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BIOCHEMICAL EFFECTS OF 3,5-DIETHOXYCARBONYL-1, 4-DIHYDROCOLLIDINE IN MOUSE LIVER*

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Abstract—3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) is a porphyrinogenic agent and is a powerful inducer of δ-aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem-biosynthetic pathway, in mouse liver. However, DDC strikingly inhibits mitochondrial as well as microsomal haem synthesis by depressing the activity of ferrochelatase *in vivo*. The drug on repeated administration to female mice has been found to elicit hypertrophic effects in the liver microsomes initially, but the effects observed at later stages denote either hyperplasia or increase in polyploidal cells. The microsomal protein concentration shows a striking decrease with repeated doses of the drug. The rate of microsomal protein synthesis *in vivo* as well as *in vitro* shows an increase with two injections of DDC but decreases considerably with repeated administration of the drug. The activities of NADPH-cytochrome c reductase and ribonuclease are not affected in the liver microsomes of drug-treated animals when expressed per mg of microsomal protein. DDC has also been found to cause degradation of microsomal haem, which is primarily responsible for the decrease in cytochrome P-450 content. The drug also leads to a decrease in mitochondrial cytochrome c levels due to inhibition of haem synthesis and also due to degradation of mitochondrial haem at later stages. The biochemical effects of the drug are compared and discussed with those reported for allylisopropylacetamide and phenobarbital.

A VARIETY of foreign chemicals which are metabolized on liver microsomes by the mixed function oxidase system have profound effects on the hepatic haem-biosynthetic pathway.[1,2] Most of them elevate the levels of δ-amino laevulinate (ALA) synthetase, the rate-limiting enzyme of the haem-biosynthetic pathway.[3–5] It has been postulated that this results in an increased rate of haem synthesis, eventually leading to enhanced cytochrome P-450 content and drug metabolism.[4,6,7] Drugs such as phenobarbital and allylisopropylacetamide, in addition to affecting the haem-biosynthetic pathway, also exert a general hypertrophic effect, especially of the endoplasmic reticulum in the liver.[8–12] Puromycin and actinomycin-D, which block ALA synthetase induction due to allylisopropylacetamide, also prevent the attendant hypertrophic phenomenon.[13] Haematin counteracts the phenobarbital-mediated increase in ALA synthetase levels as well as the increases in liver weight, microsomal protein and phospholipid contents.[7] These studies indicate a possible correlation between an enhanced rate of haem synthesis and hypertrophy. However, it has also been reported that 3-amino-1,2,4-triazole, an inhibitor of haem synthesis, prevents phenobarbital-mediated increase in cytochrome P-450 levels without significantly affecting the other biochemical and morphological changes characteristic of cellular hypertrophy.[14] Detailed studies in this laboratory with allylisopropylacetamide have shown

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that the drug may have independent effects on haem synthesis and other anabolic processes.[15] This, however, does not rule out the possibility that the drugs may influence the rate of synthesis of specific haemoproteins by virtue of their effects on the rate of haem synthesis.

3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) is a powerful inducer of ALA synthetase in mice[16,17] and the porphyrin accumulation pattern observed is similar to that of human variegate porphyria.[18] While information on the effects of DDC on hepatic haem synthesis[19] and pattern of ALA synthetase induction[20] is available, not much is known about the general biochemical effects of the drug. An interesting feature of DDC action is that it is a powerful inhibitor of haem synthesis by virtue of its depressing effect *in vivo* on the activity of ferrochelatase,[19] although the drug is a potent inducer of ALA synthetase. In the present study, the general biochemical effects of DDC in mouse liver have been discussed in relation to its effects on the haem-biosynthetic pathway. These results are compared to those reported for phenobarbital and allylisopropylacetamide.

METHODS

Chemicals. DDC was synthesized by the method of DeMatteis and Prior.[21] The final product was recrystallized twice from ethanol-water and melted at 128°. Other biochemicals were purchased from commercial sources. ^{14}C-chlorella hydrolysate (13 mCi/m-atom), ^{59}Fe-chloride (19·1 μCi/μg) and [U-^{14}C]leucine (120 mCi/m-mole) were purchased from Bhabha Atomic Research Centre, Bombay. [4-^{14}C]ALA was obtained from Amersham, Bucks, U.K.

