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Biochemical Effects of the Porphyrinogenic Drug Allylisopropylacetamide

A COMPARATIVE STUDY WITH PHENOBARBITAL

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Successive administrations of allylisopropylacetamide, a potent porphyrinogenic drug, increase liver weight, microsomal protein and phospholipid contents. There is an increase in the rate of microsomal protein synthesis *in vivo* and *in vitro*. The drug decreases microsomal ribonuclease activity and increases NADPH–cytochrome *c* reductase activity. Phenobarbital, which has been reported to exhibit all these changes mentioned, is a weaker inducer of δ-aminolaevulinate synthetase and increases the rate of haem synthesis only after a considerable time-lag in fed female rats, when compared with the effects observed with allylisopropylacetamide. Again, phenobarbital does not share the property of allylisopropylacetamide in causing an initial decrease in cytochrome *P*-450 content. Haematin does not counteract most of the biochemical effects caused by allylisopropylacetamide, although it is quite effective in the case of phenobarbital. Haematin does not inhibit the uptake of [2-^{14}C]allylisopropylacetamide by any of the liver subcellular fractions.

Several foreign chemicals are metabolized by the mixed-function oxidase system of liver microsomal material (Conney, 1967; Kuntzman, 1969). It has been suggested that the drugs induce δ-aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem biosynthetic pathway, thereby causing increased haem and cytochrome *P*-450 synthesis, which results in enhanced activity of the mixed-function oxidase system (Granick, 1966; Baron & Tephly, 1969*a*; Marver, 1969).

Phenobarbital and allylisopropylacetamide cause increased amounts of δ-aminolaevulinate synthetase (Baron & Tephly, 1969*a*; Marver *et al.*, 1966*b*). Both compounds increase the rate of haem synthesis (Marver, 1969). Phenobarbital increases cytochrome *P*-450 content and causes striking anabolic effects, such as proliferation of the endoplasmic reticulum (Orrenius *et al.*, 1965), increased synthesis of protein (Kato *et al.*, 1965) and lipid (Holtzman & Gillette, 1968), increased RNA content (Jachau & Fouts, 1966) and stabilization of polyribosomes (Cohen & Ruddon, 1971). Although detailed studies are not available, administration of allylisopropylacetamide causes an increase in liver weight, total protein and lipid contents (Marver *et al.*, 1966*b*; Lottsfeld & Labbe, 1965). Allylisopropylacetamide also causes proliferation of the smooth endoplasmic reticulum (Moses *et al.*, 1970; Biempica *et al.*, 1967; Posalaki & Barka, 1968).

It is not clear whether an increased rate of haem synthesis owing to allylisopropylacetamide or phenobarbital administration has any role in the stimulation of the anabolic effects of these drugs. Raisfeld *et al.* (1970), on the one hand, reported that the phenobarbital-induced increase in cytochrome *P*-450 content could be blocked by aminotriazole, an inhibitor of haem synthesis, without significantly interfering with the processes leading to proliferation of the endoplasmic reticulum. On the other hand, Marver (1969) and Marver *et al.* (1968) reported that externally administered haematin, the 'co-repressor' for δ-aminolaevulinate synthetase, counteracts the increased cytochrome *P*-450, protein and lipid contents caused by phenobarbital. In addition, the induction of δ-aminolaevulinate synthetase and proliferation of the endoplasmic reticulum owing to allylisopropylacetamide administration could be related events, since puromycin and actinomycin D block both the processes (Biempica *et al.*, 1967).

In the present study the biochemical effects of allylisopropylacetamide and phenobarbital in rat liver have been compared and we have examined their effects on haem synthesis to see if they have a common basis, causing the other biochemical effects.

Experimental

Materials

Allylisopropylacetamide and [2-^{14}C]allylisopropylacetamide (4.2 μCi/mg) were generously provided by Hoffmann–La Roche Ltd., Basle, Switzerland. Yeast RNA, GTP, phosphoenolpyruvate, pyruvate kinase, β-mercaptoethanol, NADPH, cytochrome *c*, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were all purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Aminopyrine was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Haemin was prepared from sheep blood by the method of Labbe & Nishida (1957). All other chemicals used were of analytical-reagent grade. [U-^{14}C]Leucine and ^{14}C-labelled *Chlorella* hydrolysate were purchased from Bhabha Atomic Research Centre, Bombay, India.

