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ADJUVANT-INDUCED ARTHRITIS AND DRUG-METABOLIZING ENZYMES

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Abstract—Rats given a single intradermal injection into the foot pad of 0.50 mg *Mycobacterium butyricum*, suspended in 0.1 ml of liquid paraffin, developed arthritis after a latent period of about 8 days. However, a decline in the activity of hepatic aminopyrine demethylase and the level of cytochrome P-450 occurred before the development of arthritis. Rats given the adjuvant preparation by the intraperitoneal route showed also a decreased activity of aminopyrine demethylase. An inverse correlation was found between the level of α_2-globulin and aminopyrine demethylase activity. Rats in which arthritis was induced by *Mycoplasma arthritidus* also showed a reduced activity of aminopyrine demethylase. The reduction of aminopyrine demethylase activity in adjuvant-treated rats was not caused by anorexia, neither could it be shown to be caused by a circulating hormonal factor. However, it was noted that the serum from many of the arthritic rats was greenish in colour and this might have been caused by the breakdown of certain haem compounds. The haem saturation of hepatic tryptophan oxidase was much less in adjuvant-induced arthritic rats than in controls. These results suggest a failure in haem biosynthesis and/or an accelerated breakdown of existing haem.

Since the production of adjuvant-induced arthritis in rats was first described [1, 2], there have been many publications concerned with the immunological components of the syndrome [2–4], with serum protein changes [5–6], and with the inflammatory response [7–8]. More recently, there have been a number of studies that have, either directly or indirectly, implicated an hepatic involvement in adjuvant disease. For example, the reduction in serum albumin [9–10] and the rise in circulating α_2-glycoproteins [9] and fibrinogen [11] all reflect altered biosynthetic activities of the liver. Further evidence comes from the finding that the activity of drug metabolizing enzymes is suppressed [12–17]. In this paper, we present further data on the hepatic enzyme changes that occur during the development of adjuvant-induced arthritis in the rat.

MATERIALS AND METHODS

Animals and production of arthritis. CFHB Wistar rats were obtained from Carworth (Europe) Ltd., Alconbury, Hunts., U.K. The rats were fed a stock, pelleted diet (Diet FFG; E. Dixon and Sons, Ware, Herts., U.K.). Adjuvant-induced arthritis was produced by a single subcutaneous (s.c.) injection of 0.50 mg of *Mycobacterium butyricum*, suspended in 0.1 ml of liquid paraffin, into the foot pad of the right hind paw [18]. Mycoplasma-induced arthritis was produced in Sprague–Dawley rats (Charles Rivers, Manston, Kent, U.K.), by the intravenous (i.v.) injection of *Mycoplasma arthritidus* ATCC 14124 (1.4×10^8 colony forming units/110 g rat).

Assessment of degree of arthritis. At autopsy, the feet were removed from each rat by cutting across the ankle joint with a scalpel and were weighed individually.

Liver aminopyrine demethylase assay and cytochrome P-450 content. The procedures used were those described by Atkin *et al.* (1972) [18].

Liver tryptophan pyrrolase activity. The tryptophan pyrrolase activity of liver homogenates and liver microsomal fraction was determined in the absence (holoenzyme activity) and presence (total enzyme activity) of added haematin, as described by Wettenberg *et al.* (1969) [19]. The activity of the apoenzyme was calculated by difference.

Serum proteins. Total protein content of serum was measured by the biuret method [20], using bovine serum albumin as standard. The separation of the serum protein was done electrophoretically. After staining with Coomassie Blue, the relative distribution of the protein was measured using a Phoroscope densitometer.

Liver 5-aminolaevulinic acid synthetase assay. Livers were perfused *in situ* with ice-cold 0.9% (w/v) saline. The liver was then removed and the 5-aminolaevulinic acid synthetase was measured as described by De Matteis [21].

