Biochemical Pharmacology, Vol. 33, No. 17, pp. 2771–2776, 1984.
Printed in Great Britain.

0006–2952/84 $3.00 + 0.00

INDUCTIVE AND REPRESSIVE EFFECTS OF RIFAMPICIN ON RABBIT LIVER MICROSOMAL CYTOCHROME P-450

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(*Received* 26 *January* 1984; *accepted* 20 *March* 1984)

Abstract—New Zealand White rabbits were treated with rifampicin at a dose of 50 mg/kg for 4 days. The total amount of microsomal hepatic cytochrome P-450 was not modified in treated, with respect to control, animals. However, further studies involving SDS-PAGE analysis, monooxygenase activity measurements and radial immunodiffusion assays indicated that rifampicin strongly affects the level of two P-450 isoenzymes. An LM_3 form was induced; this form, apparently associated with erythromycine demethylase activity and hydroxylation of progesterone preferentially in position 6β, was shown to be immunologically and functionally different from LM_{3a} and LM_{3b}. On the other hand, an LM_4 form, typically induced by β-naphthoflavone, was repressed. The concomitant inductive/repressive effect of rifampicine on two cytochrome P-450 isoenzymes makes this drug a very atypical inducer, at least in the rabbit.

Rifampicin, a macrolide antibiotic prepared from rifamycine [1–3] is established as an antituberculous drug [4], especially effective in its combination with isoniazid [5]. However, cases are reported where the combined administration of these drugs led to fulminant hepatitis, which may be associated with an enzyme-inducing effect of rifampicin [6]. The interference of rifampicin with the effectiveness of other drugs, i.e. anticoagulants [7], tolbutamide [8] and digitoxin [9] as well as its interference with steroid metabolism [10, 11] may be explained by an induction of cytochrome P-450.

Previous studies of eventual rifampicin generated P-450 induction in animals were not conclusive [12, 13]. This could be because too much attention was paid to the effect on the *total* liver microsomal P-450 rather than the effect on particular forms. Here we show that when rabbits are treated with rifampicin, whereas the total P-450 does not change, a specific form of P-450 is induced and another one is repressed.

MATERIALS AND METHODS

Microsomal preparation and characterization: rifampicin (kindly donated by Laboratories Lepetit) was intraperitoneally injected in aqueous solution (pH 7.2–8.2) in New Zealand male rabbits for 4 days. The rabbits were then starved and killed the next day. The livers were perfused *in situ* with 0.9% NaCl solution. The liver microsomal fraction was obtained according to Haugen *et al.* [14] and after homogenation in 0.01 M Tris–HCl buffer, pH 7.4, 0.1 mM EDTA, 20% glycerol (v/v) kept at −80° until further treatment. Protein concentration was determined by the Biuret method. Cytochrome P-450 and cytochrome b_5 were determined according to the procedure of Omura and Sato [15]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gel staining and scanning was carried out as described previously [16]. Spectra were recorded on a Cary 219 spectrophotometer.

