employed [4]. It has also been demonstrated that the extracellular survival of various native and modified glycoproteins is determined by the nature of the exposed sugar residues on the carbohydrate side chains, and their ability to bind with specific receptors (e.g. for galactose) in the liver [5]. Non-glycosylated proteins are not susceptible to such clearance mechanisms, and alternative pathways must exist.

Ho *et al.* [6] studied the clearance of intravenously injected asparaginase, purified from *Escherichia coli*, from the circulation of the dog, and found that the enzyme did not readily pass from the vascular system to the tissue spaces, although after 2–3 hr appreciable quantities of enzyme were found in the lymphatic system, with the highest concentration in the thoracic lymph duct. Blockade of the reticulo-endothelial system (RES) by the administration of zymosan did not affect the rate of elimination of asparaginase in either the dog or the guinea pig. The RES has, however, been implicated as being involved in the clearance of aspartate and alanine transaminases [7].

In the absence of disease of the urinary tract, injected enzyme proteins appear in the urine only in trace amounts, apart from those which have low molecular weights and are readily filterable by the glomerulus [8]. Strandjord *et al.* [9] showed that an excretory mechanism was not an important factor in the clearance of isocitrate dehydrogenase by showing that the rate of clearance of the enzyme, injected into the dog, was unaffected by hepatectomy, nephrectomy, or splenectomy.

The clearance of dextran may occur in two distinct ways. Dextrans with molecular weights lower than about 15 kD pass freely through the glomerular filter; larger molecules are more restricted in their passage, and dextrans with a molecular weight greater than 50 kD are rarely excreted intact in humans [10]. In these cases, dextran uptake by the liver is observed, with a continuous and rapid elimination from the parenchymal cells taking place [11]. Dextran is also susceptible to uptake by the RES, and has been used experimentally as an RES-blockading agent [12]. Further evidence of this property has been found by Lahnborg *et al.* [13], who reported considerable impairment of the phagocytic activity of the RES of patients given dextran as a plasma volume expander.

The processes by which dextran–enzyme conjugates are cleared from the circulation have not been ascertained to date. Blomhoff *et al.* [14], working with bovine testis β-galactosidase conjugated to dextran T10 found that uptake of both native and conjugated β-galactosidase by isolated hepatocytes and non-parenchymal liver cells occurred at similar rates. However, there was a marked increase in stability to degradation by both cell types when the enzyme was conjugated with dextran. *In vivo* experiments were also performed, where the enzyme was injected intravenously into the rat, and similar stab-

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ility properties were observed. The clearance rate of the conjugate was slightly slower than that of the native enzyme, but not markedly so. This latter observation is similar to that of Wileman [15], who found that conjugation of dextran T10 to asparaginase did not significantly alter the clearance rate of the enzyme.

In this paper we report the mechanism by which conjugates of soluble dextran with the therapeutic enzyme carboxypeptidase G_2 (CPG_2) [16] are cleared *in vivo*.

MATERIALS AND METHODS

Enzyme assay and glycosylation of CPG_2. These were carried out as previously described [4].

Preparation of ^{125}I–CPG_2. ^{125}I–CPG_2 was prepared by the Chloramine T method [17]. To 100 μl of enzyme solution (126.5 units; 0.23 mg) in 0.1 M Tris–HCl buffer, pH 7.3, was added 10 μl of Na ^{125}I (1 mCi, Amersham International, Amersham, Bucks, U.K.) and 20 μl of Chloramine T (0.01 M in 0.9% NaCl) (Sigma Chemical Co., Poole, U.K.). After 3 min incubation at 24° 20 μl of 0.01 M sodium metabisulphate and 40 μl of 1% (w/v) potassium iodide were added consecutively. The mixture was filtered through a disposable column of Sephadex G25 (PD10 column, Pharmacia, Uppsala, Sweden) previously equilibrated with phosphate-buffered saline (PBS = g/l: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.13; KH_2PO_4, 0.2, pH 7.4). The enzyme was eluted with the same buffer and collected in five fractions (0.5 ml each), which were pooled and stored at −20°. The specific activity of the iodinated enzyme was 550 U/mg, 926.5 μCi/mg on the day of preparation. This stock solution was diluted with unlabelled enzyme in 0.1 M Tris–HCl pH 7.3 containing 0.2 mM $ZnSO_4$ to give an activity of 10 μCi/ml at an enzyme activity level of 25 U/ml.

Preparation of ^{14}C–dextran 70–^{125}I–CPG_2. Double-labelled conjugate was prepared using "Lomodex 70" (Fisons, Loughborough, U.K.) to which had been added ^{14}C-carboxyl-dextran 70 (250 μCi, 208.5 mg, New England Nuclear, Dreieich, F.R.G.). The dextran was activated as described previously [4] and allowed to react with a mixture of unlabelled enzyme (1265 U, 2.3 mg in 1 ml) plus 125 μCi (75 U, 0.14 mg in 0.13 ml) of ^{125}I-labelled enzyme. Conjugate was separated as previously described. Following gel filtration, samples were withdrawn from each column fraction and ^{14}C activity counted on the basis of β-emission using "Aqualuma" scintillant (LKB, Bromma, Sweden) in an LKB Model 1215 "Rackbeta" liquid scintillation counter, in which the count windows had been previously adjusted to minimise the overlap of the ^{125}I and ^{14}C count spectra; ^{125}I activity was determined by counting samples in an LKB "Rackgamma" counter.

