The filtrate was evaporated under reduced pressure. After removal of the solvent, 5% hydrochloric acid was added to the residue and the solution allowed to stand overnight. The solid material was collected and recrystallized from ethanol–water to yield 102 mg. of product (m.p. 152–153°). Mixed melting point with an authentic specimen of 10-(3-*N*-*p*-toluenesulfonylaminopropyl)-2-chlorophenothiazine (152–153.5°) did not show depression (152–153.5°). This compound was the sulfonamide of the primary amine, The acidic aqueous layer mentioned above was adjusted to pH 10 with 10% NaOH and extracted with ether. After the ether layer was separated and concentrated, a light oil was obtained. Upon recrystallization from acetone–chloroform, a solid was obtained which melted at 59–60°; this indicated the unreacted chlorpromazine base.

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Salicylate Degradation by *Aspergillus niger*: Influence of Glucose

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Abstract □ The addition of glucose at 2.5 g./l. to a salicylate medium resulted in an increase in the rate of salicylate degradation in shake culture. Total capacity of salicylate degraded was also markedly increased (fivefold). Addition of glucose also increased the dry weight of the fungus, an increase which is essential for isolating maximum amounts of the enzyme or enzymes responsible for salicylate degradation.

Keyphrases □ Salicylate degradation—*Aspergillus niger* □ Glucose effect—*A. niger* salicylate degradation □ Fungus, mycelial weights—glucose, shake culture effects □ Colorimetric analysis—spectrophotometer

In 1966, aspirin products led in the number (24.9%) of accidental poisonings reported to the National Clearinghouse for Poison Control Centers (1). In a previous communication, the authors described the concept of producing microbial enzymes for therapeutic use in aspirin poisoning (2). Although immunological obstacles might still have to be surmounted before any such enzymes could be used *in vivo*, the value of such enzymes was stressed as presenting a potentially useful improvement over current methods used in counteracting salicylate toxicity. Methods in current use include gastric lavage, measures designed to increase the renal excretion of salicylate, exchange transfusion, peritoneal dialysis, and hemodialysis (3).

It has been reported that microorganisms are capable of growing on media containing salicylate as the sole carbon source (4–7). This has been demonstrated with the fungus, *Aspergillus niger*, in this laboratory (2). Yamamoto *et al.* (8) have isolated and purified an enzyme, salicylate hydroxylase, from a soil pseudomonad growing on a salicylate medium. The following report

Table I—Influence of Glucose on Salicylate Degradation by *Aspergillus niger* and on Growth of the Fungus

Treatments[a]		Growth[b]	Salicylate[c]
Salicylate	(1 mg./ml.)	+h	Absent
Salicylate	(2 mg./ml.)	+m	Present
Salicylate	(3 mg./ml.)	+s	Present
Salicylate	(4 mg./ml.)	+s	Present
Salicylate	(5 mg./ml.)	—	Present
Salicylate	(1 mg./ml.) + glucose	+h	Absent
Salicylate	(2 mg./ml.) + glucose	+h	Absent
Salicylate	(3 mg./ml.) + glucose	+h	Absent
Salicylate	(4 mg./ml.) + glucose	+h	Absent
Salicylate	(5 mg./ml.) + glucose	+m	Absent
Salicylate	(6 mg./ml.) + glucose	—	Present

[a] All treatments include the basic inorganic medium. [b] The symbols +s (slight), +m (moderate), and +h (heavy) refer to visual observation of the growth of the fungus in shake culture from three replicates. [c] Salicylate disappearance was measured after five days incubation of the fungus from three replicates per treatment.

presents data on maximum degradation of salicylate by the ascomycete, *Aspergillus niger*. One of the advantages of utilizing this particular fungus is that it has been extensively studied and approved for drug or food use by the U. S. Food and Drug Administration.

EXPERIMENTAL

Fungus—*Aspergillus niger* strain No. 10 was obtained from the Ohio State University stock culture collection and maintained on *Difco potato dextrose agar slants. Inoculations were carried out* at 20° using 0.2 ml. of a water suspension of spores from 5–7-day-old slants to which 10 ml. of sterile distilled water had been added.