Treatment of animals. Female mice (23–25 g) of the local Institute strain were used in all the experiments. The animals were fed *ad lib.* on stock diet obtained from Hindustan Lever Ltd., Bombay. DDC was dissolved in coconut oil and was given subcutaneously at a dose of 400 mg/kg. Haemin was dissolved in 0·1 N KOH and made up to the volume with 0·05 M potassium phosphate buffer, pH 7·5. It was given intraperitoneally at a dose of 2 mg/100 g. ALA HCl was injected in three doses of 5 mg/animal each time at intervals of 12 hr. In experiments involving different periods of drug treatment, the schedule of injections was started on different days such that all the animals were ready for sacrifice on the same day and time. This was done to avoid data variation that could arise as a result of the biochemical analyses being performed on different days.

Treatment of the livers. The animals were killed by decapitation, the livers were removed and their fresh weights recorded. They were then homogenized with 4 vol. of 0·25 M sucrose in 0·01 M Tris-HCl buffer, pH 7·4. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant was spun at 10,000 *g* for 15 min. The sediment was washed twice with 0·25 M sucrose and was used as the mitochondrial fraction. The 10,000 *g* supernatant was spun at 20,000 *g* for 10 min and the pellet was discarded. The supernatant was spun at 105,000 *g* for 60 min and the pellet after washing was suspended in 0·25 M sucrose so as to contain microsomes from 1 g liver in 1 ml. Portions of this suspension were used for the determination of microsomal protein, RNA and Phospholipid contents, as well as ribonuclease activity.

Determination of cytochrome P-450 and cytochrome c. For the estimation of cytochrome P–450, the livers were homogenized with 1·15% KCl and microsomes were iso-

lated as described earlier. The pelleted microsomes were rinsed with 1·15% KCl and suspended in 0·05 M potassium phosphate buffer, pH 7·5. Cytochrome P-450 content was estimated according to the method of Omura and Sato,[22] from the CO-difference spectrum of dithionite-reduced preparations using an extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm.

Cytochrome c content was measured in solubilised mitochondrial suspensions by measuring the difference spectra between dithionite-reduced and ferricyanide-oxidized aliquots using a Cary 14 spectrophotometer.[23]

Enzyme assays. Microsomal ribonuclease was assayed according to the method of Louis-Ferdinand and Fuller.[24] The incubation mixture contained: 0·2 ml of 0·2 M Tris-HCl buffer, pH 7·6, 1 mg of highly polymerized RNA and 6–8 mg of microsomal protein in a final volume of 1 ml. RNA was added at the end and the incubation was carried out in air for 30 min, at 37°. The reaction was arrested by the addition of 1 ml of 1 M HCl in 75% ethanol and the mixture was kept in ice for 30 min. After centrifugation, the optical density of the suitably diluted supernatant was measured at 260 nm. Appropriate controls were employed for the endogenous contribution of acid-soluble nucleotides.

NADPH–cytochrome c reductase was estimated as described by Masters *et al.*,[25] wherein the rate of reduction of cytochrome c was followed at 550 nm using a Cary 14 spectrophotometer.

Aniline hydroxylase was measured by the method of Imai *et al.*[26] For the assay, microsomes isolated from livers homogenized in 1·15% KCl were used. The reaction mixture contained: 8 mM aniline, 0·32 mM NADP, 3 mM glucose 6-phosphate, 2·5 mM $MgCl_2$, 1·3 units of glucose 6-phosphate dehydrogenase, 100 mM Tris-acetate buffer, pH 8·0, and 3–4 mg of microsomal protein in a total volume of 1 ml. The reaction was carried out for 20 min at 37° aerobically and stopped by the addition of 0·5 ml of 20% trichloroacetic acid. The *p*-aminophenol formed was measured in 1 ml of the supernatant by adding 0·5 ml of 10% sodium carbonate and 1 ml of 2% phenol in 0·2 N NaOH. The resulting blue colour was measured at 630 nm after standing for 30 min.

Measurement of the rate of protein synthesis in vivo. The various groups of animals were injected intraperitoneally with ^{14}C-chlorella hydrolysate (2 μCi/animal). After 45 min, they were killed by decapitation and the livers were removed. The microsomes were isolated from the 0·25 M sucrose homogenate after layering 5 ml of post-mitochondrial supernatant over 5 ml of 1 M sucrose and carrying out the centrifugation at 125,000 *g* for 90 min. The mitochondrial and microsomal pellets were washed and suspended in 0·25 M sucrose. Mitochondrial, microsomal as well as soluble proteins were precipitated with trichloroacetic acid (5% w/v in final concentration). The precipitates were washed once with hot trichloroacetic acid, at 90° for 20 min, twice with cold trichloroacetic acid, once with 2:1 (v/v) mixture of ethanol–ether and finally with ether. The dry residue was dissolved in 1 ml formic acid. A 0·5-ml portion was applied onto Whatman No. 3 filter paper discs for measurement of radioactivity. Protein was estimated in suitably diluted samples after neutralization.

