Biochemical Pharmacology, Vol. 22, pp. 1989–2001. Pergamon Press, 1973. Printed in Great Britain.

EFFECTS OF BROMOBENZENE AND CARBON TETRACHLORIDE ON THE SYNTHESIS AND RELEASE OF PROTEINS BY PERFUSED RAT LIVER

DONALD C. DAVIS,* MASAHISA HASHIMOTO† and JAMES R. GILLETTE

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

(*Received* 13 *November* 1972; *accepted* 19 *January* 1973)

Abstract—The effects of bromobenzene and carbon tetrachloride (CCl_4) on the hepatic incorporation of ^{14}C-L-leucine into protein were compared to ascertain the relationship between liver necrosis and impairment of radiolabeled protein synthesis and release caused by these toxicants. Male rats were treated with bromobenzene or CCl_4 and 4 or 12 hr later the livers were removed and perfused *in vitro* in a recirculating perfusion apparatus. At 4 hr after its administration, CCl_4 only slightly impaired the rate of synthesis of radiolabeled proteins (liver + serum) but markedly decreased the release of radiolabeled proteins into the serum (perfusate). At 4 hr after the administration of bromobenzene, the rates of incorporation of ^{14}C-L-leucine into proteins and the release of radiolabeled proteins were not appreciably changed. At 12 hr after bromobenzene pretreatment, the rate of radiolabeled protein synthesis was not altered when compared to that of control livers, but the release of ^{14}C-labeled proteins into the perfusate was decreased. After pretreatment of rats with phenobarbital, bromobenzene decreased the synthesis and the release of ^{14}C-labeled proteins (4 hr). β-Diethylaminoethyl diphenylpropylacetate (SKF 525-A) and 3-methylcholanthrene (3-MC) did not alter the effect of bromobenzene on the accumulation of total radiolabeled protein synthesis, while SKF 525-A, but not 3-MC, blocked the ability of bromobenzene to decrease the release of ^{14}C-labeled proteins into serum. Since bromobenzene does not alter the rate of ^{14}C-labeled protein synthesis at either 4 or 12 hr after administration, impairment of protein synthesis is probably not a primary cause of bromobenzene-induced liver necrosis. Evidence is presented suggesting that the decrease in the release of ^{14}C-labeled proteins in the perfusate is due to changes in the pattern of protein synthesis as well as to decreases in the rate of protein synthesis.

THERE IS considerable evidence that bromobenzene exerts its necrotic effects by being converted to 3,4-bromobenzene epoxide, which in turn reacts with macromolecules.[1] Since the epoxide rapidly reacts with glutathione (GSH) to form a bromobenzene–GSH conjugate,[2] little covalent binding or necrosis occurs unless GSH levels are decreased by at least 80 per cent.‡ In normal rats the GSH depletion takes about 4 hr after the administration of the toxicant.

Pretreatment of animals with phenobarbital accelerates the metabolism of bromo-

* Research Associate in the Pharmacology–Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md. 20014. Present address: Clinical Pharmacology Program, Emory University School of Medicine, Atlanta, Ga, 30322.

† Present address: Research and Development Division, Dainippon Pharmaceutical Co. Ltd., 39–94 Enoki Cho, Suita 564, Osaka, Japan.

‡ N. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* (in press).

benzene,[1,*] hastens GSH depletion,§ increases the covalent binding of bromobenzene metabolites to liver proteins,*,†,[3] and enhances the severity of bromobenzene-induced necrosis.[1] Thus, the toxic effects of bromobenzene can be manifested within 4 hr after the administration of bromobenzene to phenobarbital-pretreated animals. On the other hand, pretreatment of rats with 3-methylcholanthrene (3-MC) or the administration of β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) decreases the depletion of liver GSH, decreases covalent binding of bromobenzene metabolites to liver protein,*,†,[3] and ameliorates bromobenzene-induced necrosis.[4,5]

Hepatonecrosis caused by carbon tetrachloride (CCl_4) is also thought to be mediated by an active metabolite.[6,7] Examination of the liver by electron microscopy has revealed that within 3 hr after the administration of CCl_4 there is a dissociation of ribosomes from the rough endoplasmic reticulum and an alteration of the ribosomal particles.[8–11] This suggests that the alterations produced by CCl_4 might be reflected in an impairment of protein synthesis, since the endoplasmic reticulum is known to participate in the synthesis of protein. In accord with this view, Smuckler *et al.*[10,11] have shown that oral administration of CCl_4 (5 ml/kg) to rats impairs the synthesis of plasma proteins *in vivo*. These authors have also shown that protein synthesis *in vitro* is impaired within 30 min after the oral administration of CCl_4 (2·5 ml/kg).

The incorporation of radiolabeled amino acids into plasma proteins *in vivo* depends on a number of interrelated factors, such as the relative rates of protein synthesis between the liver and extrahepatic tissues. Thus, it is entirely plausible that a decrease in the amount of radiolabeled amino acids incorporated into plasma proteins could arise from an increase in protein synthesis in another tissue as well as a decrease in hepatic protein synthesis. For this reason, we have studied the possibility of using isolated liver preparations as a model for determining the effects of various treatments on plasma protein synthesis and release.

METHODS

Male Sprague–Dawley rats (180–300 g) were used as a source of liver and blood donors. Bromobenzene and CCl_4 were injected into rats in approximately equimolar doses (1 ml/kg, i.p. in sesame oil); the livers of animals receiving bromobenzene were removed at 4 or 12 hr thereafter and those of animals receiving CCl_4 were removed 4 hr later. Control rats received sesame oil. The livers were then perfused as described below. Experiments were conducted as paired experiments, that is, a control and an experimental liver were perfused simultaneously.