Methods

Treatment of animals. Female rats (100–110g body wt.) of the local Institute strain were used in all the experiments. The animals were fed *ad libitum* on stock diet obtained from Hindustan Lever Ltd., Bombay, India. Allylisopropylacetamide was dissolved in 0.15M-NaCl and was given subcutaneously at a dose of 400mg/kg. Sodium phenobarbital was given intraperitoneally at a concentration of 80mg/kg. Haemin was dissolved in 0.01M-KOH and made up to volume with 0.05M-potassium phosphate buffer, pH7.5. It was given intraperitoneally at a dose of 2mg/100g. The protocol of each experiment is given in the respective tables. In experiments involving different periods of drug treatment, the schedules of injections were started on different days so that all the animals were ready to be killed on the same day and time. This was done to avoid the variations that could arise as a result of the biochemical analyses being done on different days.

Treatment of the livers. The animals were killed by decapitation and the livers were removed and fresh weights recorded. They were then homogenized with 5vol. of 0.25M-sucrose. The homogenate was centrifuged at 15000*g* for 15min. A known volume of the post-mitochondrial supernatant was taken and centrifuged at 105000*g* for 1h in a Beckman L3-50 ultracentrifuge. The microsomal pellets obtained were rinsed once and suspended in 0.25M-sucrose to contain microsomal fractions (microsomes) from 1g of liver in 1ml. All the operations were carried out at 0–4°C. Portions of the microsomal suspensions were used for determination of protein, RNA and phospholipid contents.

Determination of cytochrome P-450. For this determination, the livers were homogenized with 1.15% KCl and microsomes were isolated as described above. The pelleted microsomes were rinsed with 1.15% KCl and suspended in 0.05M-potassium phosphate buffer, pH7.5. Cytochrome *P*-450 content was determined by the method of Omura & Sato (1964), from the CO-difference spectrum of dithionite-reduced preparations by using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ between 450nm and 490nm.

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. RNA content was determined by the method of Munro & Fleck (1966). Phospholipid content was determined by the method of Folch *et al.* (1957). The liver δ-aminolaevulinate and porphobilinogen contents were determined by the method of Satyanarayana Rao *et al.* (1971).

Enzyme assays. δ-Aminolaevulinate synthetase was assayed in the liver homogenates by the method of Marver *et al.* (1966*a*).

Ribonuclease activity of the microsomes isolated from the livers homogenized in 0.25M-sucrose was determined by the method of Louis-Ferdinand & Fuller (1970). The incubation mixture contained 0.2ml of 0.2M-Tris–HCl buffer, pH7.6, 1.0mg of highly polymerized yeast RNA and 6–8mg of microsomal protein in a final volume of 1ml. RNA was added at the end and the incubation was carried out in air for 30min at 37°C. After the incubation, 1ml of 1M-HCl in aq. 75% (v/v) ethanol was added and the mixture was kept in ice for 30min. After centrifugation the E_{260} of the suitably diluted supernatant was measured. Appropriate controls were employed for the endogenous contribution of acid-soluble nucleotides.

NADPH–cytochrome *c* reductase activity was determined by the method of Masters *et al.* (1967), in which the rate of reduction of cytochrome *c* was followed at 550nm by using a Cary 14 spectrophotometer.

Drug-metabolizing enzymes. Aniline hydroxylase was measured by the method of Imai *et al.* (1966). For the assay, the microsomes isolated from livers homogenized in 1.15% KCl were used. The reaction mixture contained 8mM-aniline, 0.32mM-NADP, 3mM-glucose 6-phosphate, 2.5mM-$MgCl_2$, 1.3 units of glucose 6-phosphate dehydrogenase, 100mM-Tris–acetate buffer, pH8.0, and 3–4mg of microsomal protein in a total volume of 1ml. The reaction was carried out for 20min at 37°C aerobically and stopped by the addition of 0.5ml of 20% (w/v) trichloroacetic acid. The *p*-aminophenol formed was measured in 1ml of the supernatant by adding 0.5ml of 10% (w/v) Na_2CO_3 and 1ml of 2% (w/v) phenol in 0.2M-NaOH and measuring the E_{630} after 30min.

