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METABOLISM OF JUVENILE HORMONE WITH ISOLATED RAT HEPATOCYTES*

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Abstract—The metabolism of juvenile hormone I has been examined in rat hepatocyte suspensions. The hormone penetrates rapidly into the cells where its concentration becomes higher than in the incubation medium. About 25 per cent of the hormone is metabolized during min 1 of incubation, while at 30 min of incubation, essentially all the hormone has been metabolized into several organic-soluble and water-soluble metabolites. Eventually the organic-soluble metabolites (including the diol ester, the diol acid and the acid) are recycled by re-entry and are metabolized further into water-soluble metabolites. The most important water-soluble metabolite is the mercapturic acid followed by the glucuronide. These results suggest that the low toxicity of juvenile hormone in mammals may be explained, at least in part, by metabolic detoxication.

Of the known insect hormones, only juvenile hormone (JH) analogues (juvenoids) are considered at present to have an immediate promise for insect pest control. Some of these compounds are highly active, and under controlled conditions, they disrupt metamorphosis and reproduction or cause death when applied topically at dosages at ng levels or when injected at concentrations of less than 1 ppm. They penetrate the egg chorion or insect cuticle and hence are active on contact. Some are sufficiently volatile to act as fumigants. The juvenoids affect developmental stages including eggs, full grown larvae or nymphs, prepupae, pupae and female adults. Since juvenoids are chemical compounds that eventually may be in contact with man and/or animals, it is desirable to have thorough information on their metabolism and mode of action. JH II (methyl-10,11-epoxy-3,7,11-trimethyl-10,11-epoxydodeca-2,6-dienoate) have been shown to produce no signs of toxicity when given to mice in a single oral dose of 5 g/kg of body weight, which indicates that large quantities of these compounds can be tolerated on acute ingestion by this mammalian species [1]. The juvenoid 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene (Stauffer R-20458) appears to be nontoxic when administered orally or intraperitoneally to laboratory rats and mice [2–4] due to metabolic degradation, the metabolic products being eliminated rapidly and essentially quantitatively through the urine and feces. A similar observation has been reported in steer [5]. In spite of these observations, the possibility of long range effects due to storage and metabolism cannot be excluded at present.

In the present study, as a preliminary step in the analysis of juvenoids metabolism in mammalian tissues, we report on the fate of JH I (methyl-*trans,trans,cis*-3,11-dimethyl-7-ethyl-10,11-epoxytrideca-2,6-dienoate) in isolated rat hepatocytes using high pressure liquid chromatography (h.p.l.c.) and thin-layer chromatography (t.l.c.) for the analysis of its metabolic pattern in the cells and in their surrounding medium. The hepatocyte is an ideal experimental model for studying toxifying and detoxifying reactions since it resembles *in vivo* conditions more than subcellular preparations [6], and it actively catalyzes the cytochrome P-450-linked and epoxide hydrase-linked, as well as conjugative and hydrolytic reactions [7]. Attack on juvenoids such as hydroprene and methoprene, by insect cytochrome P-450 and epoxide hydrase reactions, as well as by esterase action, is well documented [8, 9]. The present results indicate that JH I is metabolized in hepatocytes by pathways involving, in order of importance, ester hydrolysis, mercapturic acid formation and hydration of the epoxide ring primarily, and glucuronide and sulfate conjugation secondarily.

MATERIALS AND METHODS

Chemicals. Pure synthetic *Hyalophora cecropia* juvenile hormone (methyl-*trans,trans,cis*-3,11-dimethyl-7-ethyl-10,11-epoxy trideca-2,6-dienoate) was purchased from ECO Control (Cambridge, MA). Labeled *H. cecropia* JH I[10-^{3}H(N)] (specific activity, 13.5 Ci/m-mole) was obtained from New England Nuclear (Boston, MA). *Clostridium histolyticum* collagenase (grade IV) was purchased from Boehringer (Indianapolis, IN); β-glucuronidase from bovine liver (1×10^6 Fishman units/g) and *Helix pomatia* sulfatase (3000 units/ml) were purchased from the Sigma Chemical Co. (St. Louis, MO). Fraction V of bovine serum albumin (BSA) obtained from the Sigma Chemical Co. was purified by the procedure of Chen [10]. Methanol (h.p.l.c. grade) was obtained from Fisher Scientific (Pittsburgh, PA). The rest of the reagents used were of the highest purity commercially available.