In some experiments, animals were pretreated with phenobarbital, 3-MC, or SKF 525-A. Phenobarbital was administered in a dose of 80 mg/kg i.p. daily for 3 days. 3-MC was dissolved in sesame oil and three doses of 20 mg/kg were administered i.p. 12 hr apart. SKF 525-A was administered in a dose of 80 mg/kg i.p. 20 min before bromobenzene and again 8 hr later.

A recirculating isolated perfused liver preparation was employed for these studies. The apparatus and surgical procedures were those used by Heimberg *et al.*,[12,13] except: (1) tygon tubing in the perfusion apparatus was replaced with a medical grade

* N. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* (in press).

† D. J. Jollow, J. R. Mitchell, N. Zampaglione and J. R. Gillette, *Biochem. J.*, submitted for publication.

Silastic (Dow Corning Corp.) tubing which contains no plasticizers; and (2) the 12XX silk filters used in the original apparatus were replaced with Nitex brand nylon filters (Kressilk Products Inc.) with a thread diameter of 43 μm, a mesh opening of 0·0035 in., and 47 per cent open area. The perfusion medium was prepared as a single batch and divided between the control and experimental livers. The medium consisted of 40 per cent defibrinated rat blood and 60% Krebs–Henseleit bicarbonate buffer, pH 7·4.[14] Livers were removed from the animals and placed in the perfusion apparatus, which contained 79 ml of the perfusion medium. After a 20- to 30-min equilibration period, 15·0 μCi ^{14}C-L-leucine (99% pure, New England Nuclear) in a volume of 6·5 ml was added to the perfusion medium and samples were removed 3 min later and at various times thereafter. During the period of perfusion, the flow rate of perfusate through the liver and the rate of bile production by the liver were used as indices of liver function. Under the conditions employed, the livers did not become edematous, and the perfusate was evenly distributed throughout the liver. Recovery of radiolabeled material at the end of the experiment was consistently greater than 85 per cent of that added initially; thus only insignificant amounts of the radiolabeled material were either absorbed by the perfusion apparatus or metabolized to $^{14}CO_2$ and other volatile products.

The samples of perfusate were centrifuged to sediment erythrocytes and the cell-free perfusate (serum) was used for the analytical procedures. Total serum proteins were precipitated from 0·1-ml aliquotes with 6% trichloroacetic acid. The samples were allowed to stand for 10 min and then centrifuged. The supernatants were saved for determining unincorporated radiolabel. The precipitates were resuspended in 6% trichloroacetic acid and centrifuged. The resultant precipitates were then dissolved in 0·5 ml of 1 N NaOH. Fifteen-ml aliquots of a standard toluene scintillator fluid[15] were added to the protein samples and the mixtures counted in a Packard Tri-Carb liquid scintillation counter.

At the end of the experiment, the livers were flushed out with a 0·9% solution of NaCl, blotted dry and weighed. They were homogenized in 5 vol. of Krebs–Henseleit bicarbonate buffer employing a Potter-type homogenizer with a motor-driven plastic pestle. Proteins were precipitated twice from 0·1-ml aliquots of the homogenate with 6% trichloroacetic acid. One ml of NCS Solubilizer (Amersham/Searle) was added to the precipitates and the samples were dissolved by heating them at 80° for 5 min. The samples were counted by liquid scintillation spectrophotometry as described above for the serum samples.

A statistical comparison of the data was performed employing Student's *t*-test. Values of $P < 0·05$ were considered as representative of significant differences between means.

RESULTS

Serum ^{14}C-L-leucine disappearance and protein synthesis

The disappearance of ^{14}C-L-leucine from the serum at first occurred rapidly and then slowed to negligible rates towards the end of the experiments (Figs. 1a–6a). Thus at the end of almost all of the perfusions, at least 20–30 per cent of the radiolabel was still unincorporated into protein of the perfusates (Table 1, column F). At first it seemed possible that the rapid decline in ^{14}C-L-leucine might reflect merely the equilibration between plasma and liver pools of leucine and that the synthesis of

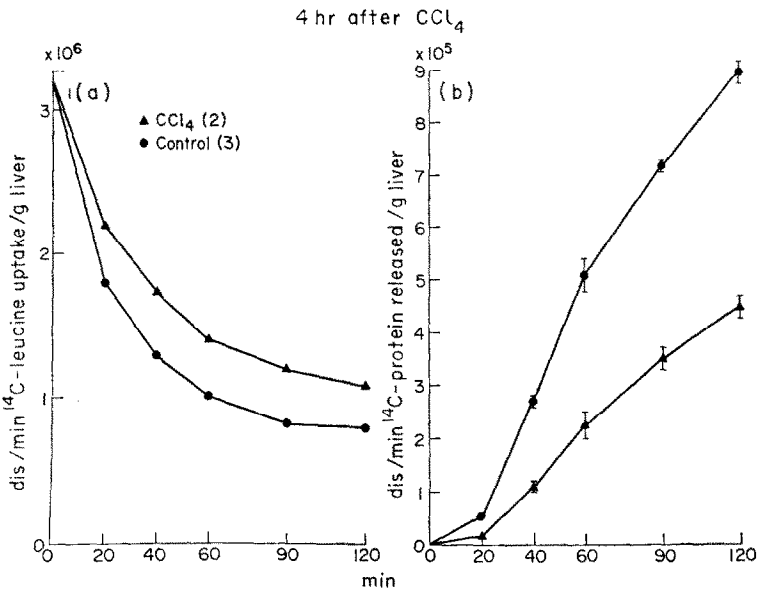

FIG. 1. Uptake of ^{14}C-L-leucine by the liver and release of radiolabeled protein into the perfusate 4 hr after CCl_4. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course of release of ^{14}C-L-leucine-labeled proteins into the perfusate.

