Utilization of acetylsalicylic acid as sole carbon source and the induction of its enzymatic hydrolysis by an isolated strain of *Acinetobacter lwoffii*

From glass distilled water that had been allowed to stand at room temperature for several days in a glass bottle open to the atmosphere, a bacterium was isolated which was capable of consuming acetylsalicylic acid (aspirin). The organism grew readily with aeration at 20–30° but not at 37° or above in the following medium (g/litre): aspirin 0·5, $(NH_4)_2SO_4$ 1·54, KH_2PO_4 1·83, Na_2HPO_4 3·91, $MgSO_4.7H_2O$ 0·0617, $FeSO_4.7H_2O$ 0·00016; pH 7·12. The bacterium was a Gram-negative, non-motile rod which grew in air; it was catalase positive, oxidase negative and urease negative; it was unable to produce acids from glucose; it gave a negative Hugh & Leifson (1953) test; it grew on nutrient and MacConkey agars; the ability to hydrolyse gelatin was uncertain; it was unable to utilize citrate, reduce nitrate, oxidize gluconate or produce H_2S; in nutrient broth it grew at 37° but not at 5°; it did not turn blood agar green; with litmus milk it reduced the indicator but produced no pH change. These results suggest that the organism is *Acinetobacter* (*Moraxella*) *lwoffii* (Cowan & Steel, 1965; Thornley, 1967). The bacterium is similar to NCIB 8250 (Vibrio O1; Fewson, 1967) but unlike that organism did not utilize citrate, L- or DL-mandelate or 4-hydroxymandelate as sole source of carbon and energy, but did utilize 2,3-dihydroxybenzoate.

In the following experiments salicylate and aspirin were determined at pH 7·12 using a Unicam SP 800 ultraviolet recording spectrophotometer after removing the bacteria by centrifugation. Complete spectral curves of suitably diluted samples were plotted from 350–200 nm for all samples and the concentrations of salicylate and aspirin were calculated from the extinction at the peaks and shoulders (Gore, Naik & others, 1968). At 296 nm salicylate absorbs maximally and obeys Beer's Law, but aspirin does not absorb. The rate of hydrolysis of aspirin was, therefore, determined by measuring the appearance of salicylate at 296 nm. At 262 nm aspirin gives a shoulder and obeys Beer's Law but salicylate, if present, interferes. The rate of disappearance of aspirin was determined at 262 nm only when the very sensitive method showed that salicylate was absent.

During initial growth of the organism in aspirin medium no salicylate was detected in the culture fluid at 296 nm and the specific rate of aspirin disappearance (at 262 nm) was equal to the first order specific rate of *spontaneous* hydrolysis of aspirin (at 296 nm). Since the ratio of increase in dry weight to the accompanying decrease in concentration of aspirin is constant (Table 1), the growth rate is equal to the rate of disappearance of, and spontaneous hydrolysis of, aspirin and does not increase exponentially with time as is usual. The growth rate is therefore governed by the the release of acetate and salicylate in the spontaneous, non-enzymatic hydrolysis of aspirin. Salicylate, and probably acetate as well, are consumed as soon as they are formed.

During the 5th and subsequent subcultures of the organism in aspirin medium, salicylate accumulated faster than in an uninoculated sterile medium and then disappeared (Table 2). The organism grew at 20–30° without a lag in the above medium containing instead of aspirin, sodium acetate (0·5 g/litre) or salicylic acid (0·5 g/litre). Bacteria repeatedly subcultured in aspirin medium therefore catalyse the hydrolysis of aspirin to acetate and salicylate which are then consumed but not necessarily immediately.

The following experiment with a cell-free extract indicates that the hydrolysis of aspirin is catalysed by a soluble intracellular esterase. After growth in aspirin medium

Table 1. *Disappearance of aspirin from the culture fluid during the initial growth of the aspirin dissimilating bacterium at 22° in a chemically defined medium containing aspirin as sole source of carbon and energy*

Time after inoculation (h)	Dry weight of bacteria (μg/ml)	Aspirin remaining (mM)	Increase in dry weight / Decrease in aspirin (μg ml^{-1} mM^{-1})
0	6·0	2·78	
24	43	2·20	64
48	74	1·72	65
72	109	1·22	70
96	148	0·61	64

Table 2. *Concentration of salicylate in the culture fluid during the 5th and 7th subcultures of the aspirin dissimilating bacterium at 22° in chemically defined medium containing aspirin as sole source of carbon and energy*

