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Microbial Transformation of the Analgesic Phenacetin and Related Compounds

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ABSTRACT. The capability of some bacterial and fungal strains for studying the metabolism of various analgesics and related compounds was investigated. It was found that phenacetin was metabolized to acetanilide by *Aspergillus niger* and to p-ethoxyaniline by *Aspergillus flavus*. Other compounds namely acetanilide, acetaminophen and acetazolamide, however were not affected under the same conditions.

The chemical activities of fungal spores were first discovered in 1958 by Gehrig and Knight. It was shown that selected microorganisms can mimic mammalian metabolism and are easier to use (Smith and Rosazza 1974, Idem 1975). A number of investigations (Casas-Campillo and Bautista 1965, Hafez-Zedan *et al.* 1970, Plourde *et al.* 1972, Schleg and Knight 1962, Singh *et al.* 1965 and 1967, Vezina *et al.* 1963 and 1968) established that non-germinating spores of fungi and actinomycetes can accomplish a wide range of conversion of steroid molecules. Microbial conversion of phenylbutazone with *Rhizopus arrhizus* to 4-hydroxyphenylbutazone studied by (Winternitz and Favero 1980). A few microorganisms have also the ability to convert tertiary amines to N-oxides, and some primary amines to nitro compounds *via* hydroxylamine intermediate (Smith and Rosazza 1975). The formation *N*-oxygenated metabolites, when *Cunninghamella echinulata* cultures were incubated with (±)−*N*−(n-propyl)-amphetamine was described (Coutts *et al.* 1979). Four oxygenated products were isolated and shown to be identical with mammalian liver metabolites. The soil microorganism *Mycobacterium smegmatis* has the ability to *N*-oxidize amino acids and incorporate the resulting *N*-hydroxyamino acid unit into larger molecules (Ratledge 1976, Snow 1970, Tateson 1970). Amphetamine and five N-alkylated homologeus were

also readily metabolized by *Mycobacterium smegmatis* (Coutts and Foster 1980). An isoquinoline alkaloid was *O*-dealkylated by *C. blakesleena,* while a natural antitumor agent transformed by *C. echinulata* (Reighard *et al.* 1981, Otten and Rosazza 1981). P-aminoazobenzene was degraded by *B. subtilis* to aniline (Idaka *et al.* 1982).

The aim of our work was to use some representative organism to study the conversion of the analgesic phenacetin and related compounds.

Material and Methods

Microorganisms

All test organisms used in this study are listed in Table (1). Bacterial strains were maintained on Brain Heart Infusion Agar (Oxoid) while fungal strains were maintained on Sabouraud Dextrose Agar (Oxoid) slants were stored in a refrigerator at 4°C prior to use. All organisms mentioned in this work were laboratory strains of the culture collection in the College of Pharmacy, King Saud University. *Aspergillus flavus* collected in our laboratory was identified by Commonwealth Mycological Institute, Ferry Lane, Kew Surrey, England. However, *Aspergillus niger* (2022), was provided by Mycological Reference Laboratories, London, England.

Table 1. Microorganisms Used for the Transformation of the Analgesic Phenacetin and Related Compounds.

No.	Test Organisms	Source
1	*Staphylococcus aureus*	Laboratory strain
2	*Escherichia coli*	Laboratory strain
3	*Proteus vulgaris*	Laboratory strain
4	*Pseudomonas aeruginosa*	Laboratory strain
5	*Salmonella* Sp.	Laboratory strain
6	*Candida albicans*	Laboratory strain
7	*Aspergillus flavus*	Isolated strain confirmed by Commonwealth Mycological Institute, England.
8	*Aspergillus niger* (2022)	Mycological Reference Laboratories, London, England.

Fermentation Medium and Inoculum

Cultures were grown in Nutrient Broth (Oxoid) supplemented with 1% glucose. The medium was sterilized by autoclaving at 121°C for 15 minutes before use.

The surface growth from slant of test organism was suspended in 5 ml of sterile supplemented nutrient broth. One ml of this suspension was used to inoculate 50 ml of supplemented nutrient broth held in 250 ml cotton-plugged Erlenmeyer flask (Stage I). The stage I flasks were incubated at 27°C in a shaker bath (Gallenkamp Co., England) operating 140 osci/min for 24-72 hours for fungi. The stage I flasks were also carried out under the same condition, however, incubated at 37°C for bacteria. From the actively growing stage I culture, one ml was transferred to 50 ml of fresh medium held in a 250 ml Erlenmeyer flask (Stage II). After 24 hours of incubation on shaker bath, 50 mg of substrate dissolved in 0.2 ml of dimethylsulfoxide was added to stage II flask. The substrate-containing flasks were incubated for an additional 24-72 hours. The culture was then extracted with an equal volume of chloroform for three times. The chloroform solution was dried over anhydrous sodium sulfate. The chloroform was evaporated to dryness under vacuum. Then samples were analysed by GC/MS and NMR spectrometry.

Controls were used in this work to ensure the metabolites were not artifacts.

Control Studies

Culture control consisted of fermentation blanks in which each organism was grown under identical conditions, as biotransformation cultures, but without substrate and substrate without microorganism. The cultures were extracted and analyzed as described for the biotransformation cultures.

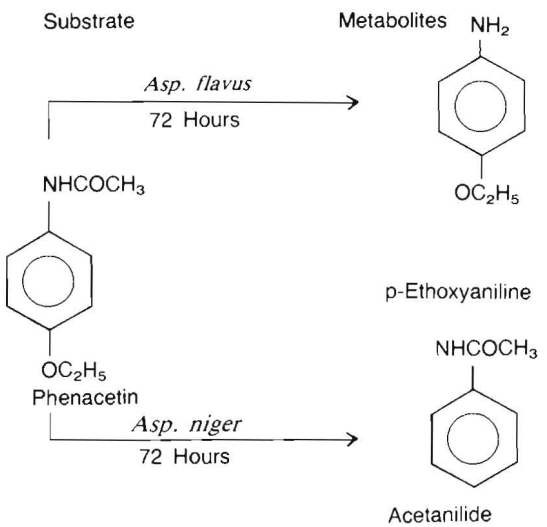
Table 2. Activities of Microorganisms on the Substrates Tested.