Enzyme persistence in vivo. Groups of 5 female Balb/c mice, 15–20 g in weight (Olac, Bicester, Oxon, U.K.) were used for persistence trials. Mice were injected intravenously via the tail vein with 1 ml of ^{125}I–CPG_2 (25 U 0.05 mg protein, 10 μCi); ^{14}C–dextran 70 (11.6 mg, 0.74 μCi) or ^{14}C–dextran 70–^{125}I–CPG_2 (25 U, 0.13 mg protein, 0.65 μCi-^{125}I; 13.3 mg carbohydrate, 0.93 μCi-^{14}C). The animals were allowed food and water (containing 1 g potassium iodide per litre as thyroid blockading agent, from 5 days prior to commencement of the experiment) *ad libitum*. Blood samples (10 μl) were taken by nicking the tail at intervals over a 72 hr period, diluted to 50 μl with 0.9% NaCl and assayed for radioactivity and enzyme activity as previously described.

Fractionation of serum samples by gel filtration. A group of twelve female Balb/c mice weighing 17–20 g each received 1 ml of ^{14}C–dextran 70–^{125}I–CPG_2 conjugate as described above. The animals were allowed food and water (containing 1 g potassium iodide per litre) *ad libitum*. Blood samples were collected from groups of 3 mice by cardiac puncture at intervals over 48 hr. The samples were pooled and delivered into tubes containing potassium–EDTA as anticoagulant. The red cells were separated by centrifugation and a 0.75 ml aliquot of the supernatant chromatographed on a column (1.6 × 90 cm) Ultrogel AcA34 (LKB, Bromma, Sweden). The sample was applied directly to the surface of the gel, following removal of the flow adaptor. The column was eluted with PBS at a flow rate of 8 ml/hr. Fractions (1 ml) were collected and 0.9 ml aliquots were withdrawn from alternate tubes for the determination of radioactivity levels.

Measurement of excretion of CPG_2, dextran 70 and dextran 70–CPG_2. Groups of 6 ex-breeder female Porton mice, weighing approximately 30 g were injected intravenously with ^{125}I–CPG_2, ^{14}C–dextran 70 or ^{14}C–dextran 70–^{125}I–CPG_2 labelled as described earlier, in 300 μl of PBS, and were placed in a metabolism cage (Techniplast, Buguggiate, Italy) for the separation of urine and faeces samples over a 48 hr period. The animals were allowed water (containing 1 g potassium iodide per litre) *ad libitum*. Food was withheld as spillage within the cage was found to absorb significant amounts of urine.

The volumes of the urine samples were measured and aliquots were counted to measure radioactivity levels. One-millilitre samples of urine were chromatographed on a column (1.6 × 90 cm) of Ultrogel AcA 34. The sample was loaded directly onto the surface of the gel, following removal of the flow adaptor. The column was eluted at a flow rate of 8 ml/hr and 1 ml fractions collected and assayed for radioactivity.

Faeces samples were weighed and extracted twice with an aliquot of an equal mass of distilled water. The extracts were pooled and solid material removed by centrifugation. Aliquots were removed for the determination of radioactivity levels, and samples chromatographed on Ultrogel AcA 34 as described above.

Measurement of levels of accumulation of CPG_2, dextran 70 and dextran 70–CPG_2 in the major organ tissues of Balb/c mice. Groups of 4 female Balb/c mice weighing 17–20 g were injected (1 ml) intravenously with ^{125}I–CPG_2, ^{14}C–dextran 70, or ^{14}C–dextran 70–^{125}I–CPG_2 as described earlier. The animals were allowed food and water (containing 1 g potassium iodide per litre) *ad libitum*. At intervals over a 48 hr period the animals were sacrificed by cervical dislocation for the collection of tissue

samples. Surface blood was removed from the samples by careful blotting. The weighed samples were made up to a total volume of 4.0 ml by the addition of "Optisolve" tissue solubiliser (LKB, Bromma, Sweden) and complete dissolution achieved by incubation overnight at 37°. All samples containing ^{14}C were bleached by the addition of 2.0 ml isopropanol and 0.5 ml of 35% hydrogen peroxide. The vials were allowed to stand overnight prior to the addition of 15 ml of "Lipoluma" scintillant (LKB, Bromma, Sweden) after which the samples were counted in an LKB model 1215 "Rackbeta" scintillation counter previously calibrated to determine the overlap of counts due to ^{125}I into the ^{14}C channel. Quench corrections were determined for individual vials by recounting the vials following addition of an internal standard solution (50 μl of U-^{14}C-glycine 2.0 μCi/ml (Amersham International, Amersham, U.K.)). The results were expressed as the number of counts per gram of tissue as a percentage of the injected dose.