Cytochrome P-450 activity measurements were carried out essentially as described by Bonfils *et al.* [16]. Erythromycin (Laboratories Roussel, Paris) demethylation was determined by formaldehyde formation, detected by the method of Nash [17, 18]; ethoxycoumarine deethylation and biphenyl (Fluka Ag, Buchs, Sg) hydroxylation were determined fluorimetrically [19, 20]. The standards used were 2 OH-biphenyl and 4 OH-biphenyl (Fluka, Ag). Aniline hydroxylation was determined as described by Schenkman *et al.* [21]. Hydroxylation of progesterone was determined by a method similar to that used by Dieter *et al* [22]: microsomes (0.5 mg protein/ml) were incubated with NADPH (Sigma) (0.8 mM) in potassium phosphate buffer (50 mM), pH 7.4, 0.5 ml final volume with 5 μl of progesterone (20 mM in benzene/methanol = 1/8 (v/v)), specific activity ^{14}C = 50 μCi/ml (^{12}C progesterone from Sigma, ^{14}C [4] progesterone from C.E.A.). The ice-cold incubation mixture was transferred to 37° and the reaction was stopped after 7.5 min by the addition of 3 ml of dichloromethane. Under these conditions, the product formation was linear as a function of time and microsomal protein concentration. After Vortex agitation (5 min) and centrifugation (10 min) at $\simeq$ 300 *g*, the organic phase was dried with sodium sulfate and evaporated at 37° under nitrogen; progesterone metabolites were redissolved in 30 μl ethyl acetate prior to thin layer chromatography on TLC plates (20 × 20 × 0.05 cm) pre-coated with silica gel 60 F 254 (Merck). The chromatograms were developed in cyclohexane/ethyl acetate/ethanol = 9/9/2. Substrate and metabolites were detected by autoradiography with a Kodak X-ray film; the radioactive material was recovered by scraping, and after two further extractions with ethyl acetate, the radioactivity was measured in 7.5 ml dimilume-30 (Packard Instr. Co., Inc., Warrenville, IL) with a delta 300 liquid scintillation counter (Searle Analytic Inc.). The identity of 6β and 16α -OH progesterone was established by comparison with authentic standards (6β OH-progesterone: Steraloids; 16α -OH pro-

gesterone: Sigma) by TLC and by gas-chromatography (to be published elsewhere). In order to check the purity of the progesterone metabolites scraped from the TLC plates, they were tested by high pressure liquid chromatography (HPLC) by means of a Gilson HPLC assembly (Model 302 pump and holochrome detector) using a Whatman Partisil PXS 5/25 ODS-3 column. We used a mixture of water/methanol = 3/7 at the flow rate of 1 ml/min. Effluent fractions of 20 drops were dried and their radioactivity was counted as described above. No significant amounts (≃3%) of impurities were found.

Radial immunodiffusion was carried out according to Thomas *et al.* [23] with antibodies raised against purified LM_{3b} and LM_4 in sheep (kindly provided by Dr C. Bonfils). The microsomal proteins were solubilized with emulgen and cholate, and 5, 10 and 15 μl of 8 mg/ml microsomal protein containing solutions were deposed onto the gel containing 0.02% IgG anti LM_{3b} or anti LM_4. The rabbit liver microsomal cytochrome P-450 isoenzymes LM_2, LM_{3b} and LM_4 were prepared as previously described [24, 25]. A sample of LM_{3c} was a gift from Prof. M. J. Coon (University of Michigan).

RESULTS

The cytochrome P-450 content and the cytochrome P-450/cytochrome b_5 ratio of microsomes prepared from rifampicin-treated rabbits is not significantly different from that of microsomes from untreated rabbits. On the other hand, the electrophoretic pattern as well as some P-450 specific activities reveal a great difference between the microsomes from rifampicin-treated and untreated rabbits. From a comparison of the microsomal electrophoretic pattern of untreated and rifampicin-treated rabbits (Fig. 1), it appears that rifampicin induces a protein with a molecular weight corresponding to an LM_3 type cytochrome P-450 isoenzyme and it represses a protein with a molecular weight corresponding to an LM_4 type P-450 isoenzyme. The assignment of the electrophoretic bands to different P-450 isoenzymes is further illustrated by the gel scanning shown in Fig. 2.

In order to quantify the induced and the repressed proteins, we used as an internal standard a protein (molecular weight *ca.* 42 KD) which is present in all microsomal preparations, but which we have not identified. The amount of this protein reflected by the intensity of its electrophoretic band, is apparently independent of drug administration. Thus, after gel scanning (Fig. 2) the relative intensity of the LM_3 and the LM_4 band were determined with respect to the 42 KD band. By this method, we found that the half-maximal inductive effect of rifampicin is obtained by treating rabbits with 15–20 mg/kg body weight/day for 4 days.