For measuring the aminopyrine demethylase activity, the same reaction mixture was used except that potassium phosphate buffer, pH7.5, was used instead of Tris–acetate buffer and the total volume was 3ml, which included 2mM-aminopyrine instead of aniline and 15mM-semicarbazide. The incubation was for 15min at 37°C and the reaction was stopped by the addition of 1ml of 20% (w/v) trichloroacetic acid. The formaldehyde formed was determined by the method of Nash (1953).

Determination of the rate of microsomal protein synthesis in vivo. The various groups of animals were injected intraperitoneally with 5μCi of ^{14}C-labelled *Chlorella* protein hydrolysate. After 45min they were killed by decapitation and the livers were removed. The microsomes were isolated from the 0.25M-sucrose homogenate as described above, suspended

in 0.25M-sucrose and precipitated with trichloroacetic acid (5%, w/v, final concentration). The precipitate was washed once with hot trichloroacetic acid (90°C) for 20min, twice with cold trichloroacetic acid, once with ethanol–diethyl ether (2:1, v/v) and finally with ether. The dry residue was dissolved in 1ml of formic acid. A 0.5ml portion was planchetted on to Whatman no. 3 filter-paper circles for measurement of radioactivity. Protein was determined in suitably diluted samples as described above after neutralization.

Determination of the rate of protein synthesis in vitro. The method of Munro *et al.* (1964) was employed. The incubation mixture contained 0.1ml of the microsomal suspension, 0.2ml of the soluble factors (pH 5.0 fraction and supernatant factors) and 0.2ml of a mixture containing the following: 19 amino acids except leucine, 0.1μmol each; ATP, 5μmol; phosphoenolpyruvate, 2.5μmol; $MgCl_2$, 2.5μmol; β-mercaptoethanol, 0.2μmol; GTP, 1μmol; pyruvate kinase, 25μg; and [U-^{14}C]leucine, 1μCi. The reaction was started by the addition of microsomes and the incubation was carried out for various time-periods. The reaction was terminated by 5% trichloroacetic acid containing 0.2% (w/v) unlabelled leucine. The radioactive precipitate was processed as described above and radioactivity was determined. RNA was also determined in 0.1ml of the microsomal suspension as described above and the results are expressed as c.p.m./mg of RNA.

Effect of allylisopropylacetamide and phenobarbital on the rate of haem synthesis. [2-^{14}C]Glycine was injected intraperitoneally into rats 8h after they had received the last injection of allylisopropylacetamide or phenobarbital. The animals were killed 1h after the tracer (10μCi/animal) administration. Haemin was isolated from the homogenates after the addition of 1ml of carrier blood and then recrystallized as described by Labbe & Nishida (1957). The recrystallized haemin was solubilized in ethyl acetate–acetic acid (3:1, v/v) and then washed with water and 3M-HCl to remove porphyrin contamination. One portion of the ethyl acetate layer was transferred to counting vials and dried *in vacuo* for radioactivity measurements. Another portion was evaporated to dryness under a stream of N_2 and the haem content determined as its pyridine haemochromogen.

Effect of haematin on the uptake of [2-^{14}C]allylisopropylacetamide by liver subcellular fractions. Haematin was given intraperitoneally at a concentration of 2mg/100g. After 30min allylisopropylacetamide was injected subcutaneously at a dose of 150 or 400mg/kg, which included 4.2μCi of [2-^{14}C]allylisopropylacetamide. The animals were killed 2h and 4h after the drug administration when the livers were removed, homogenized and the subcellular fractions isolated as described by Beattie & Stuchell (1970). The homogenate, mitochondria, microsomes and the post-microsomal supernatant were then extracted three times with ethyl acetate–acetic acid (3:1, v/v) by using 5 volumes each time. The precipitate was centrifuged and the supernatant was evaporated to dryness in a desiccator. The final residue was again dissolved in a known volume of ethyl acetate–acetic acid and portions were planchetted on to filter-paper discs for radioactivity measurements. Ethyl acetate–acetic acid treatment extracted at least 95% of the radioactivity from all of the liver fractions.

Radioactivity measurements. These were made in a Beckman LS-100 liquid-scintillation counter. The filter-paper discs were counted in vials containing 10ml of 0.5% 2,5-diphenyloxazole in toluene. The vials containing radioactive haem were also counted by using the same scintillation fluid, which became coloured owing to the solubilization of haem. Quench correction was applied by using the channels-ratio method.