Synthesis of authentic derivatives. The acid derivative of JH I or ^{3}H-labeled JH I (specific activity, 14.7 mCi/m-mole) was prepared by incubating JH I overnight in 0.5 N NaOH in 50% aqueous ethanol at room temperature, followed by extraction with benzene to remove unreacted JH I; the pH was adjusted to 5.0 by the addition of 0.5 vol. of 2 M sodium acetate buffer,

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pH 5.0, and the acid was extracted with benzene; the benzene was evaporated off under a stream of N_2 and the residue was dissolved in 100% methanol for further analysis. The yield was over 80 per cent. The diol ester was obtained by treating either unlabeled or labeled JH I (specific activity as above) with 0.05 N H_2SO_4 in 40% aqueous tetrahydrofuran for 5 hr at room temperature. The product was extracted with benzene, concentrated as above, and dissolved in 100% methanol. The yield was about 50 per cent. The diol acid was prepared from the diol ester, as indicated for the acid, with a yield of over 80 per cent. The acid, diol ester and diol acid derivatives were purified by h.p.l.c. in a Waters Associates, Inc. instrument fitted with a μBondapak-C_{18} column (30 × 0.39 cm). The elution solvent was 62% methanol in H_2O and the flow rate, 2 ml/min at 2000 psi. Fractions of 1 or 2 ml were collected into scintillation vials either for obtaining the purified compounds or for radioactivity measurements with Bray's [11] scintillation mixtures. The absorbance was monitored continuously at 242 nm using a Varian Varichrome spectrophotometer.

The mercapturic acid derivative of [^{3}H]JHI was prepared enzymatically as follows: 0.5 μCi of [^{3}H]JHI (specific activity, 14.7 mCi/m-mole) was incubated with the supernatant fluid of a rat liver (6 g) homogenate prepared in 0.1 M KH_2PO_4, pH 7.2, resulting after centrifugation at 105,000 *g* for 60 min in the 60 Ti rotor of a L2-65B Beckman centrifuge. Prior to incubation, the cytosolic fraction was dialyzed against 10 vol. of the same medium twice. Incubation was carried out at 30° for 120 min in the presence of 0.98 mM reduced glutathione (GSH). The mixture was adjusted to pH 5.0 by the addition of 2 M sodium acetate buffer, pH 5, and extracted with benzene to remove unreacted [^{3}H]JHI. The aqueous phase was concentrated in a flash evaporator at room temperature to eliminate any residual benzene. About 60 per cent of the radioactivity remained in the aqueous phase. Thin-layer chromatography of the water-soluble material, using as solvent *n*-butanol–acetic acid–H_2O (11:4:5), revealed a radioactive compound with an R_f value of 0.15 which was ninhydrin positive. A similar compound was obtained by incubating [^{3}H]JHI with GSH at room temperature overnight in 50% acetone and 0.5% $NaHCO_3$ [12], and it was concluded that both the enzymic and non-enzymic products corresponded to the glutathione–[^{3}H]JHI conjugate [GS–[^{3}H]JHI]; however, the yield of the chemical synthesis was extremely low, and the enzymatic procedure was used routinely. The GS–[^{3}H]JHI conjugate obtained enzymically was incubated with a rat kidney homogenate, as described by Booth *et al.* [13], and the cysteinyl–[^{3}H]JHI product was purified by adsorption onto activated charcoal followed by elution with methanol–ammonia mixture (9:1). The compound had an R_f of 0.91 in t.l.c. using *n*-butanol–acetic acid–water (11:4:5) as solvent. Acetylation of the cysteinyl-[^{3}H]JHI derivative was made with excess acetic anhydride–glacial acetic acid (1:1) at room temperature overnight. The material was concentrated to dryness in a flash evaporator at room temperature and the radioactivity was recovered in pure methanol for h.p.l.c. or t.l.c. analysis. The acetylated compound gave an R_f of 0.85, using as solvent *n*-butanol–propanol- 2 N NH_4OH (2:1:1).