Whereas ethoxycoumarin deethylase and aniline hydroxylase (known to be specific for the LM_{3a} isoenzyme [26]) activities were not affected by the rifampicin treatment, erythromycin demethylation

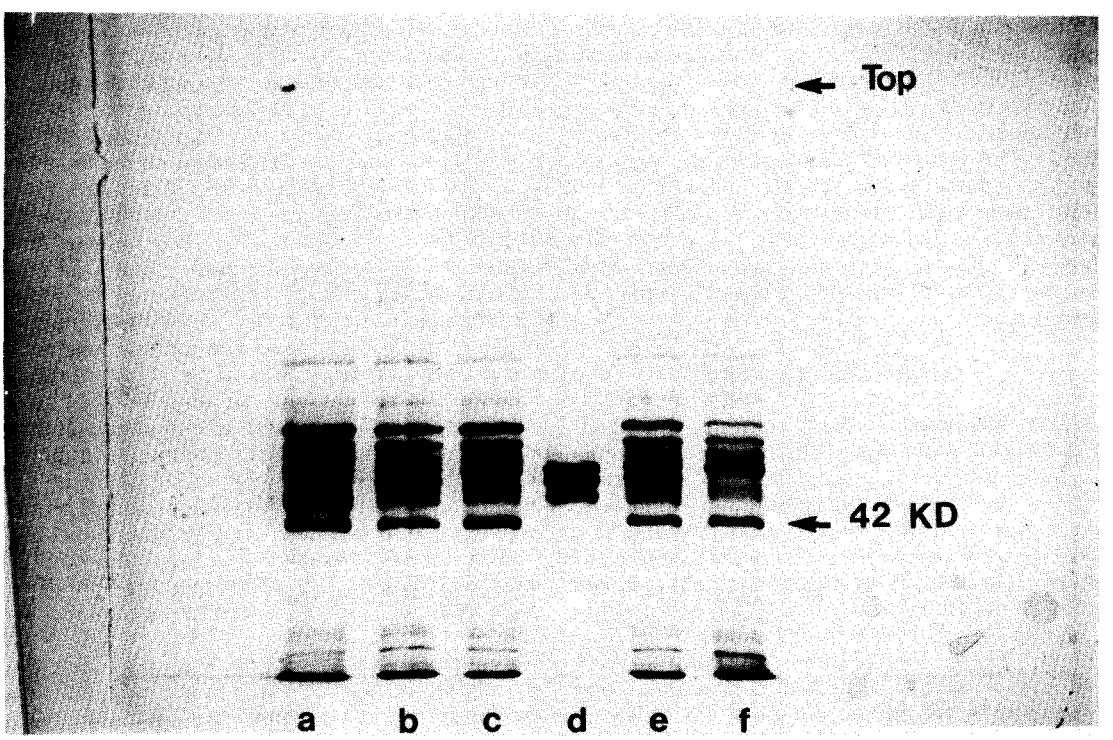

Fig. 1. SDS-polyacrylamide (8%) gel electrophoresis of rabbit microsomes prepared from (a) phenobarbital-treated (1 g/l drinking water; 8 days), (b) and (e) rifampicin-treated (50 mg/kg rabbit; 4 days), (c) untreated, (f) β-naphthoflavone-treated (80 mg/kg rabbit; 3 days) rabbits (30 μg of liver microsomal protein) and (d) of a mixture of LM_2 (20 pmol), LM_{3c} (30 pmol) and LM_4 (20 pmol). The arrow indicates the electrophoretic band used as internal standard.

