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Heterogeneousness of the Microsomal Enzymes Effecting the o- and p-Hydroxylation of Aniline

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In papers by KAMPFFMEYER and KIESE (1963, 1964a,b) evidence has been adduced for the existence of different microsomal enzymes involved in the N-hydroxylation of aniline, the N-hydroxylation of N-alkylanilines, and the p-hydroxylation of aniline. It is also hinted at there that differences exist between the enzymes that perform the o- and p-hydroxylation of aniline. PARKE and WILLIAMS and PARKE found that the ratio of p- to o-aminophenol in the urine of various animal species dosed with aniline varied widely from gerbils, 15, to cats, 0.4. In the urine of rabbits the ratio was discovered to be 6. PIOTROWSKI, in experiments on rabbits, found the ratio to be 12. In experiments with liver microsomes, species differences, it was noticed, also affected the rate of the p- and o-hydroxylation. POSNER, MITOMA, and UDENFRIEND observed that an equal rate in the p- and o-hydroxylation of aniline was achieved when microsomes obtained from cat's liver were employed. With rabbit-liver microsomes the ratio of p- to o-aminophenol was found to be "large". Similarly the p- and o-hydroxylation rate of acetanilide was seen to be equal in the case of both cat and dog microsomes, but 200 in the case of rabbit-liver microsomes.

The species-differences affecting the p- and o-hydroxylation rate of aniline, which have also been observed with 2-acetylaminofluorene (WEISBURGER, WEISBURGER, and MORRIS; WEISBURGER, WEISBURGER, MORRIS, and SOBER) could be due to various ratios of the p- and o-hydroxylating enzymes as well as to the differences in the structure of a single hydroxylating enzyme.

We have studied the p- and o-hydroxylation of aniline effected by rabbit-liver microsomes. Under the prevailing conditions of our experiments, freshly prepared microsomes produced p- and o-aminophenol in the ratio of about 5, a result very similar to that found by PARKE in rabbit urine, viz a ratio of 6 between p- and o-aminophenol. While two

weeks' storage at 2—3 °C did very little to reduce the capacity of the microsome preparations to p-hydroxylate aniline it abolished most of their capacity to o-hydroxylate aniline.

Further observations which point to the existence of a p-hydroxylating and an o-hydroxylating enzyme were made with inhibitors. Copper chloride and semicarbazide inhibited the o-hydroxylation much more than the p-hydroxylation of aniline.

MILLER and MILLER have observed that rats produce 1-hydroxy-2-acetylaminofluorene more quickly from N-hydroxy-2-acetylaminofluorene than from 2-acetylaminofluorene. They assume that the hydroxylamine is an intermediate in o-hydroxylation. It is unlikely that the main route of the o-hydroxylation of aniline by rabbit liver microsomes is via phenylhydroxylamine. The inhibitors of the o-hydroxylation used in this study do not equally affect the N-hydroxylation of aniline (KAMPFFMEYER and KIESE 1964a).

Methods

Microsomes were prepared from rabbit's livers, suspended in phosphate, and fortified with NADP etc. as described by KAMPFFMEYER and KIESE (1963). The concentration of the microsomes in the suspensions was equal to that in the homogenate. The aniline concentration was 10 mM, the incubation time 40 min. p-Aminophenol was determined according to the method of BRODIE and AXELROD with the volumes of solvents as used by KAMPFFMEYER and KIESE (1963). In order to determine the o-aminophenol this was separated from other amines by means of chromatography. As a large part of the o-aminophenol gets lost in the isolation procedure its was stabilized by acetylation before being extracted and chromatographed. After being separated from other amines the o-acetaminophenol was determined by means of the extinction of the color produced by GIBBS' reagent. The procedure was as follows. At the end of the incubation period 5 ml of the microsome suspension were treated with 0.2 ml acetic anhydride. The acetaminophenols were extracted by means of 10 ml ether freed from peroxides. An aliquot of the ether phase, as large as was possible, was separated and dried by shaking with sodium sulfate. The ether was then evaporated at 38° C. The acetic anhydride was removed in vacuo. The residue was dissolved in 0.05 ml methanol at 2° C. Of this solution 0.03 ml were used for thin-layer chromatography on silica gel according to STAHL. The upper phase of a mixture of 2 volumes benzene, 2 volumes glacial acetic acid, and 1 volume of water, which BRAY, CLOWES, and THORPE, as well as MITOMA, POSNER, REITZ, and UDENFRIEND, had employed in the paper chromatography of aminophenols, was used as solvent. During about 50 min chromatography at 2° C the solvent front ascended about 12 cm. The aminophenols were located by spraying with FOLIN's reagent (diluted with 4 volumes of water). The R_F value of o-acetaminophenol was found to be 0.125, while the p-acetaminophenol moved very little with the solvent. In the analytical procedure the chromatogramms of the microsome extracts were not sprayed with FOLIN's reagent. On each plate, however, authentic o-acetaminophenol was applied in order to be chromatographed under the conditions of the experiment. It was located by spraying with FOLIN's reagent. On the corresponding spot in the chromatogramms of the microsome extracts a 10×10 mm area of the dry silica gel was scratched off. In order to dissolve the o-acetaminophenol the powder was suspended in 1.7 ml 0.1 N sodium hydroxide and shaken