Preparation of hepatocytes. Male Sprague–Dawley rats (130–150 g), fed *ad lib.*, were used throughout. Hepatocytes were obtained by a modified collagenase procedure [14]. The liver was perfused *in situ* with 150 ml of Ca^{2+}-free Hanks' solution at the rate of 30–40 ml/min; after hepatectomy, perfusion was continued with recycling for 10–15 min with minimum essential medium (MEM, F_{12}) containing 2% BSA and 0.05% collagenase at the same rate as above. All perfusion media were maintained at 37° and bubbled with 95% O_2–5% CO_2. The liver was then transferred to a Petri dish and disintegrated further with 20 ml of Ca^{2+}-free Hanks' solution containing 2% BSA. The cell suspension was filtered through a piece of nylon stocking, and intact cells were separated from broken cells and cellular debris by differential centrifugation at low speed. The intact cells were washed three times with 20 vol. of Ca^{2+}-free Hanks' solution containing 2% BSA and collected by low speed centrifugation. The cells were resuspended in the proportion of 5 to 6 × 10^6 cells or 5.25 to 6.3 mg of cell protein/ml of the same medium. Protein was determined by the Folin procedure [15], previously washing the cells three times with Ca^{2+}-free and BSA-free Hanks' solution, and homogenization in the same medium using a hand-driven Teflon–glass homogenizer. Trypan blue staining was used to check the viability of the cells [14], and preparations containing less than 10% stained cells were used routinely. Cytochrome P-450 was measured by the procedure of Omura and Sato [16] using intact cell suspensions and dithionite as reducing agent. The cells contained 0.23 nmole cytochrome P-450/mg of protein and no cytochrome P-420 could be detected. On the assumption that about 20 per cent of the cell protein corresponds to the microsomal fraction, the level of cytochrome P-450 would be approximately 1.15 nmoles/mg of microsomal protein.

Reaction mixtures and analysis of metabolites. Reactions were carried out in small pyrex conical tubes. Incubation mixtures in a final volume of 8–10 ml contained 1.75 × 10^6 cells/ml and 1.46 μM [^{3}H]JHI (specific activity, 14.7 mCi/m-mole). The reaction was started by the addition of the hormone. Incubation was carried out at 37° with gentle shaking (50 oscillations/min). At timed intervals indicated in the figures and Table 1, aliquots of 1–2 ml were withdrawn and centrifugation was carried out immediately at 1500 *g* × 10 sec in a top bench centrifuge to separate cells and supernatant fluid. The latter was adjusted to pH 5.0 by the addition of an equal volume of 2 M sodium acetate buffer, pH 5.0, and extracted with 1 vol. benzene three times. Immediately after removal of the supernatant fluid, 1 ml benzene was added to the pelleted cells which were then thoroughly dispersed, followed by 1 ml of Ca^{2+}-free Hanks' solution and 1 ml of sodium acetate buffer, pH 5.0. Then the cells were extracted thrice with benzene, as above. Aliquots of the benzene extracts from both the supernatant fluid and the pelleted cells were analyzed for radioactivity using Bray's scintillation mixture [11] in a Beckman LC-230 liquid scintillator. Corrections for quenching were done using either an external or an internal standard. The benzene extracts were concentrated to dryness under a stream of N_2 and the residue was resuspended in a minimum volume of 100% methanol. Aliquots of the above extracts were used for either h.p.l.c. or t.l.c.

analysis.