Haem biosynthesis in vivo. Rats were given 50 μCi (300 mCi/mM) [G-^{3}H] 5-aminolaevulinic acid hydrochloride (Radiochemical Centre, Amersham, U.K.) in 0.1 ml 0.9% saline by intraperitoneal (i.p.) injection and then killed exactly 1 hr later. Livers were perfused *in situ* with ice-cold saline to remove blood. The microsomal fraction was isolated by differential centrifugation [18]. Haem was isolated from liver microsomal suspensions [21] and a portion of this extract was taken for determination of radiochemical content.

The incorporation of glycine into haem was determined in a similar manner. Rats were given 10 μCi [2-^{14}C] glycine by i.p. injection and killed 2 hr later.

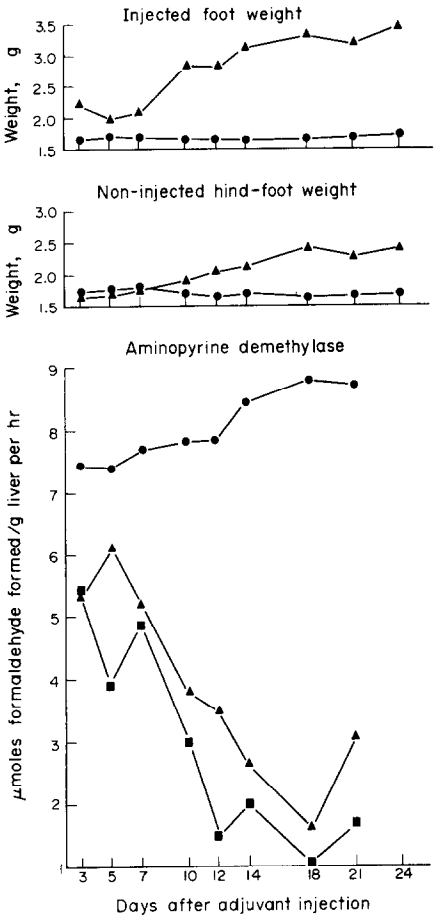

Fig. 1. The effect of adjuvant injection on the weight of the hind-feet and on the activity of aminopyrine demethylase. ● Control rats; ▲ rats given adjuvant i.d. into the foot pad; ■ rats given the adjuvant by i.p. injection.

EXPERIMENTAL AND RESULTS

Temporal relationships of the development of arthritis and the loss of drug metabolizing enzyme activity. Rats were given an injection of adjuvant into the foot or i.p. and killed at intervals up to 24 days later.

Injection of adjuvant into the foot led to the development of arthritis after a latent phase of about 8 days (Fig. 1), although an inflammatory condition developed in the injected foot immediately after the injection. However, there was a decline in hepatic aminopyrine demethylase activity before marked arthritis was visible. Thus, it would appear that the reduction in aminopyrine demethylase activity is related more to the inflammatory state than the arthritic state.

The rats given adjuvant i.p. showed similar pathology to that noted by Pearson [2]; namely, adhesion of tissues to each other and sometimes excessive retention of peritoneal fluid. However, we also found that a few rats developed minor arthritic lesions and this finding is in agreement with the work of Dolbeare [22]. The surface of the liver showed small white spots, suggestive of adjuvant deposition, and adjuvant was found coated on the abdominal surface of the diaphragm. Spleens were often enlarged and congested and sometimes showed greyish-white spots similar to those produced in arthritic rats, which had received adjuvant intradermally (i.d.).

Rats treated with an i.p. injection of adjuvant also showed a rapid decline in the activity of aminopyrine demethylase (Fig. 1) and an increase in the serum α_2-globulin level. The overall regression of the correlation coefficient for the relationship between α_2-globulin level and aminopyrine demethylase activity in control rats, rats given adjuvant i.d. and rats given adjuvant i.p., was -0.75.

Relationship between loss of aminopyrine demethylase activity and food intake in arthritic rats. Arthritic rats do not eat as much food as control rats, and the amount and quality of the eaten food can have effects on the activity of drug metabolizing enzymes [23]. It was considered appropriate, therefore, to examine the relationship between food intake and the activity of the drug metabolizing enzymes in adjuvant-induced arthritic rats (Table 1).