Test Organisms	Aceta-minophen	Aceta-nilide	Aceta-zolamide	Phenacetin	Control
S. aureus	—	—	—	—	—
E. coli	—	—	—	—	—
P. vulgaris	—	—	—	—	—
Ps. aeruginosa	—	—	—	—	—
Salmonella sp.	—	—	—	—	—
C. albicans	—	—	—	—	—
Asp. flavus	—	—	—	(P. ethoxy-aniline)	—
Asp. niger	—	—	—	(Acetanilide)	—

Results and Discussion

As it is evident from Table 2, acetaminophen, acetanilide and acetazolamide remained uneffected by the test organisms. However, it has been reported previously that acetanilide, was converted to 2-hydroxyacetanilide by *Aspergillus ochraceous* and to aniline by other different microorganisms (Smith and Rosazza 1974). The pattern of hydroxylation of acetanilide, however, by *Streptemyces* species was found to be in 2− and 4− positions. It is also reported that *Basidiomycete* hydroxylates acetanilide mainly in the 2− position.

Surprisingly enough, phenacetin has undergone biotransformation and was metabolized to acetanilide by *Asp. niger* and to *p*-ethoxyaniline by *Asp. flavus* (Scheme 1).



Scheme 1. Biotransformation of Phenacetin by *Asp. flavus and Asp. niger.*

The presence of phenacetin metabolites by *A. niger* and *A. flavus* were confirmed by GC/MS and NMR analysis.

1. *Metabolite of A. niger*

The mass spectrum showed an M^+ at m/e 135 and a base peak (100%) at m/e 93. The mass spectrum was identical with that of authentic acetanilide. The proton-NMR spectrum displayed the following signals: a singlet at 2.1 ppm ($-NHCOCH_3$) and multilet at 7.21 ppm. (Five aromatic protons). These are in agreement with that found for acetanilide.

2. *Metabolite of A. flavus*

The mass spectrum showed an M^+ at 137 and a base peak (100%) at m/e 108. The mass spectrum was identical with that of authentic *p*-ethoxyaniline. The proton-NMR spectrum displays the following signals:

a triplet centred at 1.3 ppm ($-CH_2Me$) a singlet at 3.4 ppm ($-NH_2$), quartet centred at 3.81 ppm ($-CH_2Me$) and an AB quartet centred at 6.56 ppm (four aromatic protons). These are in agreement with that found for *p*-ethoxyaniline. Deacetylation of phenacetin to *p*-ethoxyaniline by *A. flavus* was an expected metabolic pathway as it has been reported earlier that acetanilide undergoes deacetylation to aniline by *Penicellium chrysogenum* (Smith and Rosazza 1974). However, the biotransformation of phenacetin to acetanilide by *A. niger* was unusual reaction since no previous reports have indicated de-etherification as a metabolic pathway by microorganisms. This finding should be substantiated by more studies using other similar substrates.

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التحولات الجرثومية للمسكن فيناسيتين والمركبات ذات العلاقة

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تستخدم الكائنات الحية الدقيقة في دراسة طرق الأيض ونواتجها لعدد من المركبات ذات الفعالية الحيوية والتي أظهرت أن الكائنات الحية قد تشبه الثدييات في طرق أيضها. ويمكن بذلك استخدامها في الحصول على كميات كبيرة من نواتج الأيض عن طريق عمليات التخمر وأيضاً تساعد في التعرف على طرق الأيض الجديدة والتي قد تحدث في الثدييات، ومن المعروف أن الأنواع المختلفة من البكتيريا والفطريات تقوم بالعديد من عمليات الأيض مثل إدخال الهيدروكسيل وإزالة مجموعات الألكليل المتصلة بالنتروجين أو الأكسجين وكذلك إزالة مجموعة الأستيل أو إدخالها. . الخ.

ودراسة أيض المركبات ذات الفعالية الحيوية بواسطة البكتيريا والفطريات قد تساعد على اكتشاف أدوية جديدة ذات فعالية أكثر. وعلى سبيل المثال فقد تمت دراسة أيض المسكن اسيتانيليد بواسطة اسبرجيللس اكراشيس وبنسيليوم كريزوجينم وكانت نواتج الأيض هي مركب ٢ هيدروكس اسيتانيليد ومركب الأنلين.

وفي هذا البحث تم اختيار خمسة أنواع من البكتيريا استافيلوكوكس أوريس وايشير شيا كولاي وبروتيس

فولجاريس وبسيدوموناس ايروجنوزا وكانديدا البيكانز وإثنان من فطريات اسبرجيللس نيجر و اسبرجيللس فلافس لاختبار إمكانية استخدامها لتحاكي نموذج الأيض في الثدييات لدراسة التحويلات الحيوية للأنيليدات المسكنة وهي الفيناسيتين والأسيتامينوفين والأسيتانيليد.

وقد اتضح من الدراسة أن مركبات الفيناسيتين قد تحول إلى مركب الاسيتانيليد بواسطة اسبرجيللس نيجر وإلى مركب بارا أيثوكسي اثيلين بواسطة اسبرجيللس فلافس.

وسيتم عرض ومناقشة الزرعات التي استخدمت ونواتج الأيض التي فصلت وأطياف الكتلة والرنين النووي المغناطيسي لها وكذلك طبيعة عمليات الأيض في كل حالة.