The aqueous phase remaining after the benzene extraction of both the supernatant fluid and the cells was flushed with N_2 to eliminate any remaining benzene, treated with 400 units of β-glucuronidase for 24 hr at room temperature, and then extracted with benzene thrice. The benzene extracts were pooled and concentrated to dryness under N_2. The residue was dissolved in 100% methanol for further analysis. To the aqueous phase remaining after β-glucuronidase treatment, 60 units sulfatase were added and incubation and extraction carried out as for β-glucuronidase. Adequate controls showed that no detectable cleavage occurred in the absence of β-glucuronidase and sulfatase respectively. The water phase remaining after sulfatase treatment was lyophilized and the residue was taken up in 100% methanol. The methanol extract was centrifuged at 10,000 *g* for 10 min at 2–4°, and the precipitate was discarded. The methanol was then concentrated to dryness under N_2 and the residue was redissolved in about 5 ml of H_2O; the extract was acidified with glacial acetic acid and activated charcoal was added. All the radioactivity was adsorbed onto the charcoal. The latter was washed twice with diluted acetic acid and the radioactivity was extracted with methanol containing 15% concentrated ammonia [13]. The final extract was concentrated in a flash evaporator at room temperature and the residue was redissolved in 100% methanol for either h.p.l.c. analysis in the μBondapak-C_{18} column, using 62% or 40% methanol in water as solvent, or t.l.c. analysis. The latter was done on silica gel plates (20 × 20 cm) using as solvents 25% ethyl acetate in *n*-hexane for the organic-soluble metabolites and *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1) or *n*-butanol–acetic acid–H_2O (11:4:5) for the water-soluble metabolites. Radioactive spots were localized on the t.l.c. plates either by radioautography [17] or by transferring quantitatively the silica gel at 1-cm intervals into scintillation vials for radioactivity measurements in Bray's [11] mixture. All spectrophotometric procedures were done in a Unicam SP-1800 spectrophotometer using 1-ml capacity cuvettes with a light-path of 1 cm. All the results reported correspond to an average of four to seven closely agreeing separate experiments. Recoveries were essentially quantitative, 95 per cent or more.

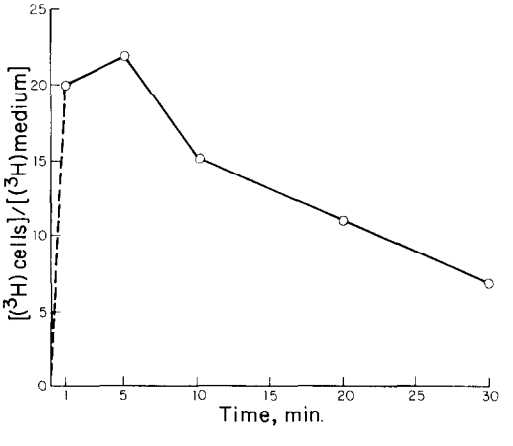

Fig. 1. Distribution of radioactivity of [^{3}H]JHI between isolated hepatocytes and their extracellular medium. See Materials and Methods for further details.

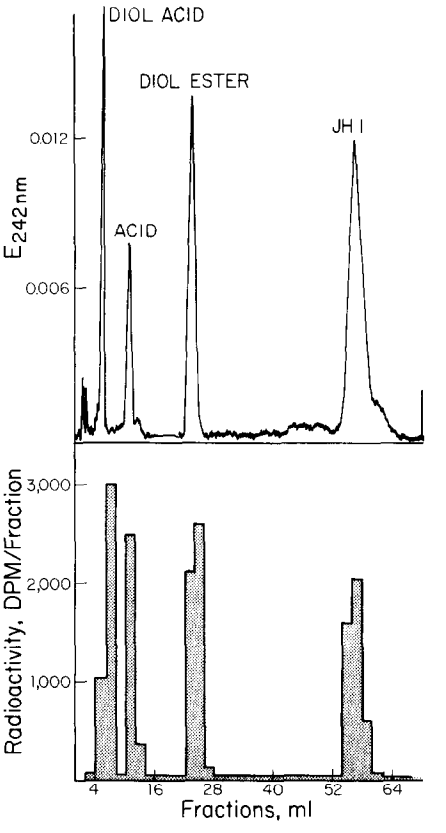

Fig. 2. High pressure liquid chromatography elution profile of [^{3}H]JHI and synthetic derivatives. The upper portion corresponds to the absorbance profile at 242 nm and the lower portion to the radioactivity profile. Chromatography was carried out in a μBondapak-C_{18} column (30 × 0.39 cm) using 62% methanol in H_2O as eluant at a flow rate of 2.0 ml/min.

RESULTS

Intracellular entry of [^{3}H]JHI. Figure 1 shows the rate of penetration of [^{3}H]JHI into isolated hepatocytes, expressed by the ratio of intracellular to extracellular concentrations of tritium. The data are based on the relationship of 1.27×10^8 cells/ml of packed cells [14, 18]. Penetration of [^{3}H]JHI is extremely rapid and a maximum is attained within the first 5 min of incubation; the ratio tends to decline fairly rapidly afterward, suggesting that [^{3}H]JHI is being metabolized into relatively more polar derivatives which diffuse out from the cells. Changes in the cell membrane structure do not appear to be the cause of the rapid decrease in intracellular radioactivity, since the trypan blue test showed that the cells are perfectly viable after the entire incubation period. The maximal concentration within the cells corresponded to 25.14 μM, assuming homogeneous distribution, as compared to the surrounding medium where the concentration dropped from the initial 1.46 μM to 1.232 μM.