protein by the livers was slow. However, other studies with livers from untreated animals showed that protein synthesis is very rapid; approximately 70 per cent of the total ^{14}C-L-leucine incorporated into liver occurred during the first 30 min of perfusion (Fig. 7). Moreover, at 30 min, only 15 per cent of the total radiolabel in liver was not incorporated into protein, and the liver to plasma ratio of unincorporated radiolabel was about 1·67. These findings thus suggest that incorporation of ^{14}C-L-leucine into

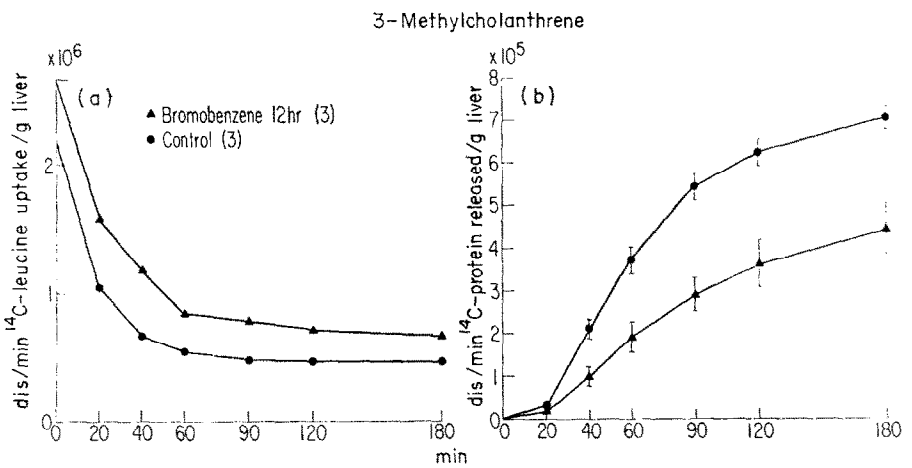

FIG. 2. Uptake of ^{14}C-L-leucine by the liver and release of radiolabeled protein into the perfusate 4 hr after bromobenzene. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course for release of ^{14}C-L-leucine-labeled proteins into the perfusate.

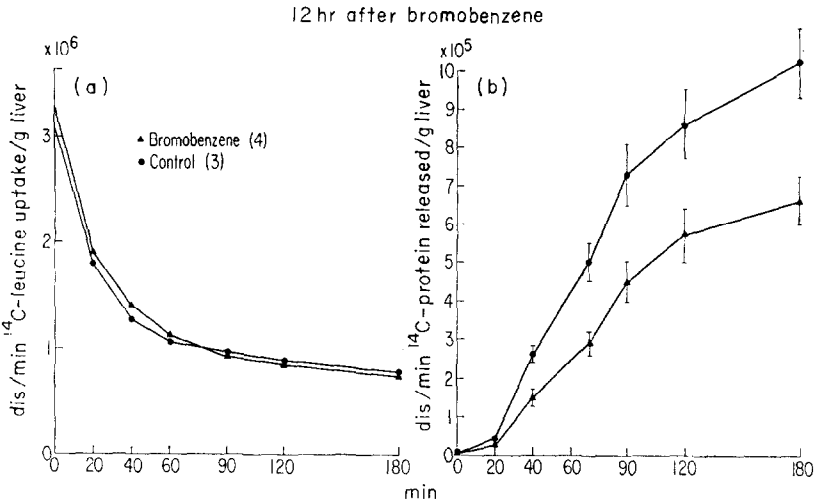

FIG. 3. Uptake of ^{14}C-L-leucine by the liver and release of radiolabeled protein into perfusate 12 hr after bromobenzene. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course for release of ^{14}C-L-leucine-labeled proteins into the perfusate.

protein had virtually stopped within the first hour of infusion, either because protein synthesis had stopped or because ^{14}C-L-leucine had been converted to metabolic products.

Since the incorporation of ^{14}C-L-leucine into protein had been completed before

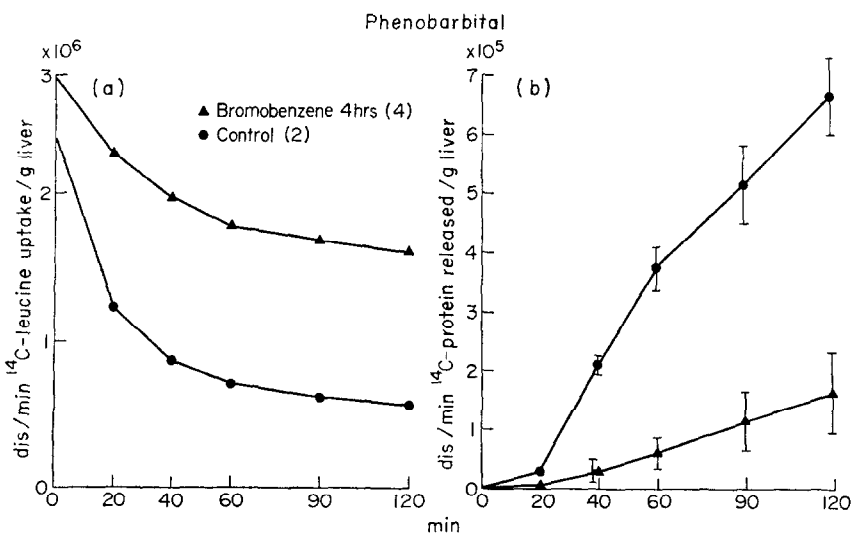

FIG. 4. Effect of phenobarbital pretreatment on uptake of ^{14}C-L-leucine by the liver and release of radiolabeled protein into the perfusate 4 hr after bromobenzene. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course for release of ^{14}C-L-leucine-labeled proteins into the perfusate.