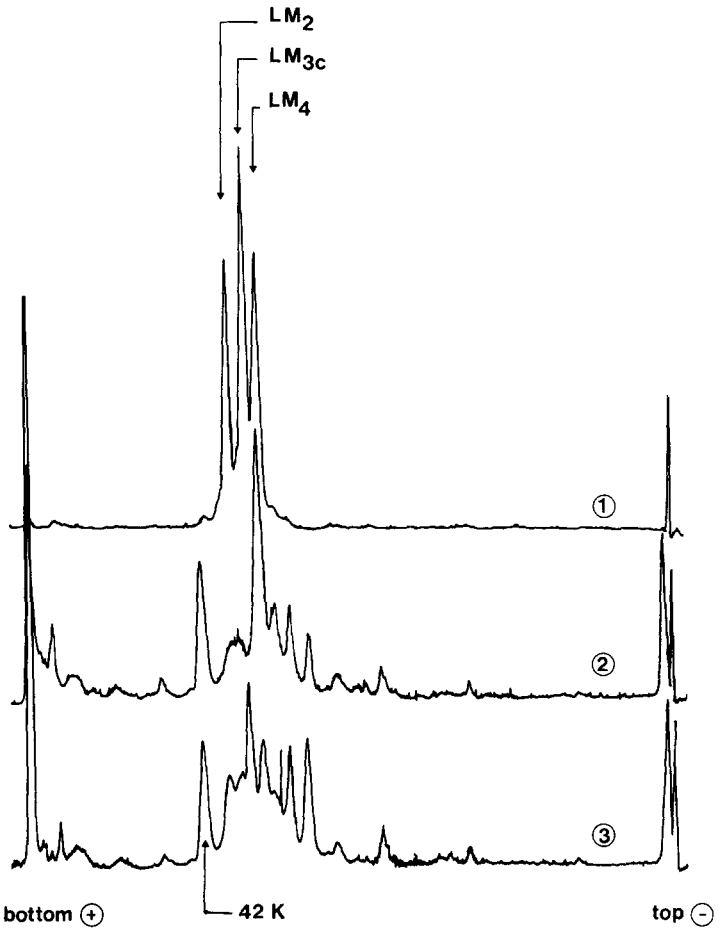

Fig. 2. Gel scanning of electrophoretic profiles shown in Fig. 1. (1) LM_2, LM_{3c}, LM_4; (2) microsomes from β-naphthoflavone-treated rabbits; (3) from rifampicin-treated rabbits.

and biphenyl-4-hydroxylation were respectively increased and decreased. As shown in Fig. 3A, erythromycin demethylase increases linearly with the relative intensity of the electrophoretic band in the LM_3 zone. Although staining intensity and concentration may not always be related quantitatively, this linear relationship indicates that the increased erythromycin demethylase activity is due to an increased concentration of an LM_3 type P-450 isoenzyme. A similar correlation is found between the repression

Table 1. Progesterone 6β and 16α hydroxylation. Effect of rifampicin administration to rabbits on the activities of the microsomal preparations, compared to the activities of selected P-450 isoenzymes

Microsomes	Progesterone hydroxylation (nmol product/mg protein/min) 6β	16α
Rifampicin; 100 mg/kg/day	4.9	0.59
Rifampicin; 50 mg/kg/day	5.1	0.72
Rifampicin; 25 mg/kg/day	3.1	0.67
Phenobarbital	0.48	0.25
Untreated	0.45	0.17
	(nmol product/nmol P-450/min)	
P-450 isoenzymes (reconstituted system)	6β	16α
LM_2	0.6	in trace
LM_{3b}	0.75	4.1
LM_4	4.2	0.9

Progesterone hydroxylase was measured as described in the Methods section. Reconstituted system as described by Bonfils *et al.* [16].