Effect of RES Blockade on the half-lives of CPG_2, dextran 70 and dextran 70–CPG_2. Groups of 4 female Balb/c mice, weighing 17–20 g were each given daily intraperitoneal injections of colloidal carbon (3.2 mg/mouse; 170 mg/kg) for 10 days. The carbon used was Pellikan Drawing Ink, Batch C11/1431a (Gunther Wagner, Hanover, F.R.G.) [18], which was diluted twofold with PBS and autoclaved. Further control groups of four mice were given daily intraperitoneal injections of PBS. At the conclusion of the 10-day period groups of RES-blockaded mice were given intravenous injections of CPG_2 (25 U, 0.4 ml, 0.05 mg protein), ^{14}C–dextran 70 (5 mg, 0.4 ml, 0.84 μCi) or ^{14}C–dextran 70–CPG_2 (25 U, 0.63 μCi, 0.4 ml, 0.11 mg protein, 15.9 mg dextran). Control groups of four non-RES-blockaded mice were similarly treated. Blood samples (10 μl) were withdrawn at intervals for the determination of enzymic activity or ^{14}C activity, as described previously. The half-lives of the injected materials were calculated by unweighted regression analysis. Further RES-blockaded mice were sacrificed in pairs over the course of the experiment and the spleens and livers dissected out for histological examination to assess the effectiveness of the RES blockade. Portions of the liver and the entire spleen were fixed in 10% phosphate-buffered neutral formalin. After embedding in paraffin wax sections cut at 5 μm were stained with haemotoxylin and eosin.

RESULTS AND DISCUSSION

Relative rates of clearance of ^{125}I-activity, ^{14}C-activity and enzymic activity

Radiolabelled native enzyme (^{125}I–CPG_2) and dextran–enzyme conjugate (^{14}C–dextran 70–^{125}I–CPG_2) were used to assess whether breakdown of these molecules occurred prior to removal from the plasma. Following intravenous injection into mice, the rate of removal of the radioactive labels on both the dextran and enzyme were followed, together with the clearance of enzyme activity. Semi-logarithmic plots of activity versus time were prepared, and the half-lives ($t_{\frac{1}{2}}$) of the components calculated by unweighted regression analysis (Figs 1a and 1b).

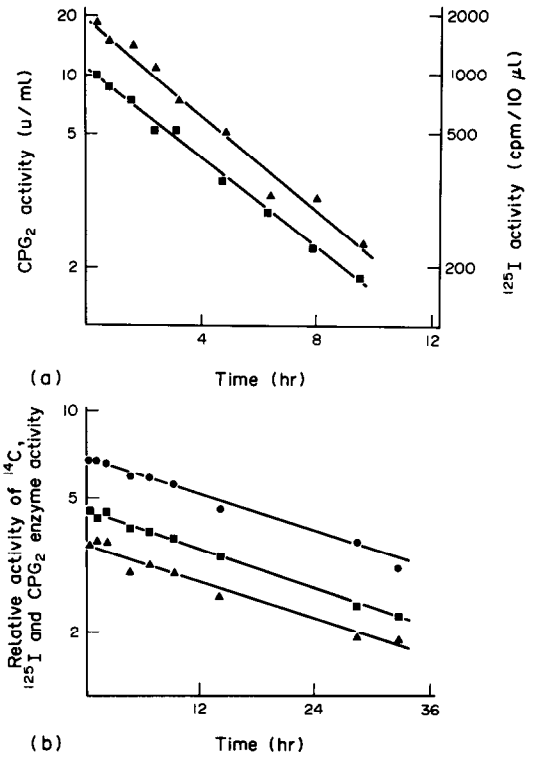

Fig. 1. The clearance rates of ^{125}I–CPG_2 (a) and ^{14}C–dextran 70–^{125}I–CPG_2 (b) from the circulation of Balb/c mice were determined by the assay of 10 μl blood samples collected from the tail vein: ▲, enzyme activity; ■, ^{125}I activity; ●, ^{14}C activity. Data points shown are means from groups of four animals.

Enzyme activity and radioactivity were cleared at equal rates in the case of native CPG_2 (Fig. 1a) and dextran 70–CPG_2 conjugate (Fig. 1b), demonstrating that native enzyme and conjugate were not broken down or catalytically inactivated prior to removal from the bloodstream. If this had been the case a more rapid disappearance of enzyme activity or ^{125}I label compared with the ^{14}C dextran label would have been expected as the free enzyme is cleared far more rapidly ($t_{\frac{1}{2}}$ = 3.1 hr) than the dextran ($t_{\frac{1}{2}}$ = 16.3 hr) [4]. The simultaneous removal of enzyme and dextran moieties has also been reported for dextran–asparaginase conjugates [2].

Fractionation of serum samples by gel filtration

Mice injected with double-labelled conjugate were sacrificed at various intervals of time up to 48 hr post-injection. Blood samples collected by cardiac puncture were chromatographed on Ultrogel AcA 34 and the elution profiles are shown in Fig. 2. The results from this experiment confirmed that the conjugate remained intact prior to removal from the bloodstream. The conjugates prepared by the CNBr-activation technique are heterogeneous in their molecular weight distribution, as are the dextrans they are prepared from. It has been previously demonstrated that the half lives of dextran–CPG_2 conjugates increased as the molecular weight of the dextran carrier increased [4] hence a gradual shift in