Analysis of metabolites. The h.p.l.c. elution patterns of pure [^{3}H]JHI and authentic derivatives are shown in Fig. 2. The upper part of Fig. 2 corresponds to the ultraviolet profile, while the lower part indicates the radioactivity profile. The retention times in order of decreasing polarities were diol acid, 3 min; acid,

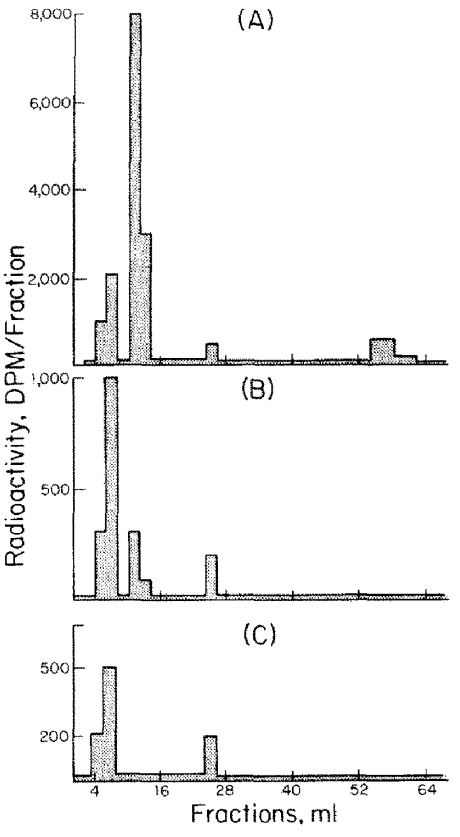

Fig. 3. High pressure liquid chromatography elution profile of ^{3}H-labeled metabolites produced by isolated hepatocytes incubated with [^{3}H]JHI for 5 min. Panel A: organic-soluble metabolites excreted into the medium; panel B: organic-soluble metabolites excreted as glucuronides; and panel C: organic-soluble metabolites excreted as sulfates. Chromatographic conditions were the same as in Fig. 2. See Materials and Methods for further details.

5.5 min; diol ester, 11.5 min; and JHI, 28 min. Due to these marked differences in retention time, the compounds were perfectly separable and easy to purify. Figure 3A shows the h.p.l.c. pattern of metabolites present in the organic-soluble fractions of the supernatant fluid after 5 min of incubation. Three peaks can be clearly distinguished having the retention times of the diol acid, acid and diol ester derivatives of [^{3}H]JHI; a fourth peak coincides with the position of [^{3}H]JHI (Fig. 2). After glucuronidase treatment of the remaining aqueous phase (Fig. 3B), the same peaks were observed, i.e. diol acid, acid and diol ester, whereas the diol acid and the diol ester were observed after sulfatase action (Fig. 3C). A similar pattern of metabolism was observed in the intracellular organic-soluble fraction, as well as after glucuronidase and sulfatase treatment. These results were confirmed by t.l.c., as shown in Table 1; however, it should be pointed out that the relative mobilities of the diol acid and the acid were reversed in t.l.c. when compared with the corresponding polarities in h.p.l.c. (Fig. 2).

In both the cells and the extracellular medium, a considerable amount of radioactivity was present in the aqueous phase after benzene extraction of the products of glucuronidase and sulfatase action. The radioactivity appeared as a single peak both by h.p.l.c. and t.l.c. Thus, the metabolite had a retention time of 4 min when eluted with 40% methanol in H_2O in the μBondapak-C_{18} column and an R_f value of 0.45 by t.l.c. using *n*-butanol–propanol–2 N NH_4OH (2:1:1) as solvent (Table 2). The metabolite and the synthetic mercapturic acid prior to acetylation had the same R_f of 0.91 with *n*-butanol–CH_3COOH–H_2O (11:4:5) as solvent, but the R_f values were slightly different when the *n*-butanol–*n*-propanol–2 N NH_4OH solvent was used. However, when both the metabolite and the synthetic mercapturic acid were acetylated, as described under Materials and Methods, both had the same R_f and could not be differentiated by co-chromatography (Table 2).