We found that the weight gain of arthritic rats was much less than that of control rats fed *ad lib.*, but was similar to the weight gain of control rats that were pair-fed to the adjuvant-treated rats. Thus, it would seem that although most of the depression in weight gain of arthritic rats is associated with the reduced food intake, the reduction in enzyme activity is directly dependent on the disease state rather than with the associated anorexia.

On the existence of humoral factors that might inhibit drug metabolizing enzymes. Adjuvant-induced arthritis is thought to be a disease of immunological aetiology and the possibility of humoral factors being released and affecting the drug-metabolizing enzyme system is one that must be considered. Such substances could act by binding to the cytochrome P-450 complex in such a way as to prevent cytochrome P-450 binding

Table 1. Effect of adjuvant-arthritis and restricted feeding on drug metabolizing enzyme activity*

Treatment	Wt. gain (g)	Liver wt (% of body wt)	Aminopyrine demethylase activity (μmole formaldehyde formed/g liver/hr)
None	71 ± 10 (6)	4.24 ± 0.23 (8)	12.41 ± 0.85 (4)
Adjuvant-treated	21 ± 6 (12)†	4.94 ± 0.20 (16)	3.70 ± 0.83 (8)‡
Control pair-fed to adjuvant-treated rats	39 ± 4 (12)†	3.44 ± 0.14 (16)	12.18 ± 0.71 (8)

* Male CFHB rats were distributed into three groups. One group was given no treatment and fed on the diet *ad lib.* The second group was given an i.d. injection of the adjuvant and the third group was given no treatment but was pair-fed to the adjuvant-treated group. After 14 days, the rats were killed, livers removed and combined in pairs for the enzyme assay. Results are given as mean ± S.E.M.; number of values in parentheses.

† Significantly different from untreated rats. $P < 0.01$.

‡ Significantly different from both untreated rats and from pair-fed control rats, $P < 0.001$.

Table 2. Subcellular localization of defect in drug metabolizing enzyme activity in arthritic rats*

Source of microsomal fraction	Source of supernatant fraction	Aminopyrine demethylase activity (μmoles formaldehyde formed/g liver/hr)
Control	Control	7.40 ± 0.25
Control	Arthritic	7.36 ± 0.34
Arthritic	Arthritic	2.31 ± 0.61†
Arthritic	Control	2.35 ± 0.66†

* The hepatic subcellular fraction was obtained from individual control and arthritic rats. The microsomal and supernatant fractions were constituted as shown in the table and incubated at 37° for 30 min, prior to the addition of substrate. The results are the mean ± S.E.M. of experiments involving four pairs of rats.

† Significantly less than incubations containing the microsomal fraction from control rats; $P < 0.001$.

with physiological ligands. Such an effect would be manifested *in vitro* by an inhibition af aminopyrine demethylase activity. An experiment was therefore done to seek such an agent in the soluble fraction of liver (Table 2) and also in serum (Table 3). Low enzyme activity was associated solely with the presence of the microsomal fraction (100,000 *g* pellet) from arthritic rats and serum obtained from rats 5, 12 and 18 days after adjuvant treatment had no greater inhibitory effect on the enzyme activity than serum from control rats. These results support those of Beck and Whitehouse [16], who studied the effects of adjuvant disease on cyclosphosphamide metabolism.

Effect of mycoplasma-induced arthritis on drug metabolizing enzyme activity and α_2-globulin levels. The activity of hepatic aminopyrine demethylase was significantly lower in the mycoplasma-treated arthritic rats than in controls (Table 4), but the loss of enzyme activity was less than had been found in adjuvant-treated rats (Fig. 1). However, less inflammation also was produced by the mycoplasma treatment than was found in the adjuvant-treated rats. Mycoplasma-induced arthritis caused a change in the distribution of various serum proteins. The α_2-fraction was increased, as were all the globulin fractions, whereas the albumin fraction decreased. The correlation factor between the level of α_2-globulin in serum and the activity of aminopyrine demethylase was -0.729 ($P < 0.01$) in male rats, but in female rats it was only -0.526 ($P < 0.05$).