Media—All studies were conducted with synthetic liquid media using reagent grade chemicals and distilled water. The basic medium contained the following composition per liter: 1.0 g. of NH_4NO_3; 1.0 g. of KH_2PO_4; 0.5 g. of $MgSO_4 \cdot 7\ H_2O$; 0.13 g. of $CaCl_2$; 0.1 g. of NaCl; and 0.01 g. of $Fe_2(SO_4)_3 \cdot nH_2O$. Sodium salicylate ($NaC_7H_5O_3$) was added at a concentration of 1 mg./ml. to the above medium. In several experiments, glucose was added at levels of 2.5 g./l., 1.5 g./l., and 0.75 g./l. Final pH values before inoculation ranged from 4.5–5.0 for all treatments.

The media were dispensed in 20-ml. aliquots in replicated 125-ml. conical flasks and placed on an Eberbach reciprocating shaker (100 oscillations/min.). Unless otherwise specified, each flask contained 20 mg. of sodium salicylate.

Measurement—Salicylate degradation was measured according to the method of Trinder (9) as modified by DeMarco and Marcus (10) utilizing a spectrophotometer.[1] At specified intervals during the incubation period, 1-ml. samples were obtained from the salicylate medium and diluted 1:10 with distilled water. Five milliliters of a 0.05% solution of $FeCl_3$ made up in 0.1 *N* HCl was then added to 5 ml. of the diluted sample. Readings were taken at 532 mμ 5 min. after formation of the ferric salicylate complex and compared against standard curve data obtained from known quantities of salicylate. The system detected salicylate in quantities as low as 25 mcg.

Glucose utilization was measured by the anthrone method of Morris (11), a very sensitive test reviewed and recommended by Hodge and Hofreiter (12). Two milliliters of a 1:100 dilution of the medium was obtained and 8 ml. of the anthrone reagent added. This was boiled for 3 min, cooled, and read at 620 mμ with the spectrophotometer and compared against a standard recovery curve. Beer's law was followed from 0–80 mcg. of glucose.

Dry-weight measurements of the fungus were obtained after filtration of mycelial pads on previously weighed Whatman No. 1 filter paper which had been stored above anhydrous $CaSO_4$.[2] The filter paper and fungus were then dried at 50° for 24 hours and again stored above anhydrous $CaSO_4$ until final weighing on a single pan analytical balance (Cenco).

Measurements of pH were made with a pH-measuring instrument.[3]

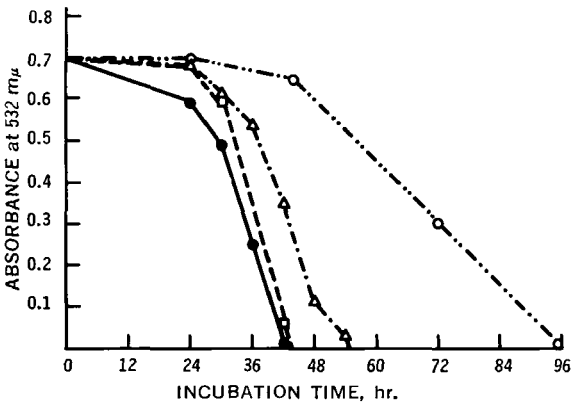

Figure 1—*Effect of glucose on salicylate degradation by* Aspergillus niger *in shake culture. All media contain sodium salicylate at 1 mg./ml. Key:* ●, *Medium A (2.5 g./l. glucose):* □, *Medium B (1.5 g./l. glucose);* △, *Medium C (0.75 g./l. glucose);* ○, *Medium D (does not contain glucose).*

RESULTS AND DISCUSSION

The authors have been primarily concerned with investigating those factors which would lead to a production of a maximum amount of fungus and enzyme in the shortest period of time. In still culture, approximately 144 hr. were necessary for *Aspergillus niger* to completely degrade 20 mg. of sodium salicylate as the sole carbon source (2). This was shortened to 96 hr. by utilizing shake culture. The addition of glucose (2.5 g./l.) to the medium (Fig. 1) increased the rate of degradation of the salicylate such that only 42 hr. were required for complete degradation of 20 mg. of sodium salicylate, an additional saving of 54 hr. In addition, glucose increased the capacity for amount of salicylate degraded fivefold within 96 hr. (Table I). Heavy to moderate growth of the fungus was observed in flasks containing 100 mg. sodium salicylate with complete degradation obtained within 5 days. The growth of the fungus also increased markedly (see dry weight data of Table II). It would seem that this increased growth could in turn result in greater production of the enzymes responsible for salicylate degradation.

