Blood Clotting System in the Pathogenesis of the Sanarelli-Shwartzman Reaction of the Rabbit

The generalized Shwartzman reaction is elicited in rabbits by means of two intravenous injections of endotoxin of Gram-negative bacteria given 24 h apart. Renal cortical necrosis is pathognomonic[1]. The demonstration of intravascular thrombin-activity[2,3] in rabbits treated in this way raised the question whether the thrombin alone can be made responsible for the morphological substratum characterizing this phenomenon. In order to clarify the importance played by the clotting mechanisms in the pathogenesis of the generalized Shwartzman reaction, the following studies were performed.

New Zealand rabbits weighing 2–2.5 kg were catheterized as described previously[4]. Resin thrombin (chromatographed and free of autoprothrombin-C) was prepared from purified bovine prothrombin[5]. *E. coli* endotoxin and Thorotrast were generously supplied by Testagar Laboratories, Detroit (Michigan). The doses used were: Thrombin-infusion: 400 U infused continuously for 4 h by intracardiac catheterization. Endotoxin: 'preparative' dose 200 γ, 'provocative' dose 2000 γ. Thorotrast 3 cm^3/kg of corporal weight.

The following experiments were carried out: *Group 1*, 2 i.v. doses of endotoxin ('preparative' and 'provocative' doses 24 h apart). Five animals: all animals developed the characteristic renal cortical necrosis. *Group 2*, preparation with Thorotrast. Provocative endotoxin injection 24 h later. Five animals: all five rabbits showed renal cortical necrosis. *Group 3*, single endotoxin injection. Five animals: no renal cortical necrosis observed. *Group 4*, single thrombin infusion: none of the five experimental animals developed cortical necrosis of the kidney. *Group 5*, double thrombin infusion, 24 h apart. None of the five animals showed the bilateral cortical necrosis. *Group 6*, preparation with thrombin infusion and provocation with endotoxin. Five animals, all developed renal cortical necrosis. *Group 7*, preparation with endotoxin, provocation with thrombin infusion. Five animals: no renal cortical necrosis in the necropsy, 48 h after the thrombin infusion.

The above experiments show that the bilateral renal cortical necrosis can only be seen in experiments having endotoxin as 'provocative' injection. Thrombin in the dose used has 'preparative' but not 'provocative' properties.

It is concluded from these experiments, that the blood clotting mechanism has to be activated as *conditio sine qua non* in order to produce the generalized Shwartzman reaction. However, the endotoxin itself enhances effects other than the activation of the coagulation system, which are demonstrated to be essential. These are thought to be mainly vasculodynamic in character[6]. *Pari passu* with both endotoxin effects, the reticulo-endothelial system has to be impaired in its phagocytic function[7] in order to fulfil all requirements for the production of the reaction.

Zusammenfassung. Chromatographiertes, Autoprothrombin-C-freies Thrombin wurde Versuchstieren allein bzw. in Verbindung mit Endotoxin oder nach Blockierung des RES mittels Thorotrast, infundiert. Die Ergebnisse zeigen, dass das Thrombin in der angegebenen Dosierung vorbereitende, nicht aber auslösende Eigenschaften besitzt.

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Metabolism of Diphenylamine in the Rat and Rabbit[1]

Diphenylamine is an effective agent for the prevention of superficial scald in apples and other fruits. The toxic effect of aromatic amines and the carcinogenic properties of certain hydroxylated and N-hydroxylated aromatic amines[2,3] are well known, and because of this, we have examined the metabolism of this substance in the rat and rabbit.

The urine of rats given 5 mg intraperitoneal doses of diphenylamine or 4-hydroxydiphenylamine or N-hydroxydiphenylamine[4] was found to contain a conjugate as the major metabolite which gave 4-hydroxydiphenylamine on hydrolysis with hydrochloric acid or enzymatically with a β-glucuronidase preparation[5,6]. The metabolite was identified by comparison with an authentic sample on thin film chromatograms. N-hydroxydiphenylamine, 2-hydroxydiphenylamine or unchanged diphenylamine could not be detected in the hydrolysed urines. The same metabolite was isolated after acid and enzymatic hydrolysis of bile following an intraperitoneal dose of diphenylamine. N-hydroxydiphenylamine rearranges and

1 This work forms part of a programme supported by Grant EF.258 of the National Institute of Health, U.S. Public Health Service, the National Health and Medical Research Council of Australia and the Council of the Australian Food Technologists Association.
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decomposes to give 4-hydroxydiphenylamine and diphenylamine under the conditions used in the acidic and enzymatic hydrolysis. Although diphenylamine was not detected in the urine hydrolysates, it is uncertain whether the original urine contains conjugates of the 4-hydroxy- or the N-hydroxy-compound or both.

