action of phenobarbital speaks in favour of an increased synthesis of enzyme protein induced by phenobarbital. Such a mechanism underlying the action of phenobarbital might explain, why stimulation of *microsomal* enzymes eventually leads to an increased activity also of *cytoplasmatic* enzymes.

It is not clear why phenobarbital did not stimulate other cytoplasmatic enzymes—lactate dehydrogenase for instance remained unaffected—and why tolbutamide was without any effect on liver dopadecarboxylase.

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REFERENCES

1. H. V. GELBOIN and L. SOKOLOFF, *Science, N.Y.* **134**, 611 (1961).
2. H. REMMER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **237**, 296 (1959).
3. A. H. CONNEY, C. DAVISON, R. GASTEL and J. J. BURNS, *J. Pharmac. exp. Ther.* **130**, 1 (1960).
4. W. KORANSKY, J. PORTIG, H. W. VOHLAND and I. KLEMPAU, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **247**, 61 (1964).
5. H. REMMER, *Proc. Int. Pharmac. Meetings*, Vol. 6, 235 (1962).
6. L. COOK, J. J. TONER and E. J. FELLOWS, *J. Pharmac. exp. Ther.* **111**, 131 (1954).
7. J. AXELROD, J. REICHENTHAL and B. B. BRODIE, *J. Pharmac. exp. Ther.* **112**, 49 (1954).
8. J. B. SCHENKMAN and H. REMMER, *Fedn Proc.* **25**, 343 (1966).
9. M. V. SIMPSON, E. FARBER and H. TARVER, *J. biol. Chem.* **182**, 81 (1950).
10. H. HERKEN, D. MAIBAUER and D. NEUBERT, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **233**, 139 (1958).

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Excretion of N-hydroxy-2-aminofluorene by guinea pigs injected with 2-acetylaminofluorene

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GUINEA PIGS are refractory to the carcinogenic action of 2-aminofluorene[1, 2] and 2-acetylaminofluorene.[3–5] In contrast to many other species they do not excrete N-hydroxy-2-acetylaminofluorene in the urine after the administration of 2-acetylaminofluorene, at any rate less than could hitherto be detected.[5, 6] Kiese *et al.*[7] found N-hydroxy-2-aminofluorene in the urine of guinea pigs intraperitoneally injected with 2-aminofluorene. Only 0·5 per cent of a dose of 100 mg/kg was recovered as free N-hydroxy derivative in the urine. Since guinea pig liver microsomes have been observed to deacetylate 2-acetylaminofluorene[8] and, even more rapidly, N-hydroxy-2-acetylaminofluorene,[9] we studied whether guinea pigs excrete N-hydroxy-2-aminofluorene after the injection of 2-acetylaminofluorene.

The role of N-hydroxy derivatives of aromatic amines as proximate metabolites in carcinogenic and ferrihaemoglobin-forming action of aromatic amines has been recently reviewed by Miller and Miller[10] and by Kiese.[11]

METHODS

N-Hydroxy-2-aminofluorene was determined in the urine after being oxidized to 2-nitrosofluorene by means of ferricyanide, as described by Kiese *et al.*[7] Silica gel HF 254 + 366 was used for thin-layer chromatography of carbon tetrachloride extracts prepared from urine. The authentic 2-nitrosofluorene used for identifying the compound observed in the urine was the same as used in the experiments of Kiese *et al.*[7] Its preparation is described in a paper by Jagow *et al.*[12]

Other arylhydroxylamines have been observed[12] to be most stable in urine if acidified to a pH between 4 and 5. The highest yield of nitroso compound was observed in the carbon tetrachloride extracts, if the oxidation of the hydroxylamines had been carried out at pH 4 to 5. This was confirmed with urine containing N-hydroxy-2-aminofluorene. The urine of the guinea pigs was therefore collected in glasses which contained a suitable amount of acetic acid and were cooled in an ice bath. Before oxidation with ferricyanide and extraction of the 2-nitrosofluorene by means of carbon tetrachloride the pH of the urine was adjusted to 4·5. Oxidation of the urine with ferricyanide at pH lower than 4 or higher than 5 was found to yield less 2-nitrosofluorene in the carbon tetrachloride extract than the oxidation at a pH between 4 and 5. If 2-nitrosofluorene dissolved in a small amount of methanol was added to guinea pig urine acidified to pH 4·5 so as to produce concentrations of one or two μg/ml it was extracted to 85 per cent into the carbon tetrachloride.