TABLE 1. DISAPPEARANCE OF ^{14}C-L-LEUCINE FROM THE PERFUSATE*

Treatment	A Perfusion time (hr)	B $T_{\frac{1}{2}}$ (min)	C $k \times 10^2$ ($min^{-1}g^{-1}$)	D Liver wt (g)	E $k \times 10^3$/g liver ($min^{-1}g^{-1}$)	F Total leucine uptake (%)
Control (3)	2	17·8 ± 0·8	3·88 ± 0·18	7·3 ± 0·4	5·32 ± 0·11	74 ± 1
CCl_4 4 hr (2)	2	22·0 ± 0·7†	3·15 ± 0·10	7·6 ± 0·4	4·15 ± 0·10†	66 ± 0†
Control (3)	3	19·5 ± 1·4	3·54 ± 0·27	8·0 ± 0·4	4·42 ± 0·29	79 ± 0
Bromobenzene 4 hr (3)	3	23·5 ± 4·0	3·01 ± 0·52	6·7 ± 0·4	4·49 ± 0·51	77 ± 2
Control (3)	3	24·0 ± 2·8	2·91 ± 0·33	7·5 ± 0·5	3·89 ± 0·64	74 ± 3
Bromobenzene 12 hr (4)	3	24·0 ± 4·0	2·88 ± 0·14	6·9 ± 0·4	4·20 ± 0·33	77 ± 2
Phenobarbital (2)	2	17·3 ± 3·2	4·09 ± 0·75	9·2 ± 0·1	4·47 ± 0·86	77 ± 1
Phenobarbitol + bromobenzene 4 hr (4)	2	29·5 ± 3·5†	2·29 ± 0·28†	8·8 ± 2·0	2·71 ± 0·65†	45 ± 11†
SKF 525-A (4)	3	15·3 ± 2·6	4·59 ± 0·70	6·9 ± 0·6	6·61 ± 0·51	77 ± 2
SKF 525-A + Bromobenzene 12 hr (4)	3	22·5 ± 3·8†	3·34 ± 0·57†	7·1 ± 0·9	4·82 ± 1·24†	73 ± 2
3-MC (3)	3	15·3 ± 3·3	4·66 ± 0·95	9·8 ± 0·2	4·77 ± 0·96	79 ± 0
3-MC + bromobenzene 12 hr (3)	3	22·5 ± 0·9†	3·15 ± 0·12	8·1 ± 1·1	3·93 ± 0·41	76 ± 3

* Numbers in parentheses after treatment groups represent the number of perfusions conducted. Values represent means ± S.D.

† Significantly different from control values at $P < 0·05$.

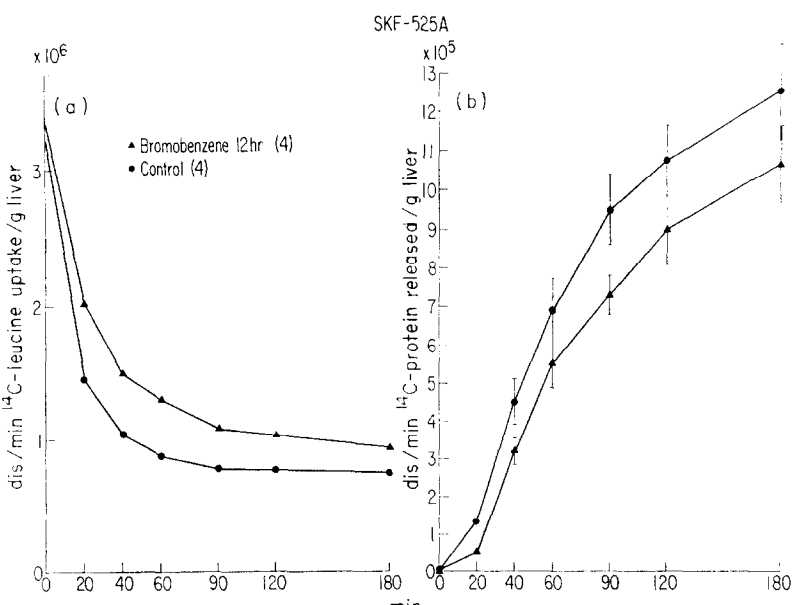

FIG. 5. Effect of SKF 525-A pretreatment on uptake of ^{14}C-L-leucine by the liver and release of radiolabeled protein into the perfusate 12 hr after bromobenzene. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course for release of ^{14}C-L-leucine-labeled proteins into the perfusate.

