Incorporation of Halophenylcysteines into Proteins.* (28480)

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Protein binding of aromatic hydrocarbons has been well documented(1) but the mechanisms of activation and reaction with proteins have not been established. Conjugation with the cysteine sulfhydryls in the binding process has often been postulated. In this laboratory Mills and Wood showed that metabolism of a synthetically-produced hydrocarbon-S-protein conjugate resulted in mercapturic acid excretion(2). Such hydrocarbon-protein conjugates were separated by Smith and Wood from liver and intestine of rats given iodobenzene-I^{131} and shown to be characteristic of no specific tissue protein(3). Peptidolysis of these conjugates yielded a series of iodophenylcysteinyl peptides. Earlier interpretations of these findings have involved a direct interaction between the protein and reactive hydrocarbon metabolites. Alternatively, the hydrocarbon-protein conjugates could develop by intrusion of some of the simpler intermediates such as an iodophenylcysteine or iodophenylglutathione derivative into one of the pathways of protein biosynthesis. Findings bearing upon this hypothesis are presented herein.

Materials and methods. Iodobenzene-I^{131} was synthesized and administered orally in corn oil to adult white rats as described previously(4). Two hours later the livers and small intestines were removed. The tissues were homogenized separately and extracted repeatedly with 0.14 M KCl to remove soluble proteins. Residual solids were extracted with M KCl from which DNA-protein was isolated after 6-fold dilution with water; the insoluble residual protein was largely lipoprotein(5). The 0.14 M KCl extract was acidified to pH 5 to precipitate RNA-protein.

Nuclei and mitochondria were isolated by the method of Hogeboom(6). The procedures of Hoagland *et al.*(7) were utilized to prepare microsomes and to wash unlabeled compounds from the cellular fractions. Labeled RNA was isolated by the 90% phenol method of Kirby(8). DNA and RNA were determined by the diphenylamine(9) and orcinol(10) methods respectively. S-*p*-chlorophenylcysteine-S^{35} was prepared from L-cysteine-S^{35}(11). All S^{35} radioactivity was counted as $BaSO_4$ following combustion by the nitric-perchloric acid method(12). I^{131} was determined in a well-scintillation counter(3).

Results and discussion. Table I shows a comparison between the radioactivity of conjugated proteins and their RNA and DNA contents. Following extraction with hot 5% trichloroacetic acid some of the radioactivity was found in the RNA fraction.

The bound radioactivity of the isolated fractions was very small compared to the dose but this has been frequently found with *in vivo* administration of hydrocarbons. On the other hand, isolation of an amino acid-RNA complex is unusual since rapid transfer to microsomes takes place in the intact cell. However, the rate of reaction of amino acid analogues in competition with natural amino acids is generally very slow; the persistence of the RNA complex is thus reasonable.

Since the above findings demonstrated incorporation of radioactive compounds derived from iodobenzene into RNA protein, the distribution of radioactivity between cellular particulates and the "pH 5 enzyme system" was investigated. Table II shows the highest activity to be in the pH 5 enzyme fraction. Since we had found S-arylcysteines in tissue homogenates(3), the possibility of such compounds incorporating into protein by a biosynthetic pathway as a means of producing hydrocarbon conjugates was postulated.

To add credence to this hypothesis, chlorophenylcysteine-S^{35} was incubated with the pH 5 enzyme system. Hydroxamate assay showed carboxyl activation at a barely significant level. However, radioactivity assays on protein and phenol-extracted soluble RNA

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TABLE I. *In vivo* Binding of Iodobenzene-I^{131} to Proteins.

Protein fraction*	Wt (g)	RNA (%)	DNA (%)	Radioactivity† Total	Radioactivity† cpm/mg
Liver					
Lipoprotein	1.727	1.5	5.2	4,050	2.3
RNA-protein	1.145	5.0	.92	29,000	25.3
DNA-protein	.407	2.9	6.5	3,710	9.1
Intestinal					
Lipoprotein	.915	2.5	2.2	1,145	1.2
RNA-protein	.566	5.8	1.0	8,700	15.5
DNA-protein	.180	2.6	6.5	720	4.0

* Lyophilized proteins were successively extracted with 2:1 chloroform-methanol at 60° to remove physically bound iodobenzene.

† Iodobenzene administered to the rats was 100 mg with a total radioactivity of 1×10^9 cpm. Total radioactivity of liver homogenate, 6.4×10^6 cpm, 0.14 M KCl extract, 6.3×10^6; initial precipitate 4.5×10^6. Total radioactivity of intestinal homogenate 9.8×10^6 cpm, 0.14 M KCl extract 3.7×10^6; initial precipitate 2.2×10^6.

fractions (Table III) suggested conjugation of the chlorophenylcysteine to the large molecules.