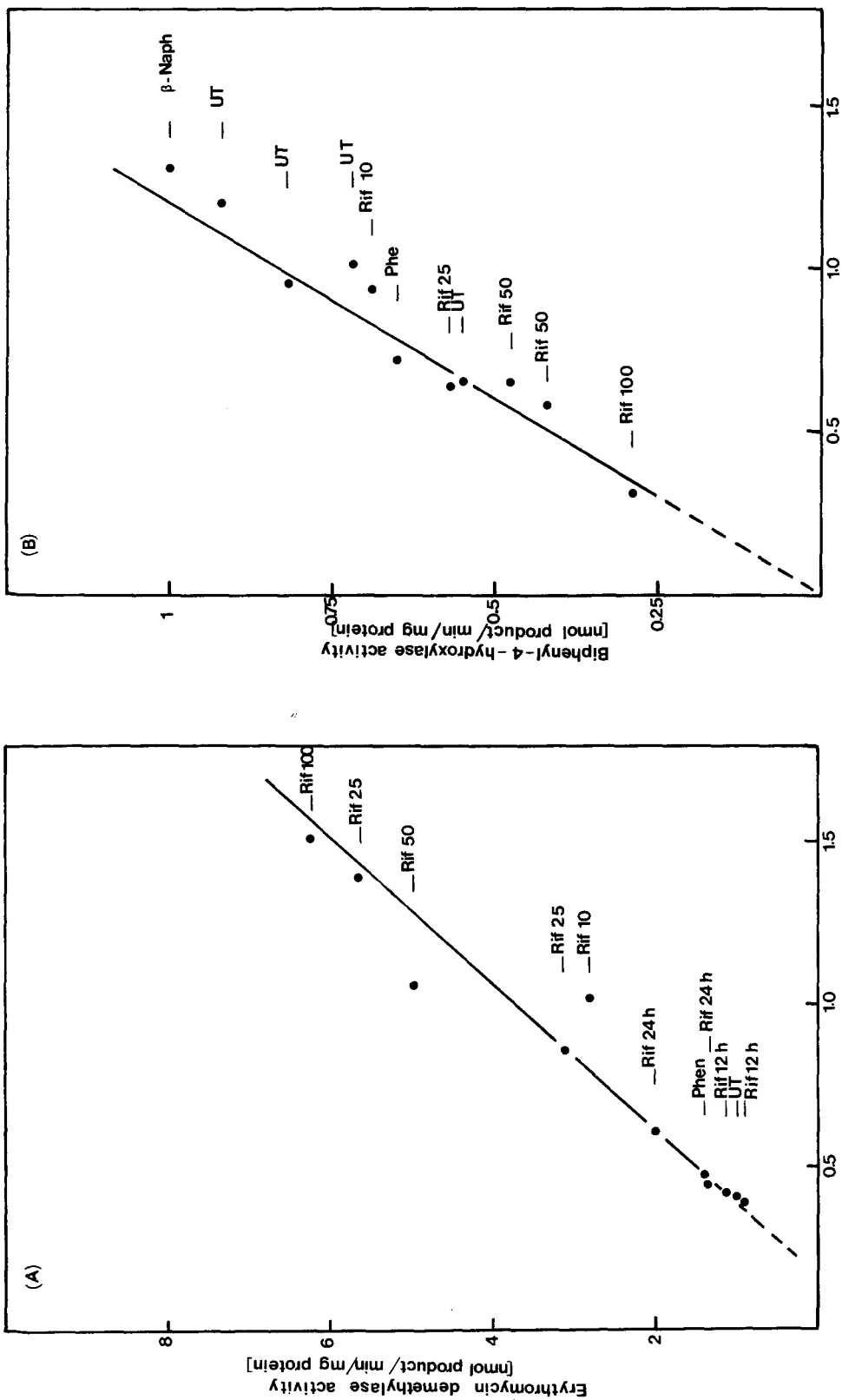

Fig. 3. Correlation between cytochrome P-450 specific activities and relative intensities of microsomal electrophoretic bands. Microsomes (1 mg protein/ml) were incubated at 37° for 10 min in phosphate buffer, 50 mM, pH = 7.4 with 0.8 mM NADPH in the presence of erythromycin, 0.4 mM or biphenyl, 0.2 mM. (A) Erythromycin demethylase and the electrophoretic LM_3 zone. (B) Biphenyl-4-hydroxylase and the electrophoretic LM_4 zone. Microsomes from untreated (UT) rabbits, and rabbits treated by a single dose of 50 mg rifampicin, the animal being sacrificed 12 or 24 hr thereafter (Rif 12 hr, 24 hr), by rifampicin for 4 days with 10, 25, 50 or 100 mg/kg rabbit (Rif 10, 25, 50, 100), by phenobarbital (Phen), or by β-naphthoflavone (β-Naph).

of the biphenyl-4-hydroxylase and the decrease of the relative intensity of the electrophoretic band corresponding to the zone of LM_4 type isoenzymes (Fig. 3B).

Another characteristic feature of rifampicin induction is the large increase of both 6β (8- to 10-fold) and 16α (3- to 4-fold) hydroxylation of progesterone, as shown in Table 1. It must be mentioned here, that although this activity has not apparently been reported to be stimulated in man under rifampicin therapy, 6β hydroxylation of cortisol is increased [10].