Results

The results presented in Tables 1–3 clearly establish that successive allylisopropylacetamide administrations stimulate the anabolic processes in the liver. There is a significant increase in liver weight, microsomal protein, RNA and phospholipid contents. The rate of protein synthesis *in vivo* shows an increase (Table 3). The increased rate of protein synthesis owing to allylisopropylacetamide administration is also evident in amino acid-incorporation studies with isolated microsomes (Fig. 1). None of these effects of allylisopropylacetamide are counteracted by externally administered haematin, which by itself exhibits a small but consistent anabolic effect in every one of the parameters investigated.

With phenobarbital administration, although the isolated nuclei (Gelboin *et al.*, 1967) and chromatin (Piper & Bousquet, 1968) have a greater capacity to synthesize RNA, the enhanced RNA content is not due to increased rate of synthesis, but may be due to increased processing or transport of ribosomal precursor RNA (Cohen & Ruddon, 1970). Also the microsomal ribonuclease activity is strikingly inhibited by successive administrations of phenobarbital (Mycek, 1971). The results in Table 4 show that successive administrations of allylisopropylacetamide also cause a striking decrease in microsomal ribonuclease activity. Haematin by itself depresses ribonuclease activity *in vivo* and it has been reported to inhibit erythrocyte ribonuclease activity (Burka, 1970).

Allylisopropylacetamide and phenobarbital cause an increase in δ-aminolaevulinate synthetase activity (Baron & Tephly, 1969*a*; Marver *et al.*, 1966*b*) and in the rate of haem synthesis (Marver, 1969). A detailed study (Table 5) indicates that a single

Table 1. *Effect of daily administration of allylisopropylacetamide and haematin on liver weight and microsomal protein content in rat liver*

The animals were given daily subcutaneous injections of allylisopropylacetamide (400mg/kg). Haematin was given intraperitoneally just before allylisopropylacetamide and was administered at a dose of 2mg/100g. The drug treatment for the different groups of animals was started on different days so that all the animals were ready to be killed on the same day. The control group consisted of animals that had received one or more injections of saline or the appropriate vehicle. The liver weight and microsomal-protein content of the control animals were 4.1±0.2g/100g body wt. and 20.8±0.5mg/g of liver respectively. The results represent the means±S.D. from four experiments in which two livers were pooled in each experiment. *$P<0.05$; **$P<0.02$; ***$P<0.001$. The data without asterisks are not statistically significant. P values were calculated with respect to the corresponding control values.

Number of days of treatment ...	1		3		5	
Treatment	Liver wt. (g/100g body wt.)	Microsomal protein (mg/g of liver)	Liver wt. (g/100g body wt.)	Microsomal protein (mg/g of liver)	Liver wt. (g/100g body wt.)	Microsomal protein (mg/g of liver)
Allylisopropylacetamide	4.2±0.3	21.5±0.2	5.5±0.2***	30.5±0.6***	6.8±0.4	33.4±1.5***
Haematin	3.8±0.1	21.0±0.4	4.5±0.3	23.5±0.8	4.8±0.4	24.5±1.6*
Allylisopropylacetamide+ haematin	4.2±0.4	22.0±1.0	5.3±0.5**	32.0±1.5***	6.5±0.3***	35.0±2.5***

Table 2. *Effect of allylisopropylacetamide and haematin on the RNA and phospholipid contents of microsomes from rat liver*

The protocol of the experiments is the same as described in Table 1. The RNA and phospholipid contents of the control animals were 3.3±0.12mg/g of liver and 210±4.5μg of P/g of liver respectively and represent the means±S.D. obtained from six experiments. The RNA values for the experimental animals represent the mean±S.D. obtained from four experiments. The values given for phospholipid are averages of two experiments. *$P<0.01$; **$P<0.02$; ***$P<0.001$. The data without asterisks are not statistically significant. P values were calculated with respect to the corresponding control values.