Effect of adjuvant-induced arthritis on hepatic tryptophan oxidase activity. The excretion of large amounts of tryptophan and its metabolites by arthritic patients have been noted by several workers [26–30]. Tryptophan is metabolised by tryptophan oxidase (L-tryptophan-O_2 oxidoreductase, E.C. 1.13.11.11) to kynurenine and the enzyme is activated by haem. We have

Table 3. Effect of serum from arthritic rats on hepatic drug metabolizing enzyme activity*

Source of rats serum	Time of pre-incubation with serum (mins)	Aminopyrine demethylase activity (μmoles formaldehyde formed/g liver/hr)
Control rat	30	5.35
18 day arthritic rat	30	5.60
12 day arthritic rat	30	5.60
5 day arthritic rat	30	5.35
Control rat	60	4.40
18 day arthritic rat	60	4.77
12 day arthritic rat	60	4.83
5 day arthritic rat	60	4.53

* Liver preparations from normal rats were incubated with serum from either control or arthritic rats. The activity of aminopyrine demethylase was determined as described in the text.

Table 4. Effect of mycoplasma-induced arthritis on drug metabolizing enzyme activity and α_2-globulin levels*

Rat Sex	Treatment	Aminopyrine demethylase activity (μmoles formaldehyde formed/g liver/hr)	Cytochrome P-450 (μmoles/mg protein)	α_2-globulin (% of total serum protein)
Male	Control	8.50 ± 0.80	—	11.7 ± 0.8
	Arthritis	5.98 ± 1.94†	—	18.7 ± 1.1‡
Female	Control	3.87 ± 0.64	0.51 ± 0.17	8.9 ± 1.3
	Arthritis	2.94 ± 0.66§	0.40 ± 0.29	16.5 ± 1.4

* The CFHB rats were killed 8 days after receiving 1.4×10^8 colony-forming units/100 g rat of Mycoplasma arthritidus by i.v. injection. Results are given as mean ± S.E.M. of eight values.

† Significantly different from controls, $P < 0.01$.

‡ Significantly different from controls, $P < 0.001$.

§ Significantly different from controls, $P < 0.02$.

Table 5. Effect of adjuvant-induced arthritis on hepatic tryptophan oxygenase activity*

Source of enzyme	Tryptophan oxygenase (nmoles/g liver/hr) Holoenzyme	Total enzyme activity	% Saturation
Control rat—Homogenate	10.7 ± 2.4	17.5 ± 1.3	61
Arthritic rat—Homogenate	11.4 ± 2.3	48.9 ± 10.2	23
Control rat—Microsomal fraction	6.1 ± 1.2	17.8 ± 3.4	34
Arthritic rat—Microsomal fraction	3.1 ± 0.4	32.1 ± 7.2	10

* Rats were killed 14 days after receiving the adjuvant treatment. Results are given as mean ± S.E.M. Each group contained three rats.

noted that many of the samples of serum from the arthritic rats were green in colour and it is thought possible that this pigment could arise from the breakdown of certain haem compounds. Other workers [24, 25] have described brownish-green discolouration of livers of rats and rabbits given porphyria-inducing drugs, such as sedormid and allylisopropylacetamide, which also cause loss of drug-metabolizing enzyme activity. If haem metabolism is deranged in adjuvant arthritis, a change in tryptophan pyrrolase activity might result.

Livers of adjuvant-treated rats were perfused *in situ* with ice-cold saline and tryptophan oxidase activity determined in rat liver homogenates and in the microsomal fraction, both in the presence and absence of added haematin. The results (Table 5) showed that the arthritic and control rats had similar enzyme activities in the absence of haem, but the degree of haem saturation was less in the arthritic rats than in controls. A possible explanation for these findings is that there is a reduction in the amount of endogenous haem in arthritic rats and that the rat adapts by producing more apoenzyme.