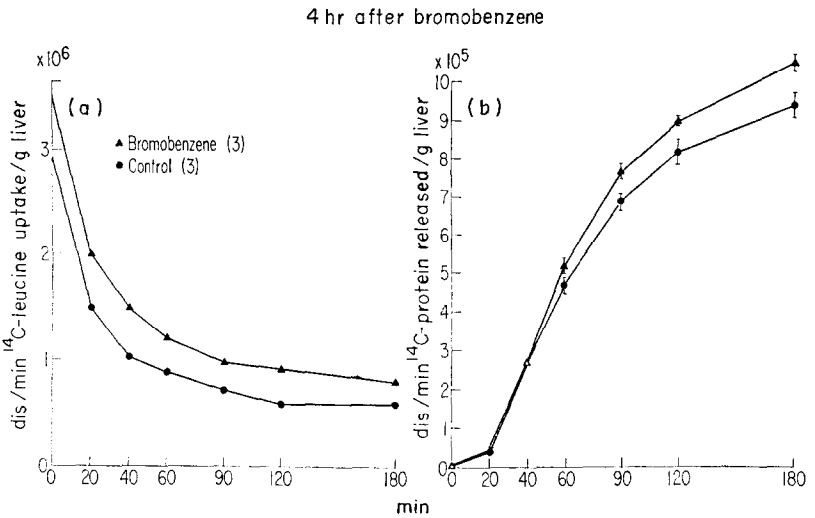

FIG. 6. Effect of 3-MC pretreatment on uptake of ^{14}C-L-leucine and release of radiolabeled protein into the perfusate 12 hr after bromobenzene. Values in (a) are mean values and values in (b) are means ± standard errors. Figures in parentheses represent the number of experiments. (a) Time course of ^{14}C-L-leucine uptake by the liver. (b) Time course for release of ^{14}C-L-leucine-labeled proteins into the perfusate.

the end of the experiments, the total amount of radiolabel incorporation could not be used as a measure of the rate of synthesis of radiolabeled proteins. However, the findings that the synthesis of radiolabeled protein was rather rapid and that the liver to plasma ratio of unincorporated radiolabel was only about 1·67 at 30 min in the

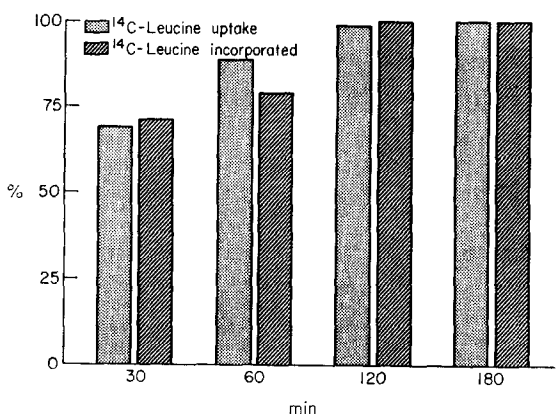

FIG. 7. Uptake of ^{14}C-L-leucine (▨) and incorporation into total protein (serum protein + liver protein) (▦) by control liver during various times of perfusion. The total incorporation of ^{14}C-L-leucine into protein was measured in systems perfused for 30, 60, 120 or 180 min. The total uptake of ^{14}C-L-leucine at these times was calculated by inserting the rate constants of control livers (Table 1, column C) into the expression: $U = (X_0 - X_\infty)(1 - \exp - kt)$, in which U is the uptake and X_0 and X_∞ are the initial and final amounts of ^{14}C-L-leucine in the perfusate. At 30 min, about 15 per cent of the total radiolabel in liver was not incorporated into protein and the liver/plasma ratio of unincorporated radiolabel was about 1·67. At 60 min, about 10 per cent of the total radiolabel in liver was not incorporated into protein and the liver/plasma ratio of unincorporated radiolabel was about 1·78.

livers from untreated animals raised the possibility that the rate of ^{14}C-L-leucine uptake by the livers might be used as a reasonable estimate of the rate of synthesis of radiolabeled proteins. To test the validity of this approach, we subtracted the amount of unincorporated radiolabel present in the plasma at the end of the experiment from the amounts found at earlier times and plotted the resultant values on semilogarithmic paper. Since a biphasic plot would be expected if the uptake represented both a distribution phase and a metabolic phase, the finding that the plots were now linear supported the view that the ^{14}C-L-leucine in the plasma equilibrated with the leucine pools within hepatocytes very shortly after beginning the infusion and that the rate of ^{14}C-L-leucine uptake could be used to estimate the rate of radiolabeled protein synthesis. Moreover, using the rate constants of ^{14}C-L-leucine uptake obtained with livers from untreated animals, we calculated the expected amounts of synthesis of radiolabeled proteins at various times and found that the calculated values closely paralleled the observed values (Fig. 7). The first-order rate constants of ^{14}C-L-leucine uptake (Table 1, column C) obtained for each liver were divided by the corresponding liver weight to estimate the rate of synthesis of radiolabeled proteins per gram of liver (Table 1, column E).

Release of ^{14}C-labeled proteins into serum

There was about a 20-min delay in the appearance of radiolabeled proteins in the perfusate, after which the proteins were released slowly into serum (Figs. 1b–6b). Theoretically, the rate of release should fit the expression, $P_s/P_t = (P_{st}/P_t)\ (1 - \exp - k_r t)$, in which P_s is the amount of ^{14}C-labeled serum protein in the perfusate at any given time, P_{st} is the total amount of ^{14}C-labeled serum protein synthesized, P_t is the total amount of ^{14}C-labeled protein synthesized, and k_r is the rate constant of release. However, in our experiments the release of ^{14}C-labeled proteins was never complete at the end of the perfusions and thus we were unable to determine the P_{st}/P_t ratio directly. Nevertheless, we were able to evaluate relative changes in the release of ^{14}C-labeled proteins by calculating the ratio (P_s/P_t) exptl/(P_s/P_t) control

TABLE 2. RELATIVE RATIOS OF ^{14}C-LABELED PLASMA PROTEINS RELEASED TO TOTAL ^{14}C-LABELED PROTEINS*

Experiment	(Released proteins/total proteins) experimental/ (Released proteins/total proteins) control			
	60 min	90 min	120 min	180 min
CCl_4	0·59	0·63	0·65	
Bromobenzene (4 hr)	0·95	0·97	0·97	0·97
Bromobenzene (12 hr)	0·68	0·63	0·66	0·63
Bromobenzene (4 hr) + phenobarbital	0·33	0·48	0·52	
Bromobenzene (12 hr) + SKF 525-A	0·71	0·74	0·79	0·84
Bromobenzene (12 hr) + 3-MC	0·45	0·47	0·51	0·56

* Values for released proteins represent the amount of ^{14}C-labeled proteins in the serum at any given time. Values for the total amount of ^{14}C-labeled protein (serum + liver) were determined at the end of the perfusion period.

at various times (Table 2). Indeed this comparison should be valid regardless of the mechanism by which serum proteins are released.