Table 1. Thin-layer chromatography of [^{3}H]JHI, synthetic [^{3}H]JHI derivatives and organic-soluble metabolites*

	R_f	
	Synthetic derivatives	Organic-soluble metabolites
JHI	0.85	0.80
Diol ester	0.29	0.25
Acid	0.39	0.36
Diol acid	0.07	0.0

* Solvent: 25% ethyl in *n*-hexane. The organic-soluble metabolites and their corresponding synthetic derivatives produced a single spot on co-chromatography.

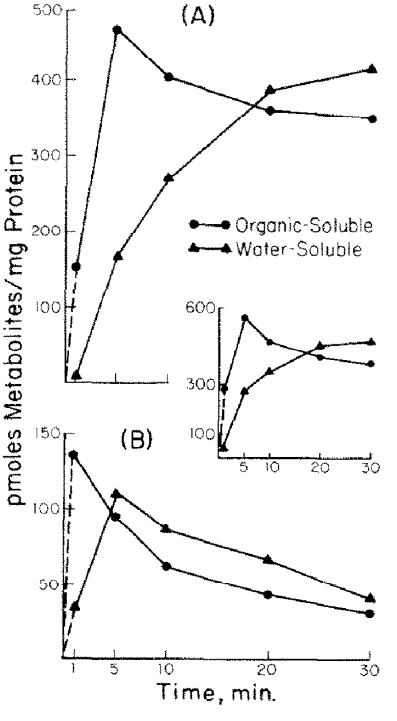

Fig. 4. Changes with time of the pattern of metabolites from [^{3}H]JHI produced by isolated hepatocytes. Panel A: extracellular metabolites; and panel B: intracellular metabolites. The inset represents the sum of the intracellular plus extracellular organic-soluble and water-soluble metabolites. See Materials and Methods for details.

Table 2. Thin-layer chromatography and high-pressure liquid chromatography characteristic of [^{3}H]JHI synthetic and metabolically produced mercapturates by isolated rat hepatocytes

Derivatives	Thin-layer chromatography*		High-pressure liquid† chromatography	
	Solvent I	Solvent II	Solvent I	Solvent II
	R_f		Retention time (min)	
Synthetic	0.91	0.55	2	3.5 (broad peak)
Metabolite	0.91	0.45	2	3.5 (broad peak)
After acetylation‡				
Synthetic		0.85	5	> 20
Metabolite		0.85	5	> 20

* Solvent I: butanol–CH_3COOH–H_2O (11:4:5). Solvent II: butanol–propanol–2 N NH_4OH (2:1:1).

† Solvent I: 62% methanol in water. Solvent II: 40% methanol in water.

‡ See Materials and Methods.

[^{3}H]JHI *metabolism as a function of time.* Time courses of production of [^{3}H]JHI metabolites with suspensions of hepatocytes are shown in Fig. 4. The pattern of changes as shown was typical for all the experiments, that is, a rapid increase during the early phase of incubation of organic-soluble metabolites in the extracellular medium (Fig. 4A) was followed by a decline which was paralleled by an increase of water-soluble metabolites outside the cells. Inside the cells, there was a rapid increase of organic-soluble metabolites within min 1 of incubation, followed by a rapid decline, whereas the water-soluble metabolites increased during the first 5 min of incubation and then declined as they diffused out of the cells (Fig. 4B) The data in the inset of Fig. 4 correspond to the intracellular and extracellular total formation of organic-soluble and water-soluble metabolites and show clearly that, as the organic-soluble metabolites (primary metabolites) are either recycled or conjugated with a concomitant decrease after 5 min of incubation, the water-soluble metabolites (conjugates) continue to increase with time.