We have compared the microsomal activities to the activities of three selected cytochrome P-450 isoenzymes. Whereas LM_2 did not show a marked activity towards progesterone, LM_{3b} had a preference for the 16α-hydroxylation and LM_4 was found to be more active in the 6β-hydroxylation. We have also verified that under our incubation conditions the three isoenzymes can support other cytochrome P-450 specific reactions.

DISCUSSION

As shown in Fig. 3, we found a strong correlation between the two cytochrome P-450 specific activities erythromycin demethylase and biphenyl hydroxylase and the relative staining intensities of the electrophoretic LM_3 and LM_4 band. In the case of the LM_4 band, the results for microsomes from different untreated rabbits are fairly scattered; this is not surprising as we did not work with a strain of inbred animals. Nevertheless, the linearity between biphenyl hydroxylase and the staining intensity of the LM_4 band is conserved. We therefore concluded that the staining intensity may be used to monitor eventual drug-induced concentration changes of LM_3 type and LM_4 type cytochrome P-450 isoenzymes. By this method we found that increasing rifampicin administration to rabbits induces an LM_3 type and represses an LM_4 type cytochrome P-450 isoenzyme. However, LM_3 and probably LM_4 also are composed of several distinct isoenzymes, and the question therefore arises, which of these isoenzymes is affected by the inductive and repressive properties of rifampicin. The increase of erythromycin demethylase activity appears suggestive of a LM_{3b} induction [16]. However, the finding that the progesterone 6β hydroxylase is much more stimulated than the 16α hydroxylase, argues against a LM_{3b} induction. Indeed, LM_{3b} is more efficient in the 16α than in the 6β progesterone hydroxylation (Table 1). Furthermore, the results presented in Fig. 3B are suggestive of a LM_4 type isoenzyme repression.

In order to clarify these points, we therefore carried out radial immunodiffusion assays. No difference in the immunodiffusion ring areas were found when solubilized microsomes from control and rifampicin-treated rabbits were run against IgG anti LM_{3b}, suggesting strongly that LM_{3b} was not the induced form. On the other hand, rifampicin treatment resulted in a 40–70% decrease (depending on dose and individual animal response) of the diffusion ring areas when the assays were carried out with IgG anti LM_4. These experiments confirm the results reported in Fig. 3B that a LM_4 isoenzyme (known to be induced by β-naphthoflavone) is repressed under rifampicin treatment.

We cannot yet definitely identify the rifampicin-induced LM_3 isoenzyme. Clearly, LM_{3a} can be ruled out on the basis of the electrophoretic profile and the absence of aniline hydroxylase stimulation. The profile of hydroxylated progesterone metabolites as well as the immunodiffusion experiments tend to rule out LM_{3b} too.

Whether rifampicin induces LM_{3c} or still another LM_3 type isoenzyme awaits further experiments. Similarly, we do not know yet whether the repression is general to LM_4 or specific to a subform of LM_4. Indeed, different LM_4 type isoenzymes have been reported [27].

According to our results, rifampicin behaves as an atypical inducer, by strongly affecting the balance between at least two forms of cytochrome P-450. It is interesting to note here, that in pigs also rifampicin appears to affect differentially specific forms of cytochrome P-450. In particular an induction of a protein (52 KD) and a repression of proteins of higher molecular weight have been reported [28]. On the other hand, in man rifampicin administration results in an increase of total cytochrome P-450 [11, 29, 30], but here a differential inductive/repressive effect on particular P-450 isoenzymes has not yet been detected. Whether such a process occurs in man or whether it is responsible for the rifampicin-mediated drug interactions observed, is still a matter of conjecture.

Acknowledgements—We greatly appreciated the helpful discussions with Dr Franck Travers, Dr Claude Bonfils and Monsieur Christian Larroque. The authors are very grateful to Professor M. J. Coon for his generous gift of LM_{3b} and LM_{3c}, to Lepetit Laboratories and Roussel Laboratories for kindly providing TAO and erythromycin, respectively. This work was supported in part by the Fondation pour la Recherche Médicale Française.

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