Determination of the rate of microsomal protein synthesis in vitro. The method described by Munro *et al.*[27] was employed for this purpose. The incubation mixture contained 0·1 ml of microsomal suspension, 0·2 ml of the soluble factors (pH 5 fraction and supernatant factors) and 0·2 ml of a mixture containing the following: 19 amino

acids except leucine, 0·1 μmole each; ATP, 5 μmoles; PEP,* 2·5 μmoles; $MgCl_2$, 2·5 μmoles; β-mercaptoethanol, 0·2 μmole; GTP, 1 μmole; pyruvate kinase, 25 μg; and [U-^{14}C]leucine, 1 μCi. The reaction was started by the addition of microsomes and the incubation was carried out at 37° for different intervals of time. The reaction was terminated by the addition of 5% trichloroacetic acid containing 0·2% cold leucine. The precipitate was processed for measurement of radioactivity as described earlier.

Effect of DDC on the rate of total haem synthesis in vivo. $^{59}FeCl_3$ (5 μCi/animal) was injected intraperitoneally into mice 6 or 12 hr after they had received the last injection of the drug. The animals were killed 1 hr after the tracer administration. Livers were pooled after perfusion with saline and then homogenized with 0·25 M sucrose. Portions of the homogenates were used for the isolation of mitochondria and microsomes. The homogenate, mitochondrial and microsomal preparations were then treated in the cold successively with excess acetone, methanol–chloroform (2:1, v/v) and once again with acetone to remove the lipids. The final preparations were suspended in water and treated with 5 vol. of ethylacetate–acetic acid (3:1, v/v) and centrifuged. The supernatant was washed with water, 3 N HCl and again with water. The ethylacetate layer was directly used for the measurement of radioactivity in haem. The method is based on the one described by Levin *et al.*[28] Chromatography [29] of the ethylacetate layer from all the preparations revealed that the entire radioactivity is associated with the haem spot.

Effect of DDC on the degradation of microsomal haem. Mice were injected with [4-^{14}C]ALA (2 μCi/animal) intraperitoneally. Six hr after the tracer administration, the animals were injected with DDC (400 mg/kg) subcutaneously. The animals were then killed at different intervals of time after DDC administration, and the livers were processed for the isolation of microsomal haem as described earlier. Portions of the final ethylacetate layer were directly transferred to counting vials, evaporated to dryness and then used for radioactivity measurements. Direct estimation of the protohaem content in the microsomal suspensions revealed that the recovery of haem in the final preparation is consistently around 71 ± 2 per cent.

Effect of DDC on the degradation of mitochondrial haem. One group of mice were injected with [4-^{14}C]ALA (2 μCi/animal) intraperitoneally and, 4 hr after the tracer administration, the first injection DDC (400 mg/kg) was given subcutaneously. To another group which had already received two injections of DDC, each at 24-hr intervals, 4 hr after the label, the third injection of DDC was given. Another group served as the control for the determination of a normal haem degradation pattern.

The control animals, as well as the animals receiving one and three injections of DDC, were killed 1·5 hr and 3 hr after 4 hr of tracer administration. The livers were processed and radioactivity in mitochondrial haem was measured as described earlier. Direct estimation of the protohaem content in the mitochondrial suspensions revealed that the recovery of haem in the final preparation is consistently around 71 ± 2 per cent. Values are expressed as a percentage of the normal value, taking the normal value as 100 at various time intervals.

Radioactivity measurements. ^{14}C was measured in Beckman LS-100 liquid scintillation counter. The filter discs were counted in vials containing 10 ml of 0·5% 2,5-diphenyloxazole (PPO) (w/v) in toluene. When haem samples were counted, quench

* PEP, Phosphoenol pyruvate.

TABLE 1. EFFECT OF DDC ADMINISTRATION ON LIVER WEIGHT, DNA CONTENT, MICROSOMAL PROTEIN, LIPID, RNA AND PHOSPHOLIPID CONTENTS IN MOUSE LIVER*

No. and days of DDC administration	Liver wt (g/100 g body wt)	DNA (mg/g liver)	Microsomal RNA (mg/g liver)	Microsomal Protein (mg/g liver)	Microsomal Lipid (mg/g liver)	Microsomal Phospholipid (μg P/g liver)
0	3·6 ± 0·2	1·98 ± 0·06	3·1 ± 0·20	33·1 ± 1·2	34·2 ± 1·1	360 ± 10·2
2	4·2 ± 0·1	1·41 ± 0·10 (P < 0·02)	3·7 ± 0·20 (P < 0·1)	36·9 ± 2·3 (P > 0·1)	39·8 ± 0·2	440
3	5·1 ± 0·1	1·56 ± 0·05	3·2 ± 0·17	28·7 ± 1·6	35·8 ± 0·1	292
5	6·8 ± 0·3	1·98 ± 0·15	3·2 ± 0·20	20·2 ± 2·3	33·0 ± 1·1	287
7	7·2 ± 0·3	2·07 ± 0·12 (P > 0·1)	3·3 ± 0·22 (P > 0·1)	18·3 ± 1·9 (P < 0·001)	35·0 ± 1·2	300