Number of days of treatment ...	1		3		5	
Treatment	RNA (mg/g of liver)	Phospholipid (μg of P/g of liver)	RNA (mg/g of liver)	Phospholipid (μg of P/g of liver)	RNA (mg/g of liver)	Phospholipid (μg of P/g of liver)
Allylisopropylacetamide	3.6±0.15	220.4	4.5±0.18*	285.8	5.0±0.2***	280.0
Haematin	3.2±0.08	201.5	3.8±0.10**	231.5	3.9±0.11	236.0
Allylisopropylacetamide+ haematin	3.8±0.18	228.0	4.3±0.20*	295.0	5.2±0.21***	293.5

Table 3. *Rate of protein synthesis by the liver microsomes in allylisopropylacetamide-treated rats in vivo*

The rats were given daily subcutaneous injections of allylisopropylacetamide (400mg/kg) at 24h intervals. At 12h after the last injection ^{14}C-labelled *Chlorella* hydrosylate (5μCi/animal) was injected intraperitoneally. After 45min the rats were killed and incorporations of radioactivity into homogenate and microsomal protein were measured. The results represent the mean±S.D. obtained from three experiments. *$P<0.01$ with respect to control. The difference between the control and allylisopropylacetamide-treated (first day) value is not statistically significant.

Treatment	Radioactivity in microsomes (c.p.m./mg of protein)
Control	256.0±63.20
Allylisopropylacetamide (first day)	349.1±62.84
Allylisopropylacetamide (second day)	410.6±10.10*

injection of allylisopropylacetamide to fed female rats causes a striking increase in the rate of haem synthesis. The significant increase in total [2-^{14}C]glycine uptake by the allylisopropylacetamide-injected liver as compared with the controls or the phenobarbital-treated ones is interesting. This could be related to the striking induction of δ-aminolaevulinate synthetase and consequently a necessity to enhance glycine concentration to saturate the enzyme, since the enzyme is reported to have a K_m value as high as 1×10^{-2}M with respect to glycine (Scholnick *et al.*, 1970). Phenobarbital does not significantly change the rate of total haem synthesis in fed female rats under these conditions. However, a significant increase is seen after the second and third injections (only the values for the first and third injections are reported in Table 5).

The results in Table 6 clearly indicate that phenobarbital does not significantly induce δ-aminolaevulinate synthetase 8h after the first injection. A significant increase in enzyme activity is, however, seen after the second injection. Haematin counteracts

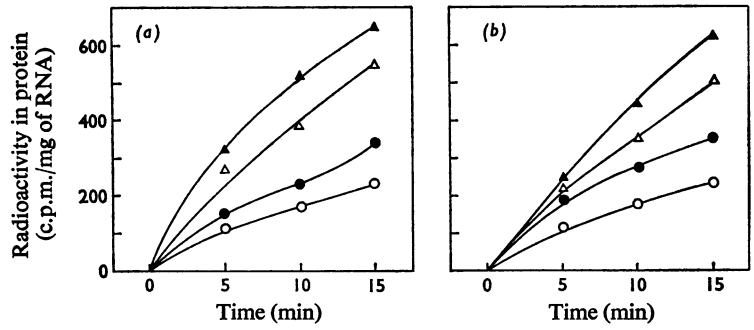

Fig. 1. *Effect of allylisopropylacetamide and haematin on amino acid incorporation by isolated microsomes in vitro*

The experimental details are given in the text. The results given are those obtained in a typical experiment. (*a*) Second day; (*b*) fourth day. ▲, Allylisopropylacetamide-treated rats; ○, control rats; △, allylisopropylacetamide+haematin-treated rats; ●, haematin-treated rats.

Table 4. *Effect of allylisopropylacetamide, phenobarbital and haematin on microsomal ribonuclease activity*

The experimental protocol is the same as described in Table 1 and the text. The control value is 0.960±0.100μg of RNA hydrolysed/30min per mg of protein and represents the means±S.D. obtained from six experiments. The other results represent the individual values obtained in each of two experiments.