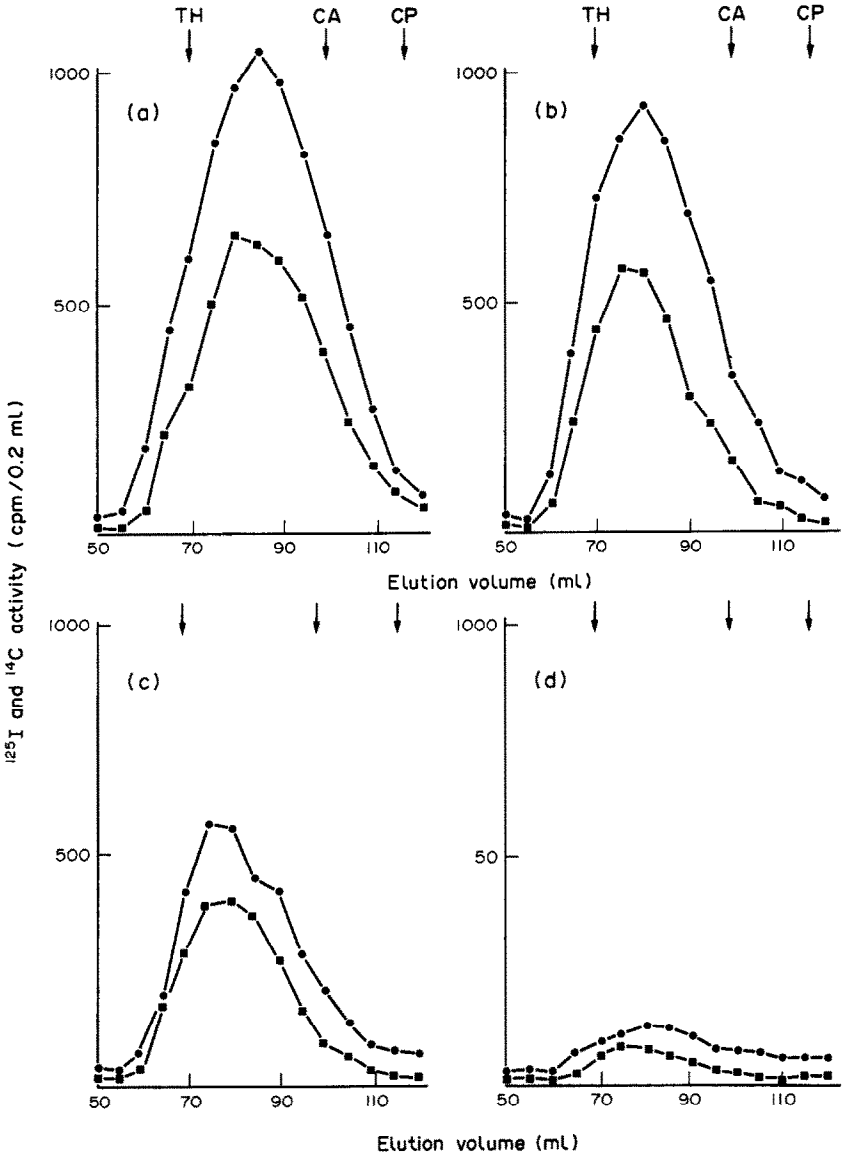

Fig. 2. Blood samples from mice injected with ^{14}C–dextran 70–^{125}I–CPG_2 were obtained 6 hr (a), 12 hr (b), 24 hr (c) and 48 hr (d) post injection, centrifuged to remove red cells and 1.0 ml serum samples chromatographed on a column (1.6 × 90 cm) of Ultrogel AcA34. 1 ml fractions were collected and assayed for ^{14}C and ^{125}I activity. Elution was with phosphate-buffered saline at a flow rate of 12 ml/hr: ●, ^{14}C activity; ■, ^{125}I activity. TH = thyroglobulin, CA = catalase, CP = carboxypeptidase G_2.

the molecular weight distribution towards the higher molecular weight conjugates, might be expected as the lower molecular weight species are preferentially cleared. This was observed as a slight shift in the molecular weight distribution of the conjugate peak seen in the elution profiles.

Excretion of CPG_2, dextran 70 and dextran 70–CPG_2

The rates of excretion of radiolabelled CPG_2, CNBr-activated dextran 70 and dextran 70–CPG_2 from Porton mice were determined from urine and faeces samples collected over a 48 hr period using a metabolism cage. The results of this experiment are presented in Table 1, and show that there were marked differences in the rates and route by which the samples were excreted. CPG_2 was rapidly excreted, with greater than 50% of the radiolabel appearing in the urine within 6 hr of injection, and almost 90% within 24 hr, which corresponded closely to the value predicted from plasma half-life data [4]. Very little appeared to be excreted in the faeces, although excretion by this route increased in the 24–48 hr period.