* The animals were injected with DDC (400 mg/kg) every 24 hr. They were killed 6 hr after the final injection. The drug treatments of the different groups were started on different days such that all the animals could be sacrificed and livers processed on the same day. The control group consisted of animals which had received a different number of injections of the vehicle, namely coconut oil, and the values represent the mean ± S. D obtained from six experiments. The values for the other groups represent the mean ± S. D obtained from three experiments, in which three livers were pooled in each experiment. The phospholipid and lipid values represent an average of two experiments. The P values have been calculated with respect to the controls receiving the vehicle only.

correction was applied using the channels ratio method. [59] Fe was measured in a well-type scintillation counter attached to a decade scaler (Nuclear Chicago).

Analytical methods. Protein was estimated according to the method of Lowry *et al.*[30] using bovine serum albumin as the standard. RNA content was determined according to the method of Munro and Fleck.[31] Phospholipid content was determined by the method described by Folsch *et al.*[32] Total lipid content was determined by the method of Handler.[33]

RESULTS

Table 1 presents data on the effects of DDC administration on liver weight, microsomal protein, RNA, lipid, and phospholipid contents as well as the liver DNA content. It can be seen that there is a striking increase in liver weight with successive doses of DDC. Microsomal RNA, protein and phospholipid contents show an initial increase. The increase observed with protein content after two injections of DDC is, however, not statistically significant. With successive doses of the drug, the increases in RNA and phospholipid contents expressed per unit weight of liver are not maintained. However, RNA and phospholipid contents per liver show an increase. Microsomal protein content shows a striking decrease with successive doses of DDC when expressed per unit weight of liver and the concentration per liver remains constant. It is also interesting to observe that the liver DNA content per unit weight of liver decreases initially but subsequently rises to normal values.

TABLE 2. EFFECT OF DDC ADMINISTRATION ON NADPH-CYTOCHROME C REDUCTASE, RIBONUCLEASE AND ANILINE HYDROXYLASE ACTIVITES IN MOUSE LIVER MICROSOMES*

No. and days of DDC administration	NADPH–cytochrome c reductase (nmoles cytochrome c reduced/mg protein)	Ribonuclease (μg RNA hydrolysed/mg protein)	Aniline hydroxylase (nmoles *p*-aminophenol/mg protein)
0	23·2 ± 1·1	25·5 ± 2·0	0·10 ± 0·001
1	26·2 ± 2·2	25·0 ± 1·2	0·07 ± 0·001
	(P > 0·1)	(P > 0·1)	(P < 0·001)
3	26·6 ± 0·9	24·7 ± 1·7	0·04 ± 0·0005
	(P > 0·1)	(P > 0·1)	(P < 0·001)

* The experimental details are given in the Methods section and Table 1. The values represent the mean ± S. D obtained from three experiments in which two livers were pooled in each experiment. P values have been calculated with respect to the controls receiving the vehicle only.

The results presented in Table 2 indicate that DDC administration does not affect the microsomal ribonuclease as well as the NADPH–cytochrome c reductase activities when they are expressed per mg of microsomal protein. However, a significant decrease in aniline hydroxylase activity has been observed.

Next, it was of interest to examine the effect of DDC on the incorporation of ^{14}C-chlorella hydrolysate *in vivo* into the proteins of the different liver subcellular fractions. It can be seen from Table 3 that, after two injections of DDC, there is a significant increase in the rate of amino acid incorporation into the total microsomal proteins. However, after seven successive doses of the drug, the rate of ^{14}C-amino acid incorporation into the microsomal proteins per unit weight of liver shows a decrease.