A male rabbit was dosed orally with an aqueous suspension of 5 g of diphenylamine in divided doses of 1 g over a period of nine days. 4-Hydroxydiphenylamine and trace amounts of 2-hydroxydiphenylamine and of diphenylamine could be detected in ether extracts of the unhydrolysed urine. Hydrolysis of the ether extracted urine with β-glucuronidase also gave these compounds.

The potassium salt of the sulphate ester of 4-hydroxydiphenylamine was isolated from rabbit urine in the following manner. After treatment of the urine with lead subacetate and removal of the excess lead with hydrogen sulphide, the filtrate was adjusted to pH 7 and concentrated to small volume under reduced pressure. On cooling the concentrated extract deposited a crystalline compound which was recrystallized from alcohol containing a small amount of aqueous potassium hydroxide. This substance was identified as follows. (a) Preliminary tests showed the presence of nitrogen, sulphur, and potassium and hydrolysis with hydrochloric acid gave 4-hydroxydiphenylamine. (b) The infra-red and ultra-violet spectra of this compound (Found: C, 45.77; H, 3.32; N, 4.10. $C_{12}H_{10}NO_4SK \cdot {}^1/_2H_2O$ requires C, 46.13; H, 3.22; N, 4.48) showed a general similarity to 4-hydroxydiphenylamine. (c) Careful examination of the infra-red spectrum in acetonitrile showed a weak band at 3350 cm^{-1}, characteristic of –NH– adsorption and almost identical to a band obtained with authentic diphenylamine (3355 cm^{-1}). The nuclear magnetic resonance spectrum in dimethylformamide showed the exchange of active hydrogen and the presence of nine aromatic protons when the sample was treated with deuterium oxide, which further substantiates the existence of a free –NH– grouping in the molecule and shows that the phenolic substitution occurs on the ring.

The structure of this compound was confirmed by synthesis according to the method of FEIGENBAUM and NEUBERG[7]. The infra-red spectrum of the synthetic material (Found: C, 44.61; H, 3.97; N, 3.93. $C_{12}H_{10}NO_4SK \cdot H_2O$ requires C, 44.84; H, 4.05; N, 4.36) was identical with that of the metabolite. Since the melting point of this sulphate ester was not characteristic, the S-benzyl-iso-thiuronium salt of both the synthetic compound (m.p. 119–121°C) and the metabolite (m.p. 118–120°C) was prepared. No depression in melting point occurred on admixture of the two salts and the infra-red spectra were identical.

The isolation of the potassium salt of the sulphate ester of 4-hydroxydiphenylamine indicates direct hydroxylation of the aromatic ring followed by conjugation. If the sulphate ester of N-hydroxydiphenylamine were formed, followed by rearrangement either *in vivo* or during the isolation procedure, the *ortho* isomer would be expected by analogy with the rearrangement of phenylhydroxylamine-*o*-sulphonic acid under acidic conditions to *o*-aminophenol and *o*-aminophenyl hydrogen sulphate *in vitro*[8]. Phenylhydroxylamine-N-sulphonic acid has been shown to rearrange to *p*-aminophenol and *p*-aminophenyl hydrogen sulphate under acid conditions *in vitro*[8], and this sulphate ester could potentially arise from such a rearrangement. However, direct conjugation of the aromatic amino group with sulphuric acid is not a common reaction *in vivo*. Experiments are in progress to quantitatively determine the metabolism of diphenylamine in the rat and rabbit and to determine whether N-hydroxylation occurs by isolation and characterization of the glucuronide fraction.

Zusammenfassung. Nach oraler Verabreichung von Diphenylamin wurde das Kaliumsalz des 4-Oxydiphenylaminsulfatesters aus dem Harn des Kaninchens isoliert. Die enzymatische Hydrolyse des Kaninchenharns ergab kleine Mengen von 2-Oxydiphenylamin, die mit Hilfe von Dünnschicht-Chromatographie identifiziert wurden. Bei der Ratte gelang nach intraperitonealer Verabreichung von Diphenylamin bei enzymatischer und saurer Hydrolyse des Harns der chromatographische Nachweis des 4-Oxydiphenylamins als Hauptprodukt der Umwandlung.

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Effect of Feeding Benzpyrene on Reproduction in the Rat[1]

SAVKUR et al.[2,3] have studied the effect of methylcholanthrene on the developing mouse embryo. They injected methylcholanthrene directly into the embryonic fluid of 10-day-old embryos and observed abnormalities at birth and resorption of other embryos. In studies in which benzpyrene crystals either were mixed with food or given by capsule to dogs, mice, chickens, ducks and cockroaches[4–7], a blue fluorescence of the skin and viscera occurred. Benzpyrene was demonstrated spectrophotometrically in the tissues. The blue fluorescence rapidly regressed when the benzpyrene was discontinued. In the first experiment with chickens, the eggs developed a bluish fluorescence and were infertile. However, in later studies the eggs were fertile[6]. In view of the apparent effect of benzpyrene on the fertility of eggs, an experiment was devised to study the effect of benzpyrene on preg-

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