In contrast, both the CNBr-activated dextran 70 and dextran–CPG_2 samples showed rather lower than predicted levels of excretion over the 48 hr duration of the experiment with only 50–70% of the injected dose being recovered. The remainder was retained predominantly in the liver and spleen (see later). CNBr-activated dextran 70 was found almost

Table 1. The excretion of ^{125}I–CPG_2, ^{14}C–dextran 70–^{125}I–CPG_2 and ^{14}C–dextran 70 from groups of six Porton mice was measured from urine and faeces samples collected in a metabolism cage

	% Injected dose excreted							
	^{125}I–CPG_2		Dextran 70–^{125}I–CPG_2		^{14}C dextran 70–CPG_2		^{14}C–dextran 70	
Time	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
0–6 hr	51.6	0.2	7.3	0	13.1	0.4	39.6	0.1
6–12 hr	21.4	0.5	16.3	0	4.5	0.6	9.3	0
12–24 hr	12.0	0.7	43.2	0	3.0	12.5	2.0	0
24–36 hr	3.8	3.6	3.9	0	1.6	15.4	0.9	0.2
36–48 hr	1.8	2.4	5.3	0	1.6	11.1	0.5	0.3
Total	98.0%		73.0%		63.8%		52.9%	
Total predicted from $t_{\frac{1}{2}}$ value (ref. 4)	100%		98%		98%		94.5%	

entirely in the urine. The bulk of the activity excreted appeared within 12 hr of injection but represented less than 50% of the injected dose. Radiolabel from the dual-labelled dextran 70–CPG_2 conjugate showed a different clearance pattern for each label. The ^{125}I–CPG_2 label appeared only in the urine and reached maximum levels between 12 and 24 hr after injection, whereas only small amounts of label from the dextran component appeared in the urine over the same period. The majority of the excreted ^{14}C–dextran label appeared in the faeces between 12–48 hr after injection. The presence of material in the faeces suggested that it was excreted via the biliary route, as might be expected in view of the uptake by the hepatic cells (see later). It is not clear, however, why CNBr-activated dextran was not similarly excreted or indeed why ^{125}I-label from the CPG_2 component of dextran 70–CPG_2 conjugate was not found in faeces samples. No enzyme activity was detected in urine or faeces samples. CPG_2 would not be active under these conditions, even if excreted intact.

Samples of urine were chromatographed on a previously calibrated column of Sephacryl S300 and the elution profiles are presented in Fig. 3 (a–c). With the exception of native CPG_2 the radioactivity excreted was associated exclusively with very low molecular weight material, none being eluted at the positions corresponding to intact dextran or dextran–CPG_2 conjugate. Native CPG_2 showed a largely similar elution profile, however a small peak was observed at the point corresponding to intact CPG_2 (83,000 daltons) and a slightly larger peak corresponding to material of similar molecular weight to the CPG_2 sub-unit (41,000 daltons). These peaks accounted for less than 2% of the radiolabelled material loaded onto the column. Similar results were recorded for aqueous extracts of faeces where radioactivity was present (data not shown).

These results demonstrated that both native CPG_2 and dextran–CPG_2 conjugates were not able to pass through the glomerular filter to a significant extent. The increase in half-lives of dextran–CPG_2 conjugate is not, therefore, simply due to the presence of the dextran carrier placing an embargo on renal filtration of the native enzyme protein as has been suggested by other workers [19].

Accumulation of CPG_2, dextran 70, and dextran 70–CPG_2 in tissue

The accumulation of radiolabelled CPG_2, CNBr-activated dextran 70 and dextran 70–CPG_2 conjugate was studied following intravenous injection. The results of this experiment are presented in Table 2 (a–d). Native CPG_2 (Table 2a) did not show significant persistence in any tissue and after 6 hr there was very little of the injected dose remaining in the body. There was little or no evidence of uptake by the macrophage-containing organs, the liver, spleen and lungs. The RES does not appear to be involved in the clearance of native CPG_2, which is in line with similar results found by Ho *et al.* [6] using asparaginase.

In contrast there was pronounced uptake of CNBr-activated dextran 70 by the liver (Table 2b) which reached peak values about 24 hr after injection and suggested that some of this material was either "stored" or was resistant to degradation. Very similar results have been reported in experiments where the distribution of dextran in mice was studied by histological techniques [20]. In such experiments the dextran was found to be present in both parenchymal and Kupffer cells although the kinetics of uptake appeared to be different, the parenchymal cells containing dextran shortly after injection, but the quantities decreasing by 24 hr. In contrast the Kupffer cells accumulated dextran from about 6 hr after injection with the quantities rising to a maximum at about 24 hr. Dextran has also been demonstrated [21, 22] to be resistant to lysosomal digestion in kidney tubules.

Uptake by the lungs occurred initially but declined steadily, whereas low initial uptake by the spleen increased, reaching maximum levels in the 24–48 hr period. The remaining tissues showed broadly similar values.

The ^{125}I–CPG_2 component of dual-labelled dextran 70–CPG_2 conjugate (Table 2c) was found to exhibit a modified distribution pattern compared to that of free CPG_2, but it was not similar to that of