TABLE 3. EFFECT OF DDC ADMINISTRATION ON ^{14}C-CHLORELLA-HYDROLYSATE INCORPORATION *in vivo* INTO THE PROTEINS OF THE MOUSE LIVER SUBCELLULAR FRACTIONS*

No. and days of DDC administration	Microsomes (cpm/g liver)	Mitochondria (cpm/g liver)	Supernatant (cpm/g liver)
0	8407 ± 270	8425 ± 150	10445 ± 311
2	10455 ± 111 (P < 0·02)	9106 ± 59 (P < 0·05)	10400 ± 273 (P > 0·1)
3	8731 ± 292 (P > 0·1)	7996 ± 119 (P < 0·1)	8975 ± 385 (P > 0·1)
7	6940 ± 156 (P < 0·05)	7291 ± 296 (P < 0·05)	12570 ± 103 (P < 0·02)

* The experimental details are given in the Methods section and Table 1. The values represent the mean ± S. D obtained from three experiments in which two livers were pooled in each experiment. P values have been calculated with respect to the controls receiving the vehicle only.

This picture can also be confirmed in cell-free studies where the U-^{14}C leucine-incorporating ability by microsomes isolated from animals receiving two and five injections of DDC was compared. It can be seen from Fig. 1 that, whereas the microsomes isolated from animals receiving two DDC injections show a greater amino acid-incorporating ability as compared to the controls, the microsomes isolated from ani-

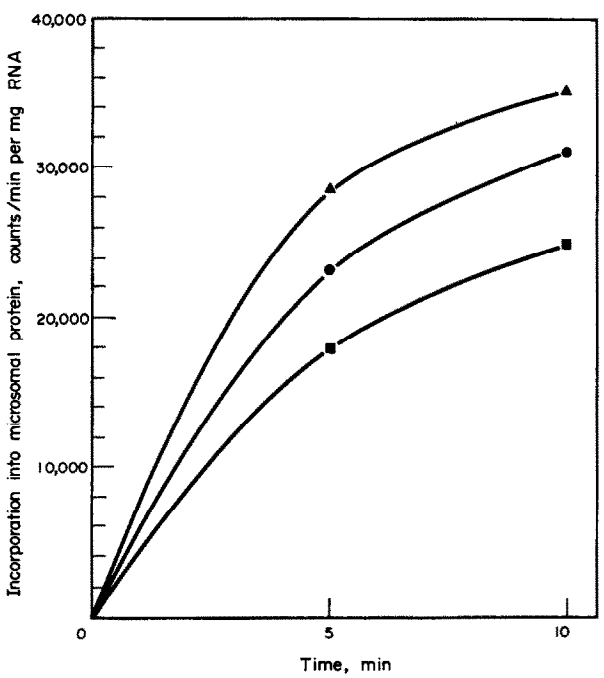

FIG. 1. Amino acid incorporation by isolated microsomes from DDC-treated mouse liver. The experimental details are given in the Methods section and Table 1. The values represent an average of two experiments. Key; ●, control; ▲, two injections of DDC; ■, five injections of DDC.

TABLE 4. EFFECT OF DDC ADMINISTRATION ON ^{59}Fe INCORPORATION INTO MITOCHONDRIAL AND MICROSOMAL HAEM OF MOUSE LIVER*

No. and days of DDC administration	Homogenate (cpm/g liver)	Mitochondria (cpm/g liver)	Microsomes (cpm/g liver)
0	2125	700	1200
1 (6 hr)	1726	583	921
1 (12 hr)	1313	493	721
2 (6 hr)	1034	368	330

* Five μCi of ^{59}Fe-citrate was injected into each animal. The other experimental details are given in the Methods section. The values given in parantheses in Column 1 represent the time at which the animals were killed after the final DDC injection. The values represent the average of two experiments in which two livers were pooled in each experiment.

mals receiving five DDC injections show a decreased capacity for cell-free protein synthesis.

It has already been mentioned that DDC depresses ferrochelatase levels *in vivo* in mice.[19] The consequence of this effect has been examined on the rate of haem synthesis in mitochondria and microsomes. The results presented in Table 4 indicate that, 6 hr after DDC administration, a small but consistent inhibition of ^{59}Fe incorporation into mitochondrial and microsomal protohaem is discernible. At 12 hr after DDC administration, the inhibition observed is significant, and this situation gets accentuated with a second injection of the drug. The results presented in Table 5 indicate that a striking decrease has been observed in cytochrome P-450 levels after a