Effect of adjuvant-induced arthritis on haem biosynthesis. The rate-limiting step in the formation of haem is the condensation of succinyl CoA and glycine by 5-aminolaevulinic acid synthetase (E.C. 2.3.1.37). The activity of the enzyme was measured [21] in liver of rats killed 4, 7, 10, 12, 15, 18 and 21 days after adjuvant treatment (Fig. 2). Although the mean activity of 5-aminolaevulinic acid synthetase was lower in the arthritic rats than in controls at day 12 and later, the variation in the values and the small differences between the means suggest that deficiency of cytochrome P-450 and therefore drug-metabolizing enzyme activity in adjuvant-induced arthritis was not the result of a defect in 5-aminolaevulinic acid synthetase. We also found that adjuvant-induced arthritis had no effect on the level of cytochrome b_5 in rat liver microsomal fraction.

The effect of arthritis on the haem biosynthesis was also determined by examining the incorporation of a tracer dose of [^{3}H]5-aminolaevulinic acid and [2-^{14}C] glycine into microsomal haem. The incorporation of label into microsomal haem was unaffected by the development of arthritis (Tables 6 and 7).

DISCUSSION

It is generally accepted that there are certain similarities between adjuvant-induced arthritis in the rat and human arthritis. This has resulted in the use of the former as a model for detecting and evaluating anti-arthritic drugs. Several studies [12–17] have shown that during the development of arthritic lesions in the rat there is a reduced capacity of the liver to metabolize oxidatively a number of drugs. Furthermore, Morton and Chatfield [14] were able to show that this effect was not merely an artefact of an *in vitro* preparation by showing that the hexobarbitone sleeping time was increased in adjuvant-induced arthritis. In the present study, we have examined the temporal relationship of the changes in the activity of drug metabolizing enzymes that follow

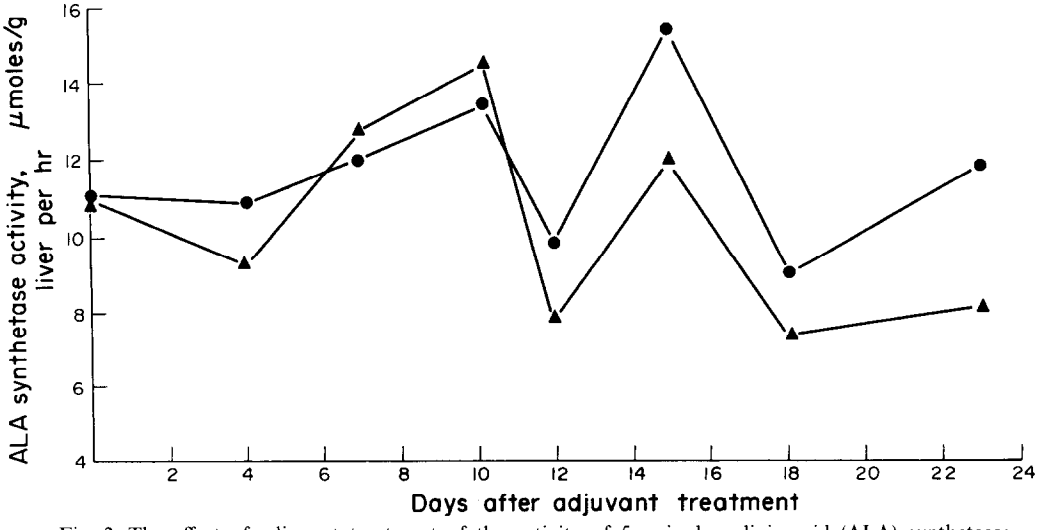

Fig. 2. The effect of adjuvant treatment of the activity of 5-aminolaevulinic acid (ALA) synthetase: ● control rats; ▲ rats given the adjuvant i.d. in the foot pad. Each result is the mean of three values.