The data of Table 3 typify the metabolite pattern both during the initial phase of incubation (1–5 min), when the concentration of [^{3}H]JHI appears not to be rate-limiting, and for longer periods of incubation up to 30 min, where over 99 per cent of the [^{3}H]JHI has been metabolized. In min 1 of incubation, about 25 per cent of the [^{3}H]JHI present in the surrounding medium was metabolized, and over 90 per cent was transformed after 5 min. The main extracellular metabolites during the first phase were the acid and the diol acid, while the diol ester, in much smaller amounts, decreased during the entire incubation period. In the latter phase (5–30 min), the diol acid production was essentially constant, while the acid decreased. All the water-soluble metabolites were in fairly low amounts during min 1 of incubation but increased rapidly as time elapsed. Seventy-two per cent of the glucuronide derivative corresponded to the diol acid after 5 min and this figure increased slightly to 79 per cent after 15 min. The acid and the diol ester also were conjugated with glucuronide, but their amounts were much lower and decreased

Table 3. [^{3}H]JHI metabolic pattern in isolated hepatocytes at various timed intervals*

Time min	Diol acid	Acid	Diol ester	Glucuronides	Sulfates	Mercapturates	JH I
			Extracellular metabolites				
1	19.4	119.3	10.8	20	1	16	510.4
5	98.8	356.8	8.5	59	30	75	43.3
10	105.4	295.2	3.8	89	44	144	9.7
20	74.3	281.8	3.9	103	54	198	3.3
30	104	245.6	2.3	100	57	233	0.9
(Glucuronides)							
5	42.5	12.1	4.3				
15	75.3	16.9	2.9				
(Sulfates)							
20	42.7	0	11.3				
			Intracellular metabolites				
1	49.7	84.1	0.9	10	7	31	16.1
5	46.5	49.9	0.7	18	10	94	2.1
10	22.9	38.3	0	11	7	74	0.9
20	19.2	27.8	0	7	4	57	1.0
30	10.6	20.7	0	5	3	40	0.5

* All values are expressed as pmoles/mg of protein. Data were obtained from h.p.l.c. analysis.

with time. As far as the sulfate conjugates were concerned, most corresponded to the diol acid (79 per cent) and a smaller percentage to the diol ester. As expected, the acid derivative was not conjugated with sulfate. The pattern of metabolism inside the cell (Table 3) shows that, during min 1 of incubation, 92 per cent of the intracellular [^{3}H]JHI already is metabolized, while over 99 per cent is metabolized at longer periods of incubation. Most of the metabolic conversion of [^{3}H]JHI during min 1 was to diol acid and acid, but their concentrations decreased afterward. Relatively smaller amounts of the glucuronide and sulfate conjugates were found and their concentrations decreased with time. Inside the cells, the main conjugation derivative and the main metabolite, as well, corresponded to mercapturic acid. The latter increased dramatically during the early phase (1–5 min), and later its concentration decreased as it diffused outside the cells.

DISCUSSION

Suspensions of rat hepatocytes metabolized [^{3}H]JHI to several organic-soluble and water-soluble metabolites with patterns somewhat resembling those described for JH I and juvenoids in whole insects [9, 19]. The possibility that some metabolites in insects could correspond to conjugates has been advanced, but their type and nature have not been reported in insects or in mammals [2,20]. In the present report, we have shown that glucuronide, sulfate, and mercapturate are conjugates produced during [^{3}H]JHI metabolism by hepatocytes. The apparent discrepancies observed during the chromatographic identification of mercapturate (Table 2) may be interpreted as follows. The synthetic mercapturic acid obtained, as indicated under Materials and Methods, was not *N*-acetylated, and this could only be accomplished by treatment with acetic anhydride; however, acetylation was apparently not restricted to the amino group of the cysteinyl residue, but esterification of the hydroxyl group resulting from conjugation of GSH with the [^{3}H]JHI epoxide also occurred. On the other hand, hepatocytes produced the *N*-acetylated mercapturate, and as a result, the R_f values for both the enzymic and the synthetic mercapturate were slightly different. Chemical acetylation of the hepatocyte-produced mercapturate only resulted in the esterification of the hydroxyl group formed by the attack of GSH on the epoxide ring; both the synthetic and the mercapturate produced by the hepatocytes became the same, with similar chromatographic behaviour by h.p.l.c. and t.l.c. (Table 2). The data also suggest that the glutathione conjugate may correspond to a C_{11}- rather than a C_{10}-derivative (Fig. 5). Whether the formation of the GS–[^{3}H]JHI conjugate in the cytosolic fraction of the hepatocytes is catalyzed mainly by GSH-transferases or whether non-enzymic processes also occur, followed by enzymic cleavage of the glutamyl and glycyl residues and *N*-acetylation of the cysteinyl residue, cannot be ascertained at present; however, the very low yields obtained in the chemical synthesis of the GS–[^{3}H]JHI derivative suggest that the process is mainly enzymatic.