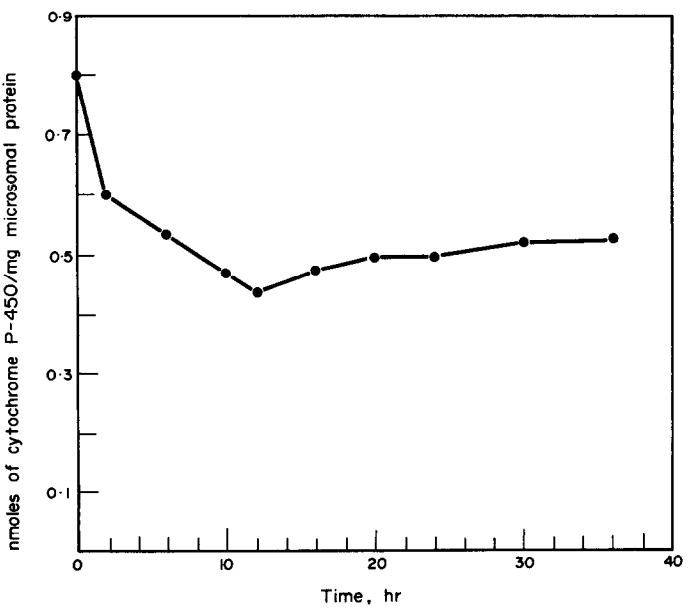

FIG. 2. changes in the levels of cytochrome P-450 content in mouse liver after a single injection of DDC. The experimental details are given in the Methods section. The animals were fasted for 36 hr before killing. The values represent an average of two experiments.

TABLE 5. EFFECT OF DDC ADMINISTRATION ON CYTOCHROME P-450 AND CYTOCHROME C CONTENTS OF MOUSE LIVER*

No. and days of DDC administration	Cytochrome P-450 (nmoles/mg microsomal protein)	Cytochrome c (nmoles/mg mitochondrial protein)
0	0·81 ± 0·02	0·12 ± 0·001
1	0·62 ± 0·01 ($P < 0·01$)	0·12 ± 0·002
3	0·51 ± 0·04 ($P < 0·01$)	0·05 ± 0·002 ($P < 0·001$)

* The experimental details are given in the Methods section and Table 1. The values represent the mean ± S. D obtained from three experiments in which two livers were pooled in each experiment. P values have been calculated with respect to the controls receiving the vehicle only.

single injection of DDC, whereas the cytochrome c levels are not affected under these conditions. However, a significant decrease in cytochrome c levels is discernible after three injections of DDC. Figure 2 indicates that the decrease in cytochrome P-450 levels can be demonstrated as early as 2 hr after DDC administration, and the levels remain depressed at least for a period of 36 hr after the drug administration. The

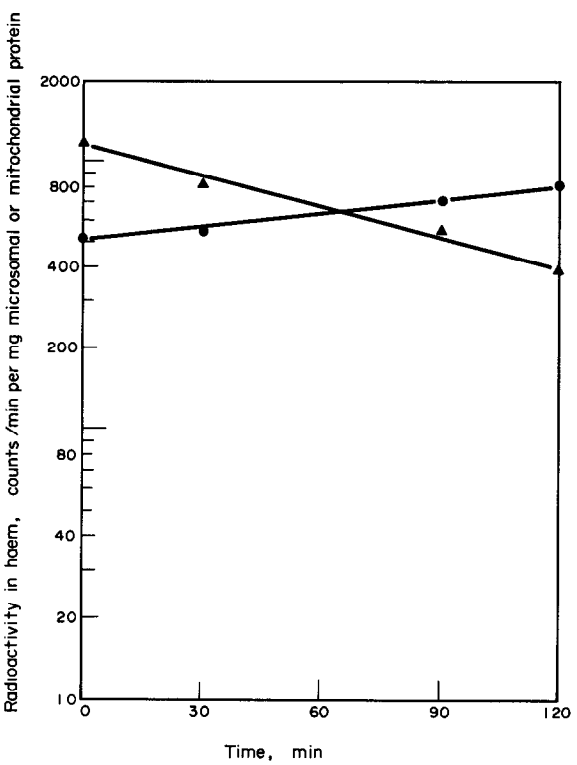

FIG. 3. Effect of DDC on the degradation of microsomal and mitochondrial haem in mouse liver. The experimental details are given in the Methods section. The values represent an average of two experiments. Key: ▲, radioactivity in microsomal haem; ●, radioactivity in mitochondrial haem.