RESULTS

For each experiment 10 guinea pigs were used. They were housed individually in Acme stainless steel metabolism cages and fed on Altromin standard diet. Food and water were available *ad libitum*. The urine of the 10 animals was pooled, and the portions collected in two 4-hr periods and in the following 16 hr were analysed separately. In 3 experiments a dose of 122·8 mg 2-acetylaminofluorene (corresponding to 100 mg aminofluorene) kg was injected intraperitoneally and in another 4 experiments a dose of 245·6 mg/kg. The 2-acetylaminofluorene was suspended in a 0·25% solution of agar agar in 0·9% sodium chloride solution.

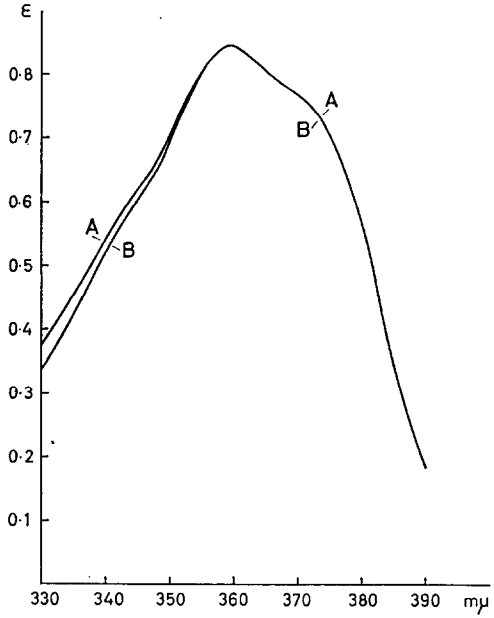

FIG. 1. 2-Nitrosofluorene extracted from the urine of guinea pigs dosed with 245·6 mg 2-acetylaminofluorene/kg after oxidation of the N-hydroxy-2-aminofluorene in the urine by means of ferricyanide. 18 ml of the carbon tetrachloride extract washed with 0·5 N sulfuric was evaporated and the residue chromatographed on silica gel. The spot with the same R_f as authentic 2-nitrosofluorene was eluted by means of 3 ml carbon tetrachloride. The extinction of this solution in a cuvette of 5 cm optical path is shown in curve A. Curve B is the extinction of a solution of 75·5 μg 2-nitrosofluorene/ml carbon tetrachloride at 1 mm optical path.

In addition to determining N-hydroxy-2-aminofluorene we were searching for N-hydroxy-2-acetylaminofluorene in the urine of the guinea pigs which had received either the lower or the higher dose. Irving's[13] chromatographic method was applied to urine samples which had been incubated with β-glucuronidase. No N-hydroxy-2-acetylaminofluorene was found.

In all experiments, however, small amounts of N-hydroxy-2-aminofluorene were determined in the urine. Mostly its concentration was highest in the first portion collected. No N-hydroxy derivative was found any longer in the urine collected 24 hr after the injection of the 2-acetylaminofluorene.

The appearance of N-hydroxy-2-aminofluorene in the urine of the guinea pigs was demonstrated by the extinction of the carbon tetrachloride extracts in u.v. light. The extinction maximum of 2-nitrosofluorene at 359 mμ was noticed in most extracts. But due to the low concentration of 2-nitrosofluorene the extinction was low. Therefore the 2-nitrosofluorene was concentrated and purified by thin-layer chromatography. Figure 1 shows the extinction of 2-nitrosofluorene obtained from 30 ml of urine excreted by guinea pigs in 4 hr after i.p. injection of 245·6 mg 2-acetylaminofluorene/kg. Only a small fraction of the 2-nitrosofluorene present in the carbon tetrachloride extract taken from urine is recovered after chromatography and elution of the spot by means of carbon tetrachloride.

The results of the determinations of N-hydroxy-2-aminofluorene in the 3 portions of urine collected after the injection of 2-acetylaminofluorene are listed in Table 1. After the dose of 245·6 mg 2-acetylaminofluorene nearly twice as much N-hydroxy-2-aminofluorene appeared in the urine as after half the dose of 2-acetylaminofluorene. The amount recovered in the urine as N-hydroxy-2-aminofluorene was only a small portion of the 2-acetylaminofluorene injected, i.e. 0·03 per cent. A much larger portion, namely 0·5 per cent, is found in the urine as N-hydroxy-2-aminofluorene if 2-aminofluorene is injected in place of 2-acetylaminofluorene.[7]

TABLE 1. N-HYDROXY-2-AMINOFLUORENE IN THE URINE OF GUINEA PIGS AFTER THE INTRAPERITONEAL INJECTION OF 2-ACETYLAMINOFLUORENE