The amount of ^{14}C-labeled protein released into serum could be changed for a variety of reasons: (1) There could be changes in the total amount of ^{14}C-L-leucine incorporated into protein (P_t is changed, but P_{st}/P_t is not). (2) There could be changes in the rate of protein synthesis. (3) There could be changes in the rate of release of ^{14}C-labeled plasma proteins (k_r is changed). (4) There could be changes in the relative proportion of ^{14}C-L-leucine incorporated into hepatic and plasma proteins (P_{st}/P_t is changed). (5) Changes could also occur for any combination of the above reasons. Although these possible explanations are frequently difficult to differentiate, it should be pointed out that the ratio, (P_s/P_t) exp/(P_s/P_t) control, should always be 1·0 when the difference in appearance of ^{14}C-labeled plasma proteins is due solely to differences in the amount of ^{14}C-L-leucine incorporated into total protein, i.e. mechanism 1. In mechanisms 2 and 3, the ratio, (P_s/P_t) exp/(P_s/P_t) control, should increase with time, reaching 1·0 only after all of the ^{14}C-labeled proteins have been released into the perfusate. Mechanism 4 can be differentiated from the others because only with this mechanism would the ratio, (P_s/P_t) exp/(P_s/P_t) control, approach a value that differs from 1·0.

Effect of CCl_4

At 4 hr after the administration of CCl_4, the livers were removed and perfused for 2 hr. The toxicant decreased the rate constant of ^{14}C-L-leucine uptake per gram of liver by about 20 per cent (Table 1, column E) and decreased the total uptake per liver by about 11 per cent (Table 1, column F). Accordingly, at the end of the perfusion, the total incorporation of ^{14}C-L-leucine per gram of liver was decreased by about 20 per cent (Table 3, column A). By contrast, the release of ^{14}C-labeled protein into the perfusate was decreased by about 50 per cent (Fig. 1b; Table 3, column C). Moreover, the per cent of the released ^{14}C-labeled protein to the total ^{14}C-labeled protein was decreased (Table 3, column D), the (P_s/P_t) exp/(P_s/P_t) control values ranging from about 0·59 of controls at 1 hr to about 0·65 of controls at 2 hr (Table 2). Thus the synthesis of radiolabeled proteins was only slightly impaired by CCl_4 administration; most of the decrease in ^{14}C-labeled protein in the perfusate was due to an apparent change in the relative rates of synthesis of plasma and hepatic proteins.

Effect of bromobenzene

When the livers were removed and perfused at 4 hr after the administration of bromobenzene, there was little if any change in the rate or total uptake of ^{14}C-L-leucine or in the synthesis and release of ^{14}C-labeled proteins (Tables 1–3; Fig. 2b). The slightly greater formation of ^{14}C-labeled protein observed in the experimental group (Table 3, column A) is probably due to the difference in liver weights of the experimental and bromobenzene-treated animals used in this experiment (Table 3). Presentation of the data in terms of dis/min/g of liver does not completely correct for variations in liver weight, because the validity of this normalization depends on relatively constant ^{14}C-L-leucine concentrations during perfusion.

When the livers were removed and perfused at 12 hr after bromobenzene administration, again there was little or no change in the rate constant or uptake of ^{14}C-L-

Table 3. Incorporation of ^{14}C-L-leucine into serum and liver protein*

Treatment	Perfusion time (hr)	Liver wt (g)	^{14}C-L-leucine incorporated (dis/min/g liver × 10^5) A Total	B Liver	C Serum	$\frac{^{14}C \text{ in serum protein}}{^{14}C \text{ in total protein}} \times 100$ D (%)
Control (3)	2	7·3 ± 0·4	22·4 ± 0·8	13·5 ± 0·5	9·0 ± 0·3	40·0 ± 0·9
CCl_4 4 hr (2)	2	7·6 ± 0·4	17·7 ± 1·8†	13·3 ± 1·5	4·5 ± 0·3†	25·3 ± 0·9†
Control (3)	3	8·0 ± 0·4	22·0 ± 0·8	12·6 ± 0·3	9·3 ± 0·5	42·5 ± 0·8
Bromobenzene 4 hr (3)	3	6·7 ± 0·4†	25·0 ± 0·5†	14·5 ± 0·4†	10·5 ± 0·3†	42·0 ± 1·1
Control (3)	3	7·5 ± 0·5	22·4 ± 1·9	12·1 ± 0·8	10·2 ± 1·7	45·6 ± 4·0
Bromobenzene 12 hr (4)	3	6·9 ± 0·4	23·0 ± 1·3	16·4 ± 1·9†	6·6 ± 1·2†	28·9 ± 6·1†
Phenobarbital (2)	2	9·2 ± 0·1	20·6 ± 0·5	13·9 ± 0·4	6·7 ± 0·9	32·4 ± 3·8
Phenobarbital + bromobenzene 4 hr (4)	2	8·8 ± 2·0	8·4 ± 4·8†	6·8 ± 3·5	1·6 ± 1·3†	19·0 ± 5·6†
SKF 525-A (4)	3	6·9 ± 0·6	25·0 ± 3·2	12·3 ± 0·8	12·6 ± 2·6	50·1 ± 4·9
SKF 525-A + bromobenzene 12 hr (4)	3	7·1 ± 0·9	22·0 ± 3·5	12·6 ± 1·0	9·4 ± 3·0	41·8 ± 7·7
3-MC (3)	3	9·8 ± 0·2	17·0 ± 1·3	10·0 ± 0·9	7·0 ± 4·0	41·2 ± 0·9
3-MC + bromobenzene 12 hr (3)	3	8·1 ± 1·1	19·1 ± 2·8	14·7 ± 1·8†	4·4 ± 1·0†	22·8 ± 2·2†