Table 6. Effect of adjuvant-induced arthritis on the incorporation of [^{3}H] 5-aminolaevulinic acid into microsomal haem*

Time of death after adjuvant injection (days)	Aminopyrine demethylase activity (μmoles formaldehyde formed/g liver/hr)		Cytochrome P-450 (nmoles/mg protein)		5-ALA incorporated into haem (dpm/mg protein)	
	Control	Arthritic	Control	Arthritic	Control	Arthritic
7	9.08 ± 0.78	6.92 ± 0.44	0.38 ± 0.02	0.35 ± 0.01	1317 ± 101	1058 ± 36
10	9.05 ± 0.67	6.04 ± 1.18	0.38 ± 0.02	0.34 ± 0.04	886 ± 132	1014 ± 59
12	9.58 ± 0.95	2.73 ± 0.70	0.33 ± 0.02	0.17 ± 0.01	873 ± 78	1075 ± 83
14	8.98 ± 0.45	1.85 ± 0.16	0.37 ± 0.05	0.05 ± 0.01	1440 ± 212	1063 ± 54
18	9.78 ± 0.55	2.04 ± 0.65	0.41 ± 0.04	0.15 ± 0.06	1210 ± 203	1201 ± 103

* Male Wistar rats were fed the adequate diet throughout. Control rats were given an i.d. injection of liquid paraffin. The other group of rats were given an injection of the adjuvant. 1 hr before each rat was killed, it was given [^{3}H]-aminolaevulinic acid i.p. Results are the mean ± S.E.M. from three rats.

adjuvant treatment and have shown that the decline in activity is an early event in the sequence of effects and invariably precedes the development of arthritis. Furthermore, the finding that liver preparations from rats given the adjuvant by the i.p. route had a decreased capacity to metabolize aminopyrine, whilst such rats had no or only minor arthritic lesions, would add support to the view of Whitehouse and Beck [15] that the failure in drug-metabolizing enzyme activity is not solely determined by the presence of arthritic lesions. They [16] were also able to show that the different types of adjuvant used in the production of arthritic lesions can have varying effects on drug-metabolizing enzyme activity.

Adjuvant-induced arthritis is currently thought to occur through a mediator that circulates in plasma. Thus, it seemed possible that this same mediator might be responsible also for the production of the lesion in drug-metabolizing enzymes. Moreover, adjuvant-induced arthritis causes changes also in plasma protein concentrations that are manifested as an increase in the globulin fraction and a decrease in albumin. Since these proteins are synthesised in the liver, we have examined the correlation coefficient for the relationship in the activity of hepatic aminopyrine demethylase with the level of α_2-globulin, which is the principle inflammatory protein. In a large number of animals killed at intervals of up to 24 days after receiving adjuvant, there was a highly significant correlation but the correlation was not as good in mycoplasma-induced arthritic rats.

There are at least three mechanisms that might account for the decrease in cytochrome P-450 concentrations seen in adjuvant-induced arthritic rats: namely, binding of a substance to the cytochrome so that it interferes with the characteristic difference spectrum that is produced by carbon monoxide; inhibition of haem and/or cytochrome P-450 formation; and accelerated destruction of existing cytochrome P-450. Experiments involving the reconstitution of the microsomal fraction and the supernatant fraction from arthritic or control rats, and experiments in which liver preparations containing aminopyrine demethylase were incubated with serum from arthritic rats, both failed to demonstrate any inhibitory effect. Thus, we have no evidence for the binding of substances to cytochrome P-450.