The results of the anthrone test (Table III) revealed that within 24 hr. after inoculation, glucose began a gradual disappearance. Increased utilization of glucose was obtained when glucose was the sole carbon source (medium A) rather than when glucose was added to salicylate as in Medium B. In the medium containing salicylate and glucose, *Aspergillus niger* did not preferentially utilize the glucose as might be expected. Instead, 6 hr. after the salicylate had been degraded, some glucose was still present in the medium.

The addition of glucose at 2.5 g./l. to the salicylate medium resulted in a decrease in pH from 5.0 to 3.0. This decrease did not occur with glucose in lesser concentrations (Table II). The utilization of glucose (2.5 g./l.) in the basic medium without salicylate also caused the pH to drop to 3. This decrease in both types of media is attributed to the production of organic acids such as citric acid and is typical for *A. niger* (13). Perlman (14) has reported that the greater the quantity of glucose utilized by *A. niger*, the higher the level of citric acid produced. According to his data, 2.64 g./l. of glucose forms 1.6 g./l. of citric acid, an efficiency of 61 %. Using Perlman's efficiency factor, one could predict that the lower levels of glucose such as in Media B and C would result in the production of much less citric acid than in Medium A. This differential production of citric acid might reasonably account for the changes in pH observed (Table II).

[1] Spectronic 20, Bausch and Lomb, Rochester, N. Y.
[2] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.
[3] Zeromatic II, Beckman Instruments, Inc., Fullerton, Calif.

Table II—Effect of Glucose on pH and Dry Weight of *Aspergillus niger* in Salicylate Medium[a]

Incubation Time, hr.	Glucose Level—pH			Glucose Level—Dry Wt., mg.		
	2.5 g./l.	1.5 g./l.	0.75 g./l.	2.5 g./l.	1.5 g./l.	0.75 g./l.
0	5.0[b]	4.9	4.9	0[b]	0	0
24	5.2	5.2	5.3	10.6	17.6	15.6
36	3.5	5.0	5.8	31.0	24.6	18.6
48	3.0	4.9	6.3	43.5	32.5	24.0
60	3.5	6.1	6.5	81.0	51.0	50.0

[a] All treatments include basic inorganic medium plus sodium salicylate at 1 mg./ml. [b] Each figure is the average of two individually determined replicates.

Table III—Weight in Milligrams of Glucose and Salicylate per Flask After Growth of *Aspergillus niger* in Shake Culture in Three Media[a]

Incubation Time, hr.	Medium A[a] Glucose[b]	Medium B[a] Glucose	Medium B[a] Salicylate[b]	Medium C[a] Salicylate
0	50	50	20	20
24	50	50	14	20
30	44	47.5	10	20
36	29	38	4	19
42	18	30	0	18
48	0	12.5	—	16
54	—	0	—	12
96	—	—	—	0

[a] All media contain basic inorganic salts. Medium A contains 2.5 g./l. glucose in addition; Medium B contains 2.5 g./l. glucose + 1 mg./ml. sodium salicylate; Medium C contains 1 mg./ml. sodium salicylate. [b] Glucose and salicylate levels determined from two replicates per treatment and comparing against standard recovery curve.

SUMMARY

Degradation of sodium salicylate by the fungus *Aspergillus niger* was accelerated by the addition of glucose (2.5 g./l.) to a basic inorganic medium containing sodium salicylate. This resulted in an increase in dry weight of the fungus, an increase which is essential for the production of useful amounts of the enzyme or enzymes which are responsible for salicylate degradation. Acetone powders of the fungus prepared by this technique have recently been found to show enzymatic activity *in vitro* (15). Studies are currently in progress on the purification and isolation of this enzyme system for its subsequent testing in mammalian tissues.

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