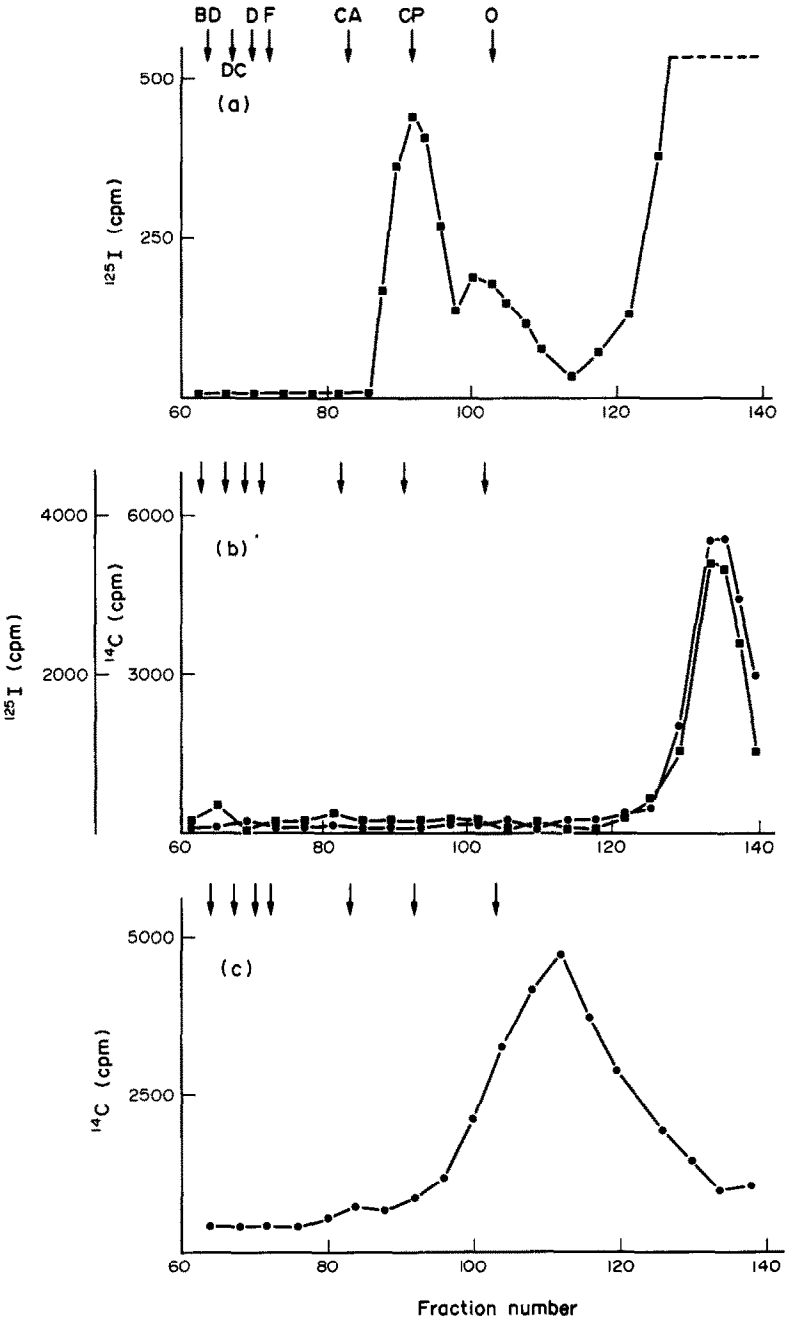

Fig. 3. Elution profiles of urine samples from mice injected with ^{125}I–CPG$_2$ (a) ^{14}C–dextran 70–^{125}I–CPG$_2$ (b), or ^{14}C–dextran 70, (c) chromatographed on a column (1.6 × 90 cm) of Sephacryl S300. 1.24 ml fractions were collected and assayed for ^{14}C and ^{125}I activity. Elution was with phosphate buffered saline at a flow rate of 12 ml/hr: ●, ^{14}C activity; ■, ^{125} activity. BD = blue dextran, DC = dextran 70–CPG$_2$, D = CNBr-activated dextran 70, F = ferritin, CA = catalase, CP = carboxypeptidase G$_2$, OA = ovalbumin.

CNBr-activated dextran 70. Initial uptake by the liver and spleen was not prolonged but rather the levels of activity found in these organs declined. No other tissues showed marked uptake.

The ^{14}C–dextran component of dual-labelled dextran 70–CPG$_2$ conjugate (Table 2d) showed a distribution pattern which was similar to that of CNBr-activated dextran 70. Liver uptake increased with time reaching a peak in the 12–24 hr period. There was little accumulation in the lungs, in marked contrast to CNBr-activated dextran 70.

These results supported the excretion results shown in Table 1, and confirmed that the two components of conjugate were degraded at different rates. The protein moiety of the conjugate is removed from the dextran component in the liver and excreted, leaving the more stable dextran to be degraded and released at a slower rate. Previous

Table 2. The tissue distribution of ^{125}I–CPG$_2$, ^{14}C–dextran 70–^{125}I–CPG$_2$ and ^{14}C–dextran 70 was determined in Balb/c mice

(a) Tissue uptake of ^{125}I–CPG$_2$ in Balb/c mice

	^{125}I cpm/g as % injected dose			
Time:	6 hr	12 hr	24 hr	36 hr
Heart	0.9 ± 0.2	0.3 ± 0.1	0.04 ± 0.01	0.02 ± 0.01
Lung	1.9 ± 0.4	0.6 ± 0.4	0.08 ± 0.01	0.05 ± 0.01
Liver	0.9 ± 0.1	0.3 ± 0.1	0.06 ± 0.01	0.03 ± 0.01
Spleen	1.1 ± 0.4	0.3 ± 0.2	0.07 ± 0.01	0.07 ± 0.05
Kidney	2.5 ± 1.0	0.7 ± 0.3	0.12 ± 0.02	0.10 ± 0.05
Muscle	0.6 ± 0.2	0.2 ± 0.2	0.02 ± 0.01	0.02 ± 0.01
Gut	1.0 ± 0.3	0.4 ± 0.3	0.04 ± 0.01	0.03 ± 0.01