* Numbers in parentheses after treatment groups represent the number of perfusions conducted. Values represent means ± S.D.
† Significantly different from control values at $P < 0·05$.

leucine (Table 1, columns E and F), or in the total amount of ^{14}C-labeled protein synthesized (Table 3, column A). But the release of ^{14}C-labeled protein into the perfusion medium was decreased (Fig. 3B). After perfusion for 3 hr, the amount of ^{14}C-labeled protein in the perfusion fluid was 35 per cent lower with livers from bromobenzene-treated rats than with those from untreated rats (Table 3, column C). As shown in Table 2, the (P_s/P_t) exp/(P_s/P_t) control values were relatively constant during the last 2 hr of perfusion. Since neither the rate of ^{14}C-L-leucine uptake nor the formation of ^{14}C-labeled protein was altered by the toxicant, the decreased amount of ^{14}C-labeled protein in the perfusion fluid is probably due to a change in the relative proportion of synthesis of ^{14}C-labeled serum and hepatic proteins.

Effect of bromobenzene in phenobarbital-pretreated rats

At 4 hr after the administration of bromobenzene to rats pretreated with phenobarbital, there were about 40 per cent decreases in the rate constant per g of liver and in the total ^{14}C-L-leucine uptake (Table 1, columns E and F). Moreover, the toxicant decreased the synthesis of the ^{14}C-labeled total protein per gram of liver by about 60 per cent, the release of ^{14}C-labeled protein into the perfusion medium by about 76 per cent, and the per cent of the total ^{14}C-labeled proteins released into the perfusate (Fig. 4b; Table 3, columns A, C and D). As shown in Table 2, the $(P_s/P_t$ exp/(P_s/P_t) control values gradually increased with time, but not as markedly as would be expected if the only effect of bromobenzene were an impairment of protein synthesis and release. Thus, bromobenzene in phenobarbital-treated animals not only impairs the rate and the amount of protein synthesis and the release of the ^{14}C-labeled plasma proteins, but also the relative proportion of synthesis of ^{14}C-labeled serum and hepatic proteins.

Effect of bromobenzene in rats receiving SKF 525-A

At 12 hr after the administration of bromobenzene, the rate constant for ^{14}C-L-leucine uptake was decreased by about 25 per cent (Fig. 5; Table 1, column E). But there was no appreciable change in the amount of ^{14}C-L-leucine uptake (Table 1, column F), in the total amount of ^{14}C-labeled protein synthesized, or in the amount of ^{14}C-labeled protein released into the perfusate (Table 3, columns A and C). Moreover, the (P_s/P_t) exp/(P_s/P_t) control values tended to increase with time, but were always higher than those obtained with livers from animals receiving bromobenzene (12 hr) alone (Table 2). Thus, SKF 525-A at least partially prevented the effects of bromobenzene on the ^{14}C-labeled serum protein synthesis, but may have enhanced its impairing effects on the release of these proteins.

Effect of bromobenzene in rats pretreated with 3-MC

At 12 hr after the administration of bromobenzene to rats pretreated with 3-MC, there was little or no change in the rate constant or the total amount of ^{14}C-L-leucine uptake (Table 1, columns E and F) or in the total amount of ^{14}C-labeled protein synthesized (Table 3, column A). However, the release of ^{14}C-labeled proteins into the perfusion fluid was decreased by about 40 per cent and the per cent of the total ^{14}C-labeled protein released into the fluid was decreased by about 44 per cent (Fig. 6b; Table 3, columns C and D). Again, the (P_s/P_t) exp/(P_s/P_t) control values tended to increase during the last 2 hr of the experiment, but the increase was too small to

be attributed solely to impaired synthesis or release of radiolabeled serum proteins (Table 2). Thus, pretreatment of the animals with 3-MC did not appreciably alter the changes in the relative rates of synthesis of radiolabeled plasma and liver proteins caused by bromobenzene.

DISCUSSION

It is clear from this study that changes in the release of radiolabeled proteins into blood plasma do not necessarily reflect changes in the rate of protein synthesis in liver. Radiolabeled amino acids are rapidly taken up by the liver and incorporated into protein, but the release of the radiolabeled protein is delayed for about 20 min and occurs slowly.

To what extent the rates of synthesis of radiolabeled protein reflect the rates of synthesis of protein in the perfused liver or its capacity to synthesize protein remains unclear. Since the specific activity of leucine in liver could not be determined without destroying the liver preparation, it was not possible to determine the specific activity of the leucine pool in hepatocytes during the synthesis of the radiolabeled proteins. However, equilibration presumably occurs very rapidly because virtually all of the ^{14}C-L-leucine taken up by the liver within the first 30 min was incorporated into liver protein (> 85 per cent). Indeed the rapid incorporation of the ^{14}C-L-leucine raises the possibility that the rate of synthesis of ^{14}C-labeled protein may be limited mainly by the transport of amino acids into liver rather than by the capacity of liver to synthesize protein. If uptake of the ^{14}C-L-leucine were the rate-limiting step, the apparently small decreases in the rate of ^{14}C-L-leucine uptake caused by various treatments would not necessarily be expected to parallel the severity of the damage to the protein synthetic mechanism.