Treatment	Microsomal ribonuclease activity (μg of RNA hydrolysed/30min per mg of protein)			
Number of days of treatment ...	1	2	3	5
Allylisopropylacetamide	0.672, 0.631	0.228, 0.290	0.265, 0.243	0.105, 0.100
Haematin	0.856, 0.843	0.565, 0.501	0.538, 0.561	0.456, 0.467
Allylisopropylacetamide+haematin	0.586, 0.572	0.285, 0.234	0.305, 0.327	0.106, 0.167
Phenobarbital	0.601, 0.601	0.354, 0.383	0.250, 0.210	0.116, 0.128

Table 5. *Effect of allylisopropylacetamide and phenobarbital on the rate of haem synthesis*

Rats were given one or three injections of allylisopropylacetamide or phenobarbital at a dose of 400mg/kg and 80mg/kg respectively at 24h intervals. At 8h after the last injection [2-^{14}C]glycine (10μCi/animal) was injected intraperitoneally into each animal. The animals were killed after 1h and the liver homogenates processed for haemin isolation after the addition of carrier blood as described in the text. The results represent the averages of two experiments.

		Radioactivity		
Treatment	No. of injections	10^{-5} × Total uptake (c.p.m./g of liver)	In haemin (c.p.m./μmol of haemin)	In haemin (% of total)
Control	—	1.72	196	0.114
Allylisopropylacetamide	1	5.90	931	0.158
Allylisopropylacetamide	3	4.61	735	0.159
Phenobarbital	1	1.74	202	0.116
Phenobarbital	3	1.79	345	0.193

Table 6. *Effect of haem on the porphyrinogenic effects of allylisopropylacetamide and phenobarbital*

Rats were given one or two injections of allylisopropylacetamide, phenobarbital and haematin at 24h intervals. The animals were killed 8h after the last injection. The δ-aminolaevulinate synthetase values represent the means ± S.D. obtained from three experiments in which two livers were pooled in each experiment. The δ-aminolaevulinate and porphobilinogen values are an average of two experiments. The control values of δ-aminolaevulinate synthetase and aminolaevulinate amounts were 16.6 ± 1.5nmol of aminolaevulinate/h per g of liver and 1.37μg of aminolaevulinate/g of liver respectively. No porphobilinogen could be detected in the control livers.

Treatment	δ-Aminolaevulinate synthetase activity (nmol of aminolaevulinate/h per g of liver)	Liver aminolaevulinate content (μg/g of liver)	Liver porphobilinogen content (μg/g of liver)
Allylisopropylacetamide (first injection)	95.6 ± 11.4	1.90	2.2
Allylisopropylacetamide (second injection)	150.3 ± 9.6	3.40	—
Phenobarbital (first injection)	16.9 ± 2.6	1.10	—
Phenobarbital (second injection)	48.0 ± 2.8	1.20	—
Haematin (second injection)	10.5 ± 2.0	1.15	—
Allylisopropylacetamide + haematin (second injection)	31.2 ± 2.8	1.31	1.1
Phenobarbital + haematin (second injection)	20.0 ± 1.6	1.16	—

the effects of phenobarbital, as well as allylisopropylacetamide, in this regard.

Although allylisopropylacetamide induces δ-aminolaevulinate synthetase and increases the rate of haem synthesis, its primary effect seems to be one of decreasing the content of cytochrome *P*-450 (Table 7). The decrease in cytochrome *P*-450 content owing to allylisopropylacetamide administration has also been reported by others and involves the breakdown of the haem moiety (De Matteis, 1970; Meyer & Marver, 1971). Cytochrome *P*-450 contents reach normal or even greater than normal values 24h after allylisopropylacetamide administration (De Matteis, 1971; Satyanarayana Rao *et al.*, 1972). The results presented in Table 7, where cytochrome *P*-450 contents have been measured 6h after each allylisopropylacetamide injection, thus represent fresh breakdown of cytochrome *P*-450 after each injection of the drug. The decrease in cytochrome *P*-450 content is also reflected in the decrease in the activities of aniline hydroxylase and aminopyrine demethylase. Even a single injection of phenobarbital causes an increase in cytochrome *P*-450 content under conditions when there is no change in δ-aminolaevulinate synthetase activity

Table 7. *Effect of haematin on allylisopropylacetamide- and phenobarbital-mediated changes in the contents of cytochrome P-450 and drug-metabolizing enzymes*

The experimental protocol is the same as described in Table 1. The animals were killed 6h after the last injection. In the case of phenobarbital, the animals were killed 12h after the last injection. Each value represents the average of two experiments in which two livers were pooled in each case. The control values represent the means ± S.D. from six experiments.