(b) Tissue uptake of CNBr-activated ^{14}C–dextran 70 in Balb/c mice

	^{14}C cpm/g as % injected dose				
Time:	6 hr	12 hr	24 hr	36 hr	48 hr
Heart	12.4 ± 3.7	8.0 ± 1.2	6.8 ± 0.9	6.2 ± 1.4	6.7 ± 1.1
Lung	15.0 ± 1.3	12.8 ± 3.9	10.4 ± 1.5	8.6 ± 2.6	6.2 ± 2.0
Liver	19.1 ± 3.0	30.4 ± 2.7	35.8 ± 5.2	28.9 ± 7.0	21.5 ± 5.7
Spleen	5.1 ± 0.9	7.3 ± 1.8	11.5 ± 2.8	12.9 ± 3.3	12.0 ± 4.0
Kidney	6.0 ± 1.0	6.4 ± 2.2	4.0 ± 1.2	3.3 ± 1.6	2.9 ± 0.8
Muscle	1.7 ± 1.2	1.5 ± 0.5	2.0 ± 0.3	3.2 ± 1.6	2.1 ± 0.4
Gut	4.5 ± 0.8	4.6 ± 1.7	5.5 ± 1.3	7.1 ± 2.3	5.0 ± 0.8

(c) Tissue uptake of dextran 70–^{125}I–CPG$_2$ conjugate in Balb/c mice

	^{125}I cpm/g as % injected dose				
Time:	6 hr	12 hr	24 hr	36 hr	48 hr
Heart	4.5 ± 1.6	4.8 ± 2.7	0.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
Lung	7.5 ± 2.5	5.2 ± 1.6	1.1 ± 0.3	0.6 ± 0.1	0.5 ± 0.4
Liver	11.6 ± 4.9	11.7 ± 4.8	6.5 ± 2.8	3.1 ± 0.9	3.1 ± 0.4
Spleen	3.3 ± 1.1	3.3 ± 0.6	1.4 ± 0.3	1.4 ± 0.4	0.7 ± 0.2
Kidney	4.3 ± 1.5	4.5 ± 1.8	0.9 ± 0.3	0.6 ± 0.2	0.5 ± 0.5
Muscle	1.0 ± 0.1	1.2 ± 0.4	0.4 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
Gut	2.4 ± 0.3	3.1 ± 1.5	0.7 ± 0.2	0.4 ± 0.1	0.3 ± 0.1

(d) Tissue uptake of ^{14}C–dextran 70–CPG$_2$ conjugate in Balb/c mice

	^{14}C cpm/g as % injected dose				
Time:	6 hr	12 hr	24 hr	36 hr	48 hr
Heart	6.5 ± 3.0	5.1 ± 2.3	5.2 ± 1.8	5.5 ± 3.4	5.9 ± 2.1
Lung	3.0 ± 0.9	3.6 ± 1.3	7.0 ± 4.3	3.7 ± 1.6	4.3 ± 1.9
Liver	19.2 ± 2.7	26.9 ± 7.5	39.4 ± 7.4	29.1 ± 1.6	23.6 ± 2.7
Spleen	5.1 ± 0.6	4.3 ± 2.8	4.4 ± 1.4	8.0 ± 2.8	5.9 ± 1.0
Kidney	4.5 ± 1.5	3.3 ± 2.2	1.9 ± 0.6	4.1 ± 2.1	2.6 ± 1.7
Muscle	1.6 ± 0.4	2.5 ± 2.5	1.6 ± 0.4	1.8 ± 0.6	1.3 ± 0.2
Gut	4.2 ± 2.3	4.1 ± 2.2	4.5 ± 1.6	3.6 ± 1.1	3.8 ± 0.9

The animals were sacrificed at intervals after a 48 hr period and tissue samples collected for the determination of radioactivity levels as described in the text. Results were calculated as the mean cpm per gram tissue, as percentage of the total injected, with standard deviation for groups of four animals.

reports [4, 23] concerning the increased stability of proteins to proteolysis following coupling to soluble dextrans may be of limited consequence when such conjugates are faced with the highly degradative conditions imposed in lysosomes.

The site of degradation of native CPG$_2$ cannot be identified for certain from these experiments. Turnover of the enzyme was rapid with no marked accumulation of radiolabel in any specific tissue. In the absence of RES uptake (see later) it is most

likely that the parenchymal cells of the liver were responsible for the degradation of native CPG_2.

The effect of blockage of the RES on the clearance of CPG_2, dextran 70 and dextran 70–CPG_2 conjugate

The effect of impairing the phagocytic activity of the RES on the clearance of CPG_2, dextran and dextran CPG_2 conjugate was studied by administering colloidal carbon to mice. The dose of colloidal carbon administered was based on that described for NIH Albino A and Swiss Webster mice [18], but histological examination of the spleen and liver of similarly treated Balb/c mice sacrificed over the course of the experiment indicated that the degree of blockade achieved was probably not absolute.