Time after inoculation (h)	Dry weight of bacteria (μg/ml)	Salicylate in medium (mM)	Salicylate in uninoculated control (mM)
	5th subculture		
0	3·4	0	0
16·3	20	0·83	0·42
41·5	118	0·81	0·81
65·3	159	0	1·30
	7th subculture		
0	1·8	0	0
15·5	16	0·76	0·39
41·5	127	0·51	0·81
66·8	155	0	1·37

at 22° the bacteria were harvested by centrifugation, resuspended in phosphate buffer (Na_2HPO_4 6·34 g/litre, KH_2PO_4 2·96 g/litre; pH 7·12), centrifuged, resuspended in distilled water, centrifuged and stored at −20° until required. Bacteria equivalent to a dry weight of 4 mg were suspended in 5 ml of phosphate buffer and disintegrated for 30 min at 0° using a MSE ultrasonic disintegrator with a stainless steel probe of diameter 19 mm. The preparation was centrifuged at 9000 *g* for 30 min at 4°. The cell-free supernatant, containing 1·0 mg of protein, was shaken at 30° with 90 μmol of aspirin in 30 ml of phosphate buffer under an atmosphere of N_2. Anaerobic conditions were maintained to prevent further metabolism (oxidation) of the hydrolysis products. From spectrophotometric determinations of salicylate at various times the first order specific rate of hydrolysis of aspirin was found to be 0·78 h^{-1} with the above cell-free extract and 0·022 h^{-1} with a boiled cell-free extract. The specific activity of acetylsalicylate hydrolase in the cell-free extract was 0·97 μmol/min mg^{-1} of protein in the phosphate buffer at 30°.

The above results indicate that the organism undergoes processes of adaptation during repeated subculture in aspirin medium such that the growth rate increases (cf. Tables 1 and 2) and the synthesis of acetylsalicylate hydrolase is induced. The rate-limiting step before adaptation is the non-enzymatic, spontaneous hydrolysis of aspirin but after adaptation is the dissimilation of salicylate, since it accumulates in the medium.

The deterioration of preparations containing salicylic acid, its salts and derivatives, especially aspirin, has long been studied. Sodium salicylate disappears slowly from

its solutions in the presence of air or oxygen with the formation of brown or black colourations or precipitates. The darkening is not very sensitive to light. The decomposition occurs in concentrated or in dilute solutions containing sodium bicarbonate or alkali, is accompanied by the absorption of molecular oxygen and is ascribed to the auto-oxidation of salicylate (Greenish & Beesley, 1915; Hilton & Bailey, 1938; Brecht & Rogers, 1940; Tomski, 1942). The discolouration is catalysed by traces of metal ions (Zwikker & Weber, 1940; Laszlovzky & Barcza, 1963) and is retarded by some substances.

Sodium salicylate preparations may also deteriorate through microbial attack. The growth of fungi in sodium salicylate solutions and its inhibition by chloroform was observed by Hanzlik & Wetzel (1920). That salicylic acid and its anion can act as a source of carbon and energy for the growth of micro-organisms (Evans, 1963) is amply demonstrated by the following recent examples: *Mycobacterium fortuitum* (Tsukamura, 1965); *Pseudomonas* species (Yamamoto, Katigiri & others, 1965; Stanier, Palleroni & Douderoff, 1966); *Trichoderma lignorum* (Vidal, Robert-Gero & others, 1967); *Aspergillus niger* (Krupka, Racle & Marderosian, 1969). Fewson (1967) reported that not only salicylate, but also aspirin, can act as sole source of carbon and energy for *Acinetobacter lwoffii* NCIB 8250 (Vibrio O1).

The spontaneous hydrolysis of aspirin in aqueous solution at physiological pH values to acetate and salicylate anions (reviewed by Stempel, 1961) might be expected to precede the growth of micro-organisms at the expense of aspirin. The experiments here described show that this indeed occurred immediately after the isolation of an aspirin-degrading bacterium from a laboratory environment. After a few subcultures in aspirin medium however, this organism produced an enzyme which hydrolysed aspirin very rapidly thus enabling it to grow and consume this substrate at a still greater rate.

The strains described here and by Fewson (1967) are variations of the widespread species *Acinetobacter lwoffii* which sometimes contaminates water and no doubt aqueous solutions. It seems likely that these organisms or related bacteria may be partially responsible for the deterioration of solutions of aspirin, salicylic acid and salicylates.

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REFERENCES

BRECHT, E. A. & ROGERS, C. H. (1940). *J. Am. pharm. Ass.*, **29**, 178–183.