Microsomal proteins and pH 5 enzyme system components which had been labeled by incubation with chlorophenylcysteine-S^{35} were incubated with fresh liver homogenate under conditions which degraded iodophenylcysteinyl-protein conjugates to peptides(3). For this experiment a pH 5 enzyme preparation containing 590 mg of protein was incubated with 600 μmoles of ATP and 1.8 mg of *p*-chlorophenylcysteine-S^{35} (6.6×10^5 cpm) for 20 minutes at 37°. The labeled pH 5 enzyme was twice reprecipitated and then homogenized with 30 ml of 0.1 M phosphate buffer, pH 7.4. Twelve ml of fresh 5% liver homogenate in phosphate buffer were added and the mixture was incubated at 37° in air for 20 minutes. The mixture was homogenized with 18% formic acid and small molecules were collected by dialysis. Chromatography of the diffusate on Dowex 50 × 4 gave a characteristic pattern of products similar to that reproducibly obtained from tissues of rats administered iodobenzene(3), (Table IV). The peptide fraction (Group 2) had a relatively high level of radioactivity. Also the protein remaining in the dialysis sack was precipitated with 55% acetone and had the highest specific radioactivity for protein.

The present experiments demonstrate the presence of a pathway for incorporation of chlorophenylcysteine into protein and are suggestive but do not delineate participation of the pH 5 enzyme system. Incorporation of amino acid analogues into protein, for example, fluorophenylalanine, is well established. The present instance is unique, however, since the chlorophenylcysteine is an amino acid analogue which is formed metabolically through conjugation of the hydrocarbon to the sulfur of various cysteine moieties.

The findings of Boyland and co-workers indicate primary participation of glutathione in the conjugation of hydrocarbons with sulfur compounds although concurrent reactions with other sulfhydryl groups are not ruled out(13). Peptidolysis of the glutathione conjugate leads to the dihydrohydroxyarylcysteine derivative which is a precursor of the mercapturic acid and is isolable from the urine.

There seems to be a good possibility that metabolically activated hydrocarbon residues react non-specifically with any available sulfhydryl group as well as specifically with glutathione. This would result in observance of

TABLE II. Distribution of Radioactivity Between Liver Particulates and pH 5 Enzyme Following Administration of Iodobenzene-I^{131}.

Cell fraction	RNA content (mg)	Nitrogen content (mg)	Specific radioactivity RNA (cpm/mg)	Specific radioactivity Protein (cpm/mg N)
Nuclei	1.8	42.0	40	1.7
Microsomes	4.4	17.0	21	5.5
Mitochondria 1	2.9	24.7	34	4.1
" 2	1.6	7.2	32	7.0
pH 5 ppt	1.8	12.6	60	8.6

Iodobenzene-I^{131} (100 mg, 2.16×10^5 cpm per μmole) was given to each of three 300-400 g rats. After 2 hr the livers were removed, pooled, and fractionated as described in section on *Methods*. Two fractions of mitochondria were obtained. Fraction 1 was separated by differential centrifugation of resuspended nuclei in medium containing 0.35 M sucrose, 0.035 M $KHCO_3$, 0.004 M $MgCl_2$ and 0.025 M KCl. Fraction 2 was obtained at the maximum gravitational field of the Servall SS-1 centrifuge.

TABLE III. Binding of Chlorophenylcysteine-S^{35} by RNA and Protein of pH 5 Precipitate.

No.	Enzyme system: Protein (mg)	Enzyme system: Chlorophenylcysteine mg	Enzyme system: Chlorophenylcysteine cpm	Isolated radioactivity: Soluble RNA (cpm)	Isolated radioactivity: Total protein ppt (cpm)	Isolated radioactivity: Chlorophenylcysteine ($m\mu$ moles/mg RNA)
I	247	.638	2.6×10^5	31	654	.07
I	247	.394	1.6×10^5	37	372	.09
II	90	.820	3.7×10^5	20	342	.04
III	110	.100	$.3 \times 10^5$	40	1896	.11
IV	140	.809	3.6×10^5	175	2502	.50
V	70	.987	5.5×10^5	100		.39
VI	300	.646	3.6×10^5	91		

In 5 ml of medium (0.35 M sucrose, 0.035 M $KHCO_3$, 0.004 M $MgCl_2$ and 0.025 M KCl) were dissolved pH 5 enzyme system precipitate and *p*-chlorophenylcysteine as shown plus 200 μmoles of ATP; incubation was for 20 min at pH 7.4 and 37°. Incubation mixture was brought to pH 5.2 with acetic acid, precipitate was redissolved and then reprecipitated at pH 5.2. Phenol extraction-ethanol precipitation method(8) was used to separate soluble RNA.

protein binding of hydrocarbons arising from any or all of the reactions noted above, *i.e.*, direct reaction through free protein sulfhydryl groups, or by any of the biosynthetic pathways for incorporation of amino acid residues into protein. On the basis of the radioactivity found in these investigations, protein binding would appear to be a minor reaction.