		Aniline hydroxylase activity		Aminopyrine demethylase activity	
Treatment	Cytochrome *P*-450 content (nmol/mg of protein)	(μmol of *p*-aminophenol/mg of protein)	(μmol of *p*-aminophenol/nmol of cytochrome *P*-450)	(μmol of HCHO/mg of protein)	(μmol of HCHO/nmol of cytochrome *P*-450)
Control	0.85 ± 0.06	0.12 ± 0.01	0.142	45.0 ± 2.1	53.0
Allylisopropylacetamide					
First day	0.76	0.11	0.141	41.0	53.9
Third day	0.51	0.08	0.158	32.6	63.8
Fifth day	0.48	0.06	0.135	27.4	57.1
Haematin					
First day	0.84	0.12	0.140	45.0	53.6
Third day	0.79	0.11	0.142	44.8	56.8
Fifth day	0.69	0.09	0.139	38.5	55.8
Allylisopropylacetamide+haematin					
First day	0.74	0.01	0.138	40.3	54.5
Third day	0.60	0.08	0.143	33.5	55.8
Fifth day	0.44	0.05	0.123	25.8	58.6
Phenobarbital					
First day	1.02	0.14	0.137	49.0	48.0
Third day	2.46	0.42	0.170	112.2	45.2
Phenobarbital+haematin					
First day	0.84	0.11	0.131	47.1	56.1
Third day	1.21	0.20	0.165	55.3	45.7

or rate of haem synthesis. Again haematin counteracts the phenobarbital-mediated, but not the allylisopropylacetamide-mediated, effects on drug-metabolizing-enzyme activities. Another interesting feature of the comparative effects of allylisopropylacetamide and phenobarbital is that both the drugs cause an increase in NADPH–cytochrome *c* reductase activity. Haematin is effective only in the case of phenobarbital (Table 8).

Finally, Marver (1969) reported that haematin inhibits the uptake of phenobarbital into liver microsomes. A similar study with [2-^{14}C]allyliso-

Table 8. *Effect of haematin on allylisopropylacetamide- and phenobarbital-mediated changes in NADPH–cytochrome c reductase activity*

The injection schedule is the same as described in Table 1. The animals were killed 12h after the last injection. The results are expressed as % of the control. The absolute value for the control is 66.8±0.8nmol of cytochrome *c* reduced/min per mg of protein and represents the means±S.D. obtained from six experiments. The other values represent the averages of two experiments in which two livers were pooled in each experiment.

Treatment	NADPH–cytochrome *c* reductase activity (% of control)
Control	100±4.8
Allylisopropylacetamide	
First day	111
Second day	276
Third day	315
Fifth day	330
Haematin	
First day	97
Second day	107
Third day	140
Fifth day	127
Allylisopropylacetamide+haematin	
First day	125
Second day	235
Third day	310
Fifth day	335
Phenobarbital	
First day	120
Third day	262
Phenobarbital+haematin	
First day	105
Third day	142

Table 9. *Effect of haematin on [2-^{14}C]allylisopropylacetamide uptake by the liver subcellular fractions*

Rats were given intraperitoneal injections of haematin (2mg/100g). After 30min allylisopropylacetamide was injected subcutaneously at a dose of 150mg/kg, which included 4.2μCi of [2-^{14}C]allylisopropylacetamide/animal. The animals were killed 2h and 4h after allylisopropylacetamide injection and the incorporation of radioactivity into the subcellular fractions was measured. The values given are averages of two experiments and livers from two animals were pooled in each case.

Treatment	Time (h)	Radioactivity (c.p.m./g of liver) in: Homogenate	Mitochondria	Microsomes	Cytosol
Allylisopropylacetamide	2	6328	64	458	4440
Allylisopropylacetamide	4	1196	78	120	900
Allylisopropylacetamide+haematin	2	6240	58	482	4208
Allylisopropylacetamide+haematin	4	1144	70	130	930

propylacetamide reveals that haematin has no inhibitory effect on the uptake of the drug by any of the subcellular fractions examined at the time-intervals studied. A striking decrease in radioactivity of the liver homogenate at the 4h time-period as compared with 2h, is an index of the rate of clearance of the drug from the liver (Table 9). Similar results were obtained at two amounts of allylisopropylacetamide administration, namely 150 and 400mg/kg body wt.