The administration of CCl_4 caused only a 20 per cent decrease in the rate of protein synthesis, as estimated from the rate constant of ^{14}C-L-leucine uptake. Thus, the marked decrease in radiolabeled plasma proteins is probably not due solely to impaired protein synthesis. Although the decrease in ^{14}C-labeled proteins was caused partly by impairment of release, most was probably due to a change in the relative proportions of plasma and hepatic proteins synthesized. Although Smuckler *et al.*[10,11] found that CCl_4 caused a greater impairment of protein synthesis than we found, their doses of CCl_4 were five times as large as ours.

In contrast, bromobenzene administered to un-pretreated animals did not decrease either the total synthesis of ^{14}C-labeled proteins or their rates of synthesis as estimated from the rate constants of ^{14}C-L-leucine uptake. Nevertheless, with livers perfused at 12 hr, but not with those perfused at 4 hr after bromobenzene administration, the release of labeled proteins into the perfusion fluid was decreased. Since the (P_s/P_t) exp/(P_s/P_t) control values remained remarkably constant with time, the decrease is not due to an impairment of the release mechanism but probably due to an alteration in the pattern of protein synthesis.

Since the covalent binding of bromobenzene metabolites to liver proteins becomes maximal at 12–16 hr after administration of the toxicant,[3] it seemed possible that the decrease in release of ^{14}C-labeled protein caused by bromobenzene might be related to liver necrosis. However, in rats pretreated with 3-MC, which prevents bromobenzene-induced liver necrosis, bromobenzene did not affect either the total synthesis of radiolabeled proteins or their rates of synthesis, but still caused a decrease in the

release of labeled proteins into the perfusion medium. Again the (P_s/P_t) exp/(P_s/P_t) control values remained remarkably constant with time. Thus, the decrease in the release of ^{14}C-labeled proteins does not appear to be related to necrosis.

The effects of bromobenzene in animals also receiving SKF 525-A are difficult to interpret. Although the rate of synthesis of ^{14}C-labeled protein is not altered by administration of bromobenzene alone, it is decreased by administration of the toxicant to animals also receiving SKF 525-A. Despite the decrease in rate of synthesis, however, the total synthesis of ^{14}C-labeled proteins was not significantly decreased. Moreover, the effects of SKF 525-A differed from those of 3-MC in that SKF 525-A diminished the bromobenzene-induced decrease in the release of ^{14}C-labeled proteins into the perfusion fluid. Thus the data appear to be inherently inconsistent. Close examination of the data reveals that the rate constants of ^{14}C-L-leucine uptake for the control livers in this experiment are unusually large compared with those in the other experiments, suggesting the possibility that the null hypothesis was erroneously rejected.

The impairing effects of bromobenzene in animals pretreated with phenobarbital were so severe that the livers could not be perfused much longer than 2 hr without a decrease in plasma and bile flow rates. Moreover, the administration of bromobenzene now decreased the rate of synthesis of ^{14}C-labeled proteins, the total amount of ^{14}C-labeled protein synthesized, and the amount of the proteins released into the perfusion fluid.

In conclusion, these studies suggest that liver perfusion experiments may provide an insight into the interrelationships between radiolabeled amino acid uptake, the rate of synthesis of radiolabeled proteins, the relative rates of synthesis of plasma and serum proteins, and the release of serum proteins. But they also suggest that such experiments do not necessarily elucidate relationships between amino acid uptake and impairment of protein synthetic mechanisms, nor do such studies necessarily predict the severity of liver damage caused by hepatotoxicants. These studies also point out that decreases in the formation of radiolabeled serum proteins *in vivo* should be interpreted with care.

REFERENCES

1. B. B. Brodie, W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette, *Proc. natn. Acad. Sci. U.S.A.* **68,** 160 (1971).
2. E. Boyland, *Concepts in Biochemical Pharmacology* (Eds. B. B. Brodie and J. R. Gillette), Vol. XXVIII, part 2, p. 584. Springer, Heidelberg (1971).
3. W. D. Reid and G. Krishna, *Expl. molec. Path.*, in press.
4. W. D. Reid, B. Christie, M. Eichelbaum and G. Krishna, *Expl. molec. Path.* **15,** 363 (1971).
5. J. R. Mitchell W. D. Reid, B. Christie, J. Moskowitz, G. Krishna and B. B. Brodie, *Res. Commun. Chem. Path. Pharmac.* **2,** 877 (1971).
6. T. F. Slater, *Nature, Lond.* **209,** 36 (1966).
7. R. O. Recknagel, *Pharmac. Rev.* **19,** 145 (1967).
8. Ch. Oberling and Ch. Rouiller, *Ann. Anat. Path.* **1,** 401 (1956).
9. M. Bassi, *Expl. Cell Res.* **20,** 313 (1960).
10. E. A. Smuckler, O. A. Iseri and E. P. Benditt, *J. exp. Med.* **116,** 55 (1962).
11. E. A. Smuckler and E. P. Benditt, *Biochemistry, N.Y.* **4,** 671 (1965).
12. M. Heimberg, N. B. Fizette and H. Klausner, *J. Am. Oil Chem. Soc.* **41,** 774 (1964).
13. M. Heimberg, I. Weinstein, H. Klausner and M. L. Watkins, *Am. J. Physiol.* **202,** 353 (1962).
14. H. A. Krebs and K. Henseleit, *Hoppe-Seyler's Z. physiol. Chem.* **210,** 33 (1932).
15. A. K. Cho, B. J. Hodshon and B. B. Brodie, *Biochem. Pharmac.* **19,** 1817 (1970).