In the liver, carbon particles were present in the cytoplasm of a number of Kupffer cells in all parts of the lobules and periportally at 0 and 24 hr. The number of positive cells had decreased in the liver of animals sacrificed at 48 hr.

Carbon-containing reticulo-endothelial cells in the spleen were found predominantly in the lymphoid tissue immediately surrounding the Malpighian corpuscles. By 48 hr the number of cells with carbon had decreased considerably.

The effect of blockading the RES on the clearances of CPG_2, dextran 70 and dextran 70–CPG_2 conjugate is illustrated in Fig. 4(a–c). The RES blockade had no discernible effect on the rate of clearance of native CPG_2, even in the presence of free CNBR-activated dextran 70, but the rates of clearance of both CNBr-activated dextran 70 and dextran 70–CPG_2 conjugate were halved, providing clear evidence of the involvement of the RES in the clearance mechanisms of these materials, and further reinforcing the results of the excretion and tissue distribution results described in this paper.

No clear picture emerges as to why the circulation half-lives of the dextran–CPG_2 conjugates increase as their molecular weights increase [4]. The results of Duncan *et al.* [24] suggest the opposite, in that the rate of uptake of macromolecules, which do not adsorb to cell membranes, by isolated macrophages increases with increasing molecular weight. Our results indicate that the key factor is the presence of dextran, which prevents parenchymal cell uptake and breakdown of the enzyme. In conjugated form the major effect is to divert the enzyme to the macrophages and uptake is further reduced as molecular weight of conjugate increases.

Our conclusion is that dextran conjugates may be of value for maintaining therapeutic enzymes and other agents in circulation for extended periods, thus prolonging their availability for cytotoxic action at the tumour site. They may also provide a method for targeting drugs to the liver, particularly the Kupffer cells. Although a number of other drug carriers, such as liposomes and synthetic microspheres, are currently used in experimental applications [26, 27], dextrans may prove to have some advantage. Their current application as plasma volume expanders make them clinically acceptable and their high coupling capacity may mean that relatively small amounts of dextran carrier would be required. This latter point may be important in light of one view that repeated administration of dextran might lead to lysosomal storage disorders. However, evidence available to date suggests that in kidney proximal tubule cells, the lysosomal pathway and protein

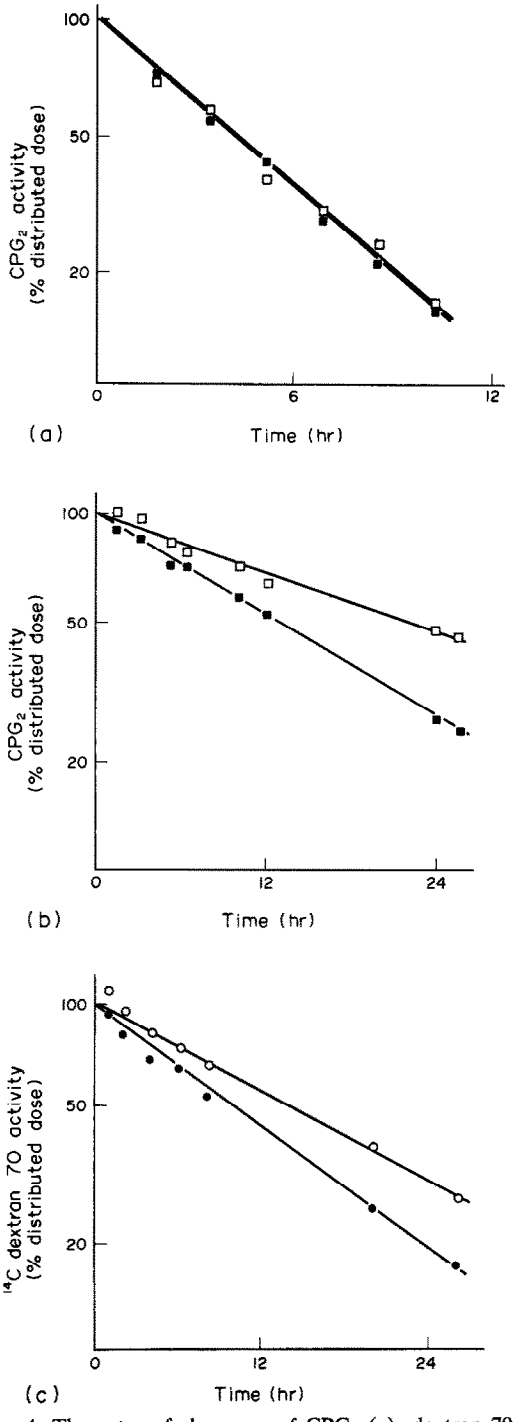

Fig. 4. The rates of clearance of CPG_2 (a), dextran 70–CPG_2 (b) and ^{14}C–dextran 70 (c) from the circulation of normal Balb/c mice (closed symbols) and RES-blockaded Balb/c mice (open symbols) were determined by the assay of 10 μl blood samples collected from the tail vein: ■□, CPG_2 activity; ●○, ^{14}C activity. Data points are means from groups of four animals.

absorption remain unaffected, despite ultrastructural changes [20, 21, 28].

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