for half an hour. After centrifuging 1.5 ml of the supernatant were mixed with 0.2 ml 0.5 N hydrochloric acid, 1 ml 0.2 M borate pH 9.25, and 0.2 ml of GIBBS reagent (0.01 g 2,6-dichloroquinonechloroimide in 100 ml ethanol). After one hour the solution was centrifuged and the extinction of the blue color measured at 607 mμ.

Results

1. The effect of the age of the microsome preparations upon the o- and p-hydroxylation of aniline

Our first experiments concerned with the microsomal o-hydroxylation of aniline showed a very wide variation of results. It was very soon noticed that older preparations were less active than fresh ones. From the data of Table 1 it may be seen that the capacity of rabbit-liver microsomes to o-hydroxylate aniline decreased to half the activity of

Table 1. *The effect of the age of rabbit liver microsome preparations upon the o- and p-hydroxylation of aniline*

The microsomes were stored at 2—3° C suspended in one fifth of the homogenate volume, as they were routinely prepared (KAMPFFMEYER and KIESE 1963)

	o-Aminophenol					p-Aminophenol			
	Age of the microsome preparations days	Number of experiments	Average concentration produced μg/ml	Difference from I	p	Number of experiments	Average concentration produced μg/ml	Difference from I	p
I	1	8	3.07 ± 0.7			12	14.7 ± 1.75		
II	4—9	4	1.38 ± 0.64	1.69 ± 0.95	0.105	4	11.5 ± 1.01	3.2 ± 2.02	0.14
III	10—19	4	0.96 ± 0.56	2.11 ± 0.87	0.035	4	12.6 ± 0.36	2.1 ± 1.89	0.29

freshly prepared microsomes within a week and to one third of this activity in two weeks. The effect of the age of the preparations is demonstrated in spite of a rather large standard error. It would have been more impressive if the decrease in activity of the same preparation in the course of time could have been followed.

In contrast to the o-hydroxylating activity the p-hydroxylating activity proved rather stable. In two weeks it decreased by only 10—30%$_0$ (Table 1).

2. The effect of some substances upon the o- and p-hydroxylation of aniline by microsomes

Several substances which have been found to affect N- and p-hydroxylation (KAMPFFMEYER and KIESE 1964) were tested as to their effect upon the o- and p-hydroxylation of aniline. The effect of triparanol and SKF 525 A (diphenylpropylacetic ester of diethylaminoethanol) upon the o-hydroxylation could not be determined. Both substances

were not separated by chromatography from o-acetaminophenol and interfered with the determination of the o-acetaminophenol. With 4 compounds a series of experiments was carried out. The results are contained in Table 2. They show that the o- and p-hydroxylation are not affected differently by iproniazid and N-ethylmaleimide. Copper chloride and semicarbazide, however, inhibit the o-hydroxylation of aniline more strongly than the p-hydroxylation.

Table 2. *The effect of some substances upon the o- and p-hydroxylation of aniline by rabbit liver microsomes*

The figures at the head of the column indicate the concentration of o- and p-aminophenol found in the microsome suspensions after 40 min incubation without any addition. The other figures indicate the change in activity produced by the substance added in % of the unaffected activity. The data are the means and standard error of 5—12 experiments. Columns IV and V show the results of the *t*-test

I		II	III	IV	V
		o-Aminophenol 3.07 µg/ml	p-Aminophenol 14.7 µg/ml	II—III	*p*
Copper chloride	10^{-3} M	-47.6 ± 5.8	-26.2 ± 3.3	21.4 ± 6.7	0.006
Semicarbazide	10^{-2} M	-59.5 ± 6.1	-14.6 ± 3.3	44.9 ± 6.9	< 0.001
Iproniazid	10^{-3} M	-38.5 ± 6.9	-51.8 ± 3.3	-13.3 ± 7.6	0.1
N-Ethylmaleimide	5×10^{-3} M	-55.8 ± 4.5	-43.0 ± 5.2	12.8 ± 6.9	0.06

Summary

A study of the p- and o-hydroxylation of aniline by rabbit-liver microsomes has shown that these reactions are differently affected by the storage of the microsomes and by copper chloride or semicarbazide. The results point to the existence of two enzymes involved in the p- and o-hydroxylation of aniline.

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