Time after injection (hr)	Urine (ml)	N-Hydroxy-2-aminofluorene	
		μg/ml urine	μg/kg guinea pig
	122·8 mg 2-Acetylaminofluorene/kg averages of 3 experiments		
0–4	56	0·95	8·7
4–8	44	0·88	7·9
8–24	253	0·35	18·4
			35·0
	245·6 mg 2-Acetylaminofluorene/kg averages of 4 experiments		
0–4	60	1·3	16·1
4–8	66	1·1	15·5
8–24	266	0·53	31·8
			63·4

The amounts of N-hydroxy-2-aminofluorene determined in our experiments are probably smaller than the amounts actually excreted. In particular the fraction conjugated with glucuronic acid could not be estimated. N-hydroxy-2-aminofluorene is not stable in urine of pH 4·5 at 37 °C. After incubation with β-glucuronidase for 2 hr under nitrogen at 37 °C less N-hydroxy-2-aminofluorene is found in the urine than before the incubation.

SUMMARY

Small amounts of N-hydroxy-2-aminofluorene were observed in the urine of guinea pigs intraperitoneally injected with 2-acetyl-aminofluorene. The N-hydroxylation product was identified and determined after being oxidized to 2-nitrosofluorene. After the injection of 122·8 mg as well as 245·6 mg/kg 0·03 per cent of the dose was found in the urine as N-hydroxy-2-aminofluorene.

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REFERENCES

1. M. F. ARGUS and F. E. RAY, *Proc. Am. Ass. Cancer Res.* **2**, 92 (1956).
2. A. W. BREIDENBACH and M. F. ARGUS, *Q. J. Fla. Acad. Sci.* **19**, 68 (1956).
3. M. E. URQUHART, *Br. J. Cancer* **9**, 611 (1955).
4. E. K. WEISBURGER and J. H. WEISBURGER, *Adv. Cancer Res.* **5**, 331 (1958).
5. E. C. MILLER, J. A. MILLER and M. ENOMOTO, *Cancer Res.* **24**, 2018 (1964).
6. J. A. MILLER, J. W. CRAMER and E. C. MILLER, *Cancer Res.* **20**, 950 (1960).
7. M. KIESE, G. RENNER and I. WIEDEMANN, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **252**, 418 (1966).
8. M. KIESE and G. RENNER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **252**, 480 (1966).
9. C. C. IRVING, *Fedn Proc.* **24**, 152 (1965).
10. E. C. MILLER and J. A. MILLER, *Pharmac. Rev.* **18**, 805 (1966).
11. M. KIESE, *Pharmac. Rev.* in press.
12. R. VON JAGOW, M. KIESE and G. RENNER, *Biochem. Pharmac.* In press.
13. C. C. IRVING, *Cancer Res.* **22**, 867 (1962).

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The effect of pH on glycogenolysis in turtle heart*

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CATECHOLAMINES, and several other agents, are known to enhance the activity of the phosphorylase enzyme system, which initiates the metabolic breakdown of glycogen.[1] One of the main reactions—the activation of phosphorylase *b* to the more active phosphorylase *a* by the phosphorylase *b* kinase—was reported to be affected also by hydrogen ion concentration. At low pH, e.g. 6·2, the substrate affinity of the phosphorylase *b* kinase is much less than at higher pH, such as 8·2.[2] Most of the data on the pH effect were obtained under aerobic conditions. It was decided, therefore, to investigate the effect of pH on the glycogenolytic response during anoxia, as well as its possible modification by exogenous glucose. Isolated turtle hearts were chosen as the experimental preparation, since they can be used under aerobic and anerobic conditions for prolonged periods of time. The production of lactic acid, the terminal metabolite in the anaerobic glycogenolytic scheme, was selected as the measure of glycogenolytic activity.

METHODS

Isolated turtle hearts of either sex of the species *Chrysemis picta* and *Pseudymus elegans* were used in the study. The experiments were carried out partly during the summer months (July to September) and partly in winter (December). The original procedure for removal and preparation of heart and perfusion as described by Hardman *et al.*[3] was followed. A constant rate of 24 beats per minute was maintained by suprathreshold stimuli originating from an A.E.L. model 104A stimulator and transmitted with the help of silver electrodes which were attached to the base of the ventricle. The borate–acetate salt solution of Mines,[4] adjusted to the desired pH, was used in all but one series. In order to test whether the presence of acetate in the buffer can alter the rate of lactate release, a series of aerobic hearts was run with the Tris buffer. The perfusing fluid for the anaerobic hearts contained 1×10^{-3} M sodium cyanide; glucose, when included, was used in a final concentration of 0·1%. The hearts were perfused at each of three pH values for at least 30 min; a 2-min perfusate sample was then collected and the pH altered to the next level. Lactic acid in the perfusate was determined by the Barker-Summerson method.[5] Most samples were also analyzed by a gas chromatographic method.[6]

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