No evidence of metabolic attack by the NADPH-linked cytochrome P-450 system under the present conditions could be observed and the only microsomal enzymes involved in [^{3}H]JHI metabolism are epoxide hydrase, UDP-glucuronosyltransferases, and possibly esterases.

It is evident from the results shown in Fig. 4 that the pattern of organic-soluble metabolites changes with the duration of the reaction and that eventually all the added [^{3}H]JHI becomes metabolized to water-soluble and other relatively more polar organic-soluble metab-

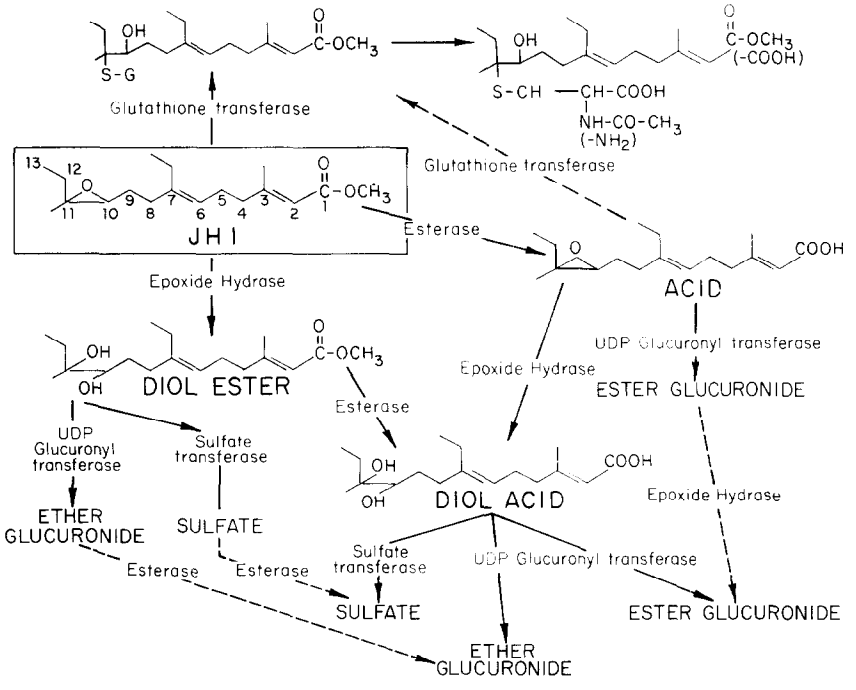

Fig. 5. Proposed metabolic pathways of JHI in isolated hepatocytes. Assignment of a C_{11}-configuration to the glutathione-conjugate is tentative. The dotted lines indicate alternative pathways.

olites. This implies that the organic-soluble metabolites released initially from the cells are recycled by re-entry and further metabolism. Conjugation with glucuronide is substantiated by the effect of β-glucuronidase and is not limited to the diols but also occurs at the carboxylic moiety of the acid derivative (Table 3). Sulfate conjugation, on the other hand, is restricted to the diol ester and the diol acid and seems to be less important than the glucuronide pathway. A proposed pathway of JHI metabolism in isolated rat hepatocytes is shown in Fig. 5. It is obvious from the data shown that further metabolic interconversions are possible. Thus, not only the hormone itself may be conjugated with GSH, but also the acid. Similarly, the GS–JH I conjugate may be transformed later on into the GS-acid derivative by the action of esterases. Other interconversions may occur at the glucuronide level; thus, the ester glucuronide of the acid may become the ester glucuronide of the diol acid by the action of epoxide hydrase. Other possibilities involve conversion of the sulfate conjugate from the diol ester to the diol acid. As a result, what appears to be a relatively simple metabolic conversion of JH I to more polar compounds is really a very complex process whereby, through different pathways, it is possible to obtain the same products.

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