During the course of our experiments, we noticed that the sera from many of the arthritic rats had a greenish tinge. It has been shown that allylisopropylacetamide and some other acetamides will produce green pigments in liver as well as reducing the levels of cytochrome P-450. Thus, it seemed possible that these two green pigments may have similar origins. In their work on allylisopropylacetamide, De Matteis and his colleagues [21, 25] demonstrated that there was a rapid loss of cytochrome P-450, whilst at the same time there was an increase in the activity of 5-aminolaevulinic acid synthetase. De Matteis [25] suggested that 'the green pigments could arise from damage to the microsomal structure so that a labile pool of microsomal haem (such as the P-450 haem) becomes sensitive to random methine bridge oxidation, resulting in the production of non-physiological biliverdin isomers that cannot be converted to bilirubin'. He suggested, furthermore, that changing the chemical constitution of the haem may render it unable to fulfil its normal function of feed-back control at the level of 5-aminolaevulinate synthetase. In our experiments, we have shown that the adjuvant

Table 7. Incorporation of [2-^{14}C]glycine into microsomal haem of adjuvant-treated rats*

Days after injection	Cytochrome P-450 (nmoles/mg protein)		Incorporation of glycine (dpm/mg protein)	
	Control	Arthritic	Control	Arthritic
10	0.39 ± 0.05	0.18 ± 0.04	19.30 ± 4.07	27.77 ± 4.18
14	0.39 ± 0.06	0.14 ± 0.08	23.13 ± 2.84	22.93 ± 3.35
18	0.34 ± 0.05	0.11 ± 0.05	24.17 ± 2.46	20.48 ± 5.30

* Male CFHB rats were injected i.d. into the right hind foot with either liquid paraffin or *M. butyricum* in liquid paraffin. Rats were killed on days. 10, 14 and 18 after injection. Two hrs before death, the rats were given an i.p. injection of [2-^{14}C] glycine (10 μCi). Results are the mean ± S.E.M. of five values, each on tissue from a single rat.

treatment had no significant effect on the activity of 5-aminolaevulinate synthetase. In further studies we found that the incorporation of [2-^{14}C]glycine and [G-^{3}H] 5-aminolaevulinate into microsomal haem was similar in both arthritic and control rats, when the results were expressed as dpm incorporated/mg of liver protein. Nevertheless, since we did not isolate purified cytochrome P-450, the possibility exists that adjuvant-induced arthritis may affect the incorporation of haem into apocytochrome P-450. It has been shown that this step is a second control step in the biosynthetic pathway leading to cytochrome P-450 synthesis [31].

De Matteis studied the effect of allylisopropylacetamide on cytochrome P-450 breakdown in two ways [21]. First, he showed that in the presence of cycloheximide, which prevented P-450 synthesis, allylisopropylacetamide accelerated the loss of cytochrome P-450 over a 6-hr period. Secondly, he pre-labelled the haem pools by giving [4-^{14}C] 5-amino-laevulinate and [G-^{3}H] 5-aminolaevulinate at different times so that the ^{3}H and ^{14}C would be expected to be in different haem pools. He was then able to show that 2-allylisopropylacetamide caused a loss of haem from the fraction with the more rapid turnover. In adjuvant-induced arthritic rats the changes in the concentration of cytochrome P-450 occur over a much longer timespan (several days) than those produced by allylisopropylacetamide (2–3 hr). Thus, the changes in concentration occur over a longer time period than the half-life of cytochrome P-450. Furthermore, there is considerable animal to animal variability in the rate at which arthritis develops and in the level of cytochrome P-450. Therefore, the interpretation of the results of decay experiments is difficult as the pool-size of the cytochrome P-450 at the beginning of the experiment is not known.

Adjuvant-induced arthritis in the rat is widely used as an experimental system for testing drugs that may be useful for treating rheumatoid arthritis in man and any effect on drug metabolizing enzymes will have implications on drug screening programmes. First, drugs may be prematuraly discarded because of toxicity in the adjuvant-treated rat (that is failure to metabolize the drug); and secondly, compounds of the cyclophosphamide type, which need to be converted to an active metabolite, may remain undetected [16]. However, the finding in this paper that drug-metabolizing enzyme activity is reduced also in mycoplasma-induced arthritis raises the possibility that a defect in drug-metabolizing enzyme activity may be present in human rheumatoid patients.

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