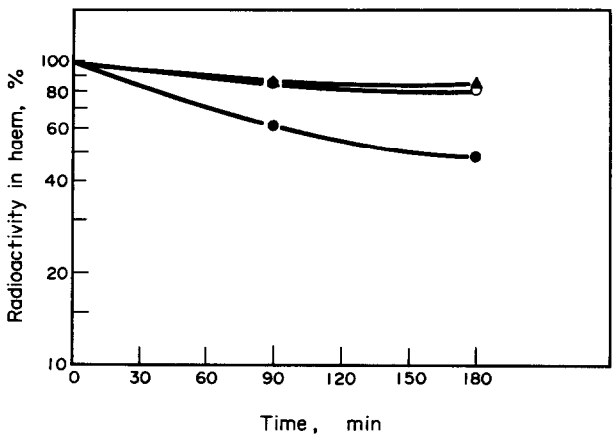

FIG. 4. Effect of DDC on the degradation of mitochondrial haem. The experimental details are given in the Methods section. The values represent an average of two experiments. The 100 per cent values represent the specific radioactivity as a percentage of total uptake, after 4 hr of labeling. Key: ▲, 100 per cent value for the degradation under normal conditions, 0·85; ○, 100 per cent value for the degradation with a single injection of DDC, 0·85; ●, 100 per cent value for the degradation with three injections of DDC, 0·0896. The difference in 100 per cent values between the normal or single injection of DDC and three injections of DDC represents the inhibition of heme synthesis (90 per cent) observed with three injections of DDC.

possibility that the initial decrease in cytochrome P-450 levels could be due to the breakdown of haem was investigated as the same has been reported in the case of allylisopropylacetamide treatment.[34,35] The results presented in Fig. 3 indicate that DDC does cause a loss of radioactive haem from microsomes. DDC does not cause degradation of mitochondrial haem with a single injection, but subsequently with three injections, it brings about degradation of mitochondrial haem as indicated in Fig. 4. To further prove that the initial decline in cytochrome P-450 levels is due to

TABLE 6. EFFECT OF HAEM ON DDC-MEDIATED CHANGES IN CYTOCHROME P-450 AND CYTOCHROME C CONTENTS OF MOUSE LIVER.*

Treatment	Cytochrome P-450 nmoles/mg microsomal protein)	Cytochrome c (nmoles/mg mitochondrial protein)
Vehicle	0·80 ± 0·02	0·11 ± 0·002
DDC (3 injections)	0·49 ± 0·01	0·04 ± 0·001
	(P < 0·01)	(P < 0·001)
DDC + haem	0·48 ± 0·01	0·08 ± 0·002
(3 injections)	(P > 0·1)	(P < 0·01)
Haem (3 injections)	0·72 ± 0·03	0·16 ± 0·003
	(P < 0·1)	(P < 0·01)
ALA (3 injections)	0·74 ± 0·03	0·14 ± 0·004
	(P < 0·1)	(P < 0·02)

* DDC (400 mg/kg) and haem (1 mg/animal) were injected every 24 hr. The animals were killed 6 hr after the last injection. ALA (5 mg/animal) was injected at 2-hr intervals and the animals were killed 6 hr after the last injection. The values represent the mean ± S. D obtained from three experiments in which three livers were pooled in each experiment. The P values were calculated with respect to the control receiving the vehicle only, except in the case of DDC ± haem treatment where the P value given in the columns for cytochrome P-450 and cytochrome c has been calculated with respect to the values recorded for DDC treatment.

haem breakdown and not to inhibition of haem synthesis, the effect of exogenously administered haematin was studied under these conditions. The results presented in Table 6 indicate that exogenously administered haematin does not counteract the decreases in cytochrome P-450 levels due to DDC administration. It is interesting to observe that haematin does counteract the decrease in cytochrome levels due to the successive administration of DDC. It is also significant to note that administration of ALA to a normal animal increases the cytochrome c content of mitochondria, while causing a slight decrease in cytochrome P-450 levels.

DISCUSSION

There are certain interesting features concerning the general biochemical effects of DDC when compared to the results reported for allylisopropylacetamide and phenobarbital.[8–12] While allylisopropylacetamide and phenobarbital show hypertrophic effects on successive administration, as evidenced by the increase in liver weight, microsomal protein, lipid, RNA and phospholipid contents, DDC appears to exert a similar effect during initial stages which is not evident at later stages. On the other hand, there is an initial decrease in the DNA content followed by an increase with subsequent injections of DDC when the DNA content is expressed per unit weight of liver. However, there is an increase in DNA content per liver from day 0 to 7 and the increase is about 2-fold by day 7. While the increase in DNA content at later stages of drug treatment, with no transient accumulation of fat, can denote hyperplasia, it can also be due to an increase in polyploidal cells. Thus, it is possible that the increased cell division may mask any increase in RNA and phospholipid contents on a unit weight basis. The picture observed with the microsomal protein content needs an additional comment. The microsomal protein actually decreases with the progressive increase in liver weight due to DDC administration. This would mean that the rate of microsomal protein synthesis is not keeping pace with the increased cell division or that the microsomal protein is undergoing enhanced degradation. The effect of DDC on microsomal protein degradation has not been studied in the present investigation. An inhibition of microsomal protein synthesis is evident both *in vivo* (Table 3) as well as *in vitro* (Fig. 1) during the later stages of DDC administration.