COWAN, S. R. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria*, Cambridge: Cambridge University Press.

EVANS, W. C. (1963). *J. gen. Microbiol.*, **32**, 177–184.

FEWSON, C. A. (1967). *Ibid.*, **46**, 255–266; **48**, 107–110.

GORE, A. Y., NAIK, K. B., KILDSIG, D. O., PECK, G. E., SMOLEN, V. F. & BANKER, G. S. (1968). *J. pharm. Sci.*, **57**, 1850–1854.

GREENISH, H. G. & BEESLEY, A. E. (1915). *Pharm. J.*, **94**, 201–202.

HANZLIK, P. J. & WETZEL, N. C. (1920). *J. Pharm. exp. Ther.*, **14**, 25–42.

HILTON, J. & BAILEY, K. C. (1938). *J. chem. Soc.*, 631–633.

HUGH, R. & LEIFSON, E. (1953). *J. Bact.*, **66**, 24–26.

KRUPKA, L. R., RACLE, F. A. & DER MARDEROSIAN, A. (1969). *J. pharm. Sci.*, **58**, 270–272.

LASZLOVSZKY, J. & BARCZA, L. (1963). *Gyogyszereszet*, **7**, 192–6. *Chem. Abs.*, **60**, 3953a (1964)
STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). *J. gen. Microbiol.*, **43**, 159–271.
STEMPEL, E. (1961). *Am. J. Pharm.*, **133**, 226–234.
THORNLEY, M. J. (1967). *J. gen. Microbiol.*, **49**, 211–257.
TOMSKI, H. W. (1942). *Pharm. J.*, **184**, 32.
TSUKAMURA, M. (1965). *J. gen. Microbiol.*, **41**, 309–315.
VIDAL, G., ROBERT-GERO, M., HARDISSON, C. & LE BORGNE, J. (1967). *C. R. Acad. Sci. (Paris)*, **264**, 2594–2596.
YAMAMOTO, S., KATAGIRI, M., MAENO, H. & HAYAISHI, O. (1965). *J. biol. Chem.*, **240**, 3408–3417.
ZWIKKER, J. J. L. & WEBER, H. A. (1940). *Pharm. Weekblad.*, **77**, 569–79; *Chem. Zentr.*, 1940, II, 1617; *Chem. Abs.*, **36**, 5616[6] (1942).

Sympathomimetic action of tetanus toxin

Clinical reports by Kerr, Corbett & others (1968) revealed that in tetanus, there may be labile hypertension, tachycardia and irregularity of cardiac rhythm. We have looked for evidence of action of the toxin on the sympathetic nervous system. Tetanus toxin (Haffkein's Institute), 20 000 MLD/ml, given in amounts of 0·01 to 1·0 ml, produced 15–25% reduction of flow in the hind limb of the white rat perfused with oxygenated Ringer-Locke solution at 37°. The reduction was approximately doubled after pretreatment with cocaine (1 mg), but there was no reduction of flow in animals pretreated with reserpine (1 mg), 24 h before, or with phenoxybenzamine (1 mg) 45 min before the toxin.

A triphasic response was seen in the systemic blood pressure of the dog treated with (1·0 ml, of toxin per kg). There was an immediate sharp rise of 10 mm of mercury, then a similar fall below the pre-injection level and later a more sustained pressor effect of 30–40 mm of mercury for 15–20 min. There was no tachyphylaxis. Pretreatment with phenoxybenzamine (10 mg/kg) abolished the delayed pressor effect without affecting the earlier two phases. Similar observations were made on the rat. In about one quarter of the dogs, administration of toxin produced only a depressor response; when these animals were pretreated with mepyramine, the injection of toxin produced a sharp transient pressor effect; this was followed by a more sustained pressor effect which was abolished by pretreatment with phenoxybenzamine.

Injection of low doses of toxin (0·01–0·5 ml) in the perfused frog or rabbit heart produced positive inotropic and chronotropic effects with higher doses of toxin (0·5 ml) there was a subsequent depression. These effects were blocked by suitable doses of β-adrenergic blocking agents like dichloroisopropyl noradrenaline or pronethalol.

The toxin also produced contractions of the smooth muscles of the dog spleen and the guinea-pig vas deferens which were antagonized by phenoxybenzamine.

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REFERENCE

KERR, J. H., CORBETT, J. L., PRYS-ROBERTS, CRAMPTON-SMITH, A. & SPALDING, J. M. K. (1968). *Lancet*, **2**, 236–241.