Because of competitive reaction with glutathione, direct binding through the sulfhydryls of protein would be expected to be very small as observed here. Likewise, incorporation of an amino acid analogue into protein has generally been found to be small in comparison to the amount of incorporation of a natural amino acid into the protein structure. In addition, the protein conjugates are constantly breaking down to yield hydrocarbon-cysteinyl peptides(3) so that, at any time of interruption of the process, the amount of protein-bound hydrocarbon is necessarily very small. Whether the presence of such modified proteins in cells can exert a physiological effect remains to be seen.

TABLE IV. Distribution of Chlorophenylcysteinyl-Sulfur Following Incubation of Labeled pH 5 Precipitate System with Liver Homogenate.

Soluble protein fractions of homogenate	Radioactivity: Total cpm	Radioactivity: cpm/mg N
I Precipitated on dialysis	2440	44.6
II KCl extract of I above	27	12.2
III Alkaline KCl extract of I above	132	10.3
IV 55% acetone precipitate of soluble fraction	492	107.0
Diffusate fraction* by groups	cpm	
1	None	
2 Large peptides	1536	
3 Minor components	150	
4–5 " "	820	
6 " "	170	
7 *p*-Chlorophenylcysteine	6000	

* Dowex-50 × 4 Na+ form column, fractions grouped according to previous selections(3).

Summary. Incorporation of labeled iodobenzene and *p*-chlorophenylcysteine into the conjugated proteins and "soluble RNA fractions" of rat liver and intestine has been demonstrated. The possibilities for production of hydrocarbon-protein conjugates by either direct reaction with the protein or initial reaction to form an amino acid analogue are recognized.

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Cholesterol Esterification by Adrenal Homogenates.* (28481)

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In certain animal species the cholesterol in the adrenal gland is present largely in the ester form(1,2), and it has been postulated that these cholesterol esters are derived from the lymph esterified cholesterol(3). It has recently been shown, however, that homogenates of rat adrenal tissue are able to esterify cholesterol(4), suggesting that the rat adrenals may form their own cholesterol esters. We now wish to report the extension of these studies with homogenates of adrenal tissue from other species.

Materials and methods.† The animals were killed and the adrenal glands rapidly removed and placed in ice-cold saline. After carefully removing the adipose tissue from the glands, they were homogenized, using 1 ml 0.25 M sucrose per 20 mg adrenal tissue. The homogenates were centrifuged at 1,000 $\times$ *g* for 10 minutes and the supernatant A collected. This fraction, unless otherwise noted, was centrifuged at 20,000 $\times$ *g* for 20 minutes, and the supernatant from the resulting sample centrifuged at 85,000 $\times$ *g* for 20 minutes. The remaining pellet, numbered 3, was then resuspended in the original volume of 0.25 M sucrose and incubated in 2 ml homogenate and 1 ml KH_2PO_4 buffer containing the cofactors and either 1-C^{14}-palmitic acid or 4-C^{14}-cholesterol. The labeled lipids were dissolved in propylene glycol to a final volume of less than 0.25% per flask. All incubations were done at 37°C in a Dubnoff metabolic shaker in room air.

At the end of the incubation period, 2 ml of medium was removed and extracted twice with 5 ml portions of chloroform:methanol (2:1 v/v) and once with 5 ml petroleum ether. The extracts were pooled, dried under nitrogen, and the residue dissolved in hexane and placed on a silicic acid column(5). The cholesterol esters were eluted with 35 to 50 ml of 15% benzene in hexane and free cholesterol was eluted with 10 ml ether and then 10 ml methanol. Aliquots of each fraction were dried under air, dissolved in toluene phosphor(6) and the radioactivity was determined in a liquid scintillation counter. Cholesterol, free and esterified, was determined on an aliquot of each fraction by the method of Courchaine, Miller and Stein(7). Proteins were determined using a modification of the Lowry method(8).

Results. As seen in Table I, supernatant A of rat and guinea pig adrenal was quite active in esterifying cholesterol while rabbit adrenal was slightly less active. Supernatant A of bovine adrenal, however, was relatively deficient in cholesterol-esterifying activity. With added 1-C^{14}-palmitic acid and unlabeled cholesterol, pellet 3 of both rat and guinea pig adrenal was very active (Table II) while bovine adrenal displayed only a fraction of this cholesterol-esterifying ability. With 4-C^{14}-cholesterol, pellet 3 of rat and rabbit adrenal was able to esterify cholesterol to a significant amount (Table III), while pellet 3 of bovine adrenal was relatively deficient in this regard.

Discussion. Using a system which is optimal for esterification of cholesterol by homogenates of rat adrenal tissue(4), similar

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