The other feature of DDC administration is that it has no effect on the activities of microsomal ribonuclease and NADPH–cytochrome c reductase when they are expressed per mg of microsomal protein (Table 2). However, the activities increase per liver. This is in contrast to phenobarbital and allylisopropylacetimide which have been found to depress ribonuclease levels *in vivo*.[15,36] The decrease in ribonuclease levels in phenobarbital treatment has been attributed to be one of the reasons for the increase in RNA content under these conditions. There is a decrease in aniline hydroxylase activity even with a single injection of DDC, which becomes more pronounced after three injections. This decrease is expected, as cytochrome P-450 decreases with DDC treatment.

DDC also has some interesting effects on haemoprotein metabolism. It decreases cytochrome P-450 content immediately (Fig. 2), and a significant decrease in cytochrome c levels is observed only after the second or third injection (Table 5). The initial decrease in cytochrome P-450 levels is due to the degradation of microsomal haem

(Fig. 3), whereas the decrease at later stages can be accounted for by the inhibition of haem synthesis by DDC. The decrease in cytochrome c content is not observed with a single injection of DDC, as there is no degradation of mitochondrial haem (Fig. 4). When the mitochondrial haem degradation was followed after three injections of DDC, the degradation was evident, as indicated by Fig. 4. Thus, the fall in cytochrome c content by about 50 per cent after three injections can be explained as follows. Primarily, inhibition of haem synthesis is involved, but with successive injections, haem degradation also takes place which accounts for the large decrease. In the case of cytochrome P-450, the initial event is the degradation, followed by inhibition of haem synthesis. The recent work of Abritti and DeMatteis[37]* supports our contention. Their data indicate degradation of liver haem by DDC, and they propose that this is an additional mechanism by which the drug might potentiate its main effect on ALA synthetase, the main effect being the inhibition of haem synthesis.[37] In the case of allylisopropylacetamide, the degradation of cytochrome P-450 haem has been linked up with the induction of ALA synthetase.[34,35,38,39] As it does not inhibit haem synthesis, the cytochrome P-450 levels after showing an initial decrease regenerate back to more than normal levels.[38,40]

The fact that availability of haem is limiting for cytochrome c synthesis is shown by the data in Table 6, where exogenous haematin is able to counteract the decrease in cytochrome c levels, whereas it is not utilised for spectrally identifiable cytochrome P-450 synthesis. Exogenous haem, when given in excess, is able to counteract the effects of DDC on cytochrome c levels, since enough haem may be available even if a part of exogenous haem is degraded by DDC. It is interesting to observe that administration of exogenous haemin, as well as ALA, the rate-limiting precursor, to normal mice increases the cytochrome c content but not the cytochrome P-450 content. This result indicates that availability of haem may be rate limiting for cytochrome c synthesis but not for cytochrome P-450 synthesis under normal circumstances. Recently, Druyan and Kelly[41] have reported that exogenous ALA does not affect the levels of cytochrome c and cytochrome P-450 in rat liver. In the present study, a higher and repeated doses of ALA has been injected than that used by Druyan.

Regarding the question of whether the response of the endoplasmic reticulum to the administration of drugs is mediated by their effects on haem synthesis, there appears to be a superficial correlation, in the sense that phenobarbital and allylisopropylacetamide accelerate the rate of haem synthesis and produce hypertrophic effects,[15] whereas DDC as an inhibitor of haem synthesis fails to produce hypertrophy. However, a careful examination would reveal that DDC brings about a significant inhibition of haem synthesis as early as 12 hr after its administration. However, the early effect of DDC is to enhance microsomal RNA and phospholipid contents as well as the rate of protein synthesis. The subsequent inhibition of protein synthesis does not appear to be due to lack of haem synthesis but is possibly due to the accumulation of the porphyrins. Recently, it has been shown that coproporphyrin inhibits amino acid incorporation into proteins in chick embryo liver cell culture systems.[42] It is, however, clear that haem may have a direct role in the synthesis of a specific mitochondrial haemoprotein such as cytochrome c. It has been consistently observed that successive doses of DDC, while resulting in a decreased rate of ^{14}C-

* G. Abritti and F. DeMatteis, unpublished work quoted in Ref. 37.

amino acid incorporation into the microsomes *in vivo*, lead to a small but significant increase in the labeling of post-microsomal supernatant proteins (Table 3). It is tempting to speculate that, so far as the endoplasmic reticulum is concerned, haem might be involved in the binding of haemoproteins to the membranes. Lack of haem could prevent the integration of these proteins into the membrane resulting in the release of the apo-proteins into the supernatant, eventually leading to their degradation.

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