Discussion

The present results clearly establish that allylisopropylacetamide shares several of the biochemical effects of phenobarbital. Both the drugs elicit an increase in liver weight, microsomal protein, RNA and phospholipid contents as well as the rate of protein synthesis *in vivo* and *in vitro*. In addition both the drugs depress ribonuclease activity and increase NADPH–cytochrome *c* reductase activity.

Regarding the effect on haem synthesis, allylisopropylacetamide and phenobarbital increase the activity of δ-aminolaevulinate synthetase and the rate of haem synthesis (Baron & Tephly, 1969*a*; Marver, 1969; Marver *et al.*, 1966*b*). Detailed studies indicate that allylisopropylacetamide causes an almost immediate increase in δ-aminolaevulinate synthetase activity. A striking increase in the rate of haem synthesis was observed 8h after allylisopropylacetamide administration. This time-period has been chosen in this study to avoid the complication arising from the initial breakdown of the haem moiety of cytochrome *P*-450 owing to allylisopropylacetamide injection. At least in fed female rats phenobarbital has a delayed effect on δ-aminolaevulinate synthetase activity and the rate of total haem synthesis as compared with that of allylisopropylacetamide. However, phenobarbital brings about an increase in cytochrome *P*-450 content under conditions where there is no change in δ-aminolaevulinate synthetase activity or in the rate of total haem synthesis. It is generally held that the availability of haem is rate-limiting for the synthesis of cytochrome *P*-450 and the drugs increase the cytochrome *P*-450 content by enhancing the rate of haem synthesis through the induction of δ-aminolaevulinate synthetase (Granick, 1966; Marver, 1969; Baron & Tephly, 1969*a*,*b*, 1970). However, the results of the present investigation indicate that at least under certain conditions the availability of haem need not be rate-limiting for cytochrome *P*-450 synthesis. We have also shown that in hexachlorobenzene feeding cytochrome *P*-450 content can increase without eliciting a change in δ-aminolaevulinate synthetase activity or in the rate of total haem synthesis (Rajamanickam *et al.*, 1972). Song *et al.* (1971) have shown that in young rats phenobarbital causes an increase in cytochrome *P*-450 content without affecting δ-aminolaevulinate synthetase. Bock *et al.* (1971) have shown a similar effect in fed adult rats. Recently, De Matteis & Gibbs (1972) have also subscribed to the view that the primary effect of drugs affecting cytochrome *P*-450 content need not be at the level of haem synthesis.

The present results clearly indicate that the gross effects of allylisopropylacetamide and phenobarbital on δ-aminolaevulinate synthetase activity and the rate of haem synthesis cannot explain the similar biochemical effects, especially the anabolic effects exerted by them. In other words, the sequence of action does not appear to be one of increasing the rate of haem synthesis and consequently the rate of microsomal protein synthesis. In view of the report that a substantial portion of the microsomal proteins appear to be haemoproteins (Black *et al.*, 1971), it is important to understand the effect of haem on the rate of microsomal protein synthesis. The situation in liver may be different when compared with the role of haem in haemoglobin synthesis. The counteracting effect of externally administered haematin on the biochemical changes elicited by phenobarbital is not because of its function as a 'co-repressor' of δ-aminolaevulinate synthetase, but is due to its ability to inhibit phenobarbital uptake at the microsomal level. This contention is supported by the fact that haematin does not counteract the biochemical changes brought about by allylisopropylacetamide other than δ-aminolaevulinate synthetase induction and has no effect on the uptake of the drug by any of the subcellular fractions examined. However, externally administered haematin consistently lowered the amino acid-incorporating ability of isolated microsomes from allylisopropylacetamide-injected rats to a small extent (Fig. 1).

It will be of interest to examine how phenobarbital and allylisopropylacetamide, which seem to act differently in the early stages, subsequently elicit similar biochemical effects. One possibility is that allylisopropylacetamide may elicit the formation of mRNA for δ-aminolaevulinate synthetase during the initial stages. Later stages involving repeated administrations of the drug may result in increased rRNA formation and/or stabilization. Phenobarbital may primarily increase the formation of apo-cytochrome *P*-450, which may subsequently elicit increased formation of δ-aminolaevulinate synthetase and haem. As suggested by Cohen & Ruddon (1970), phenobarbital may also accelerate the processing or transport of ribosomal precursor RNA.

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