have been very encouraging. Pine blocks treated with ammoniacal solutions of acrylonitrile have shown complete resistance to three commonly used test fungi in the soil-block test.

Other treatments that do not rely on the toxicity of chemicals are possible and merit investigation. There is a real need in many instances for rot-proof and termite-proof wood that is free from impregnants that are likely to contaminate other materials. Further developments in this type of treatment, therefore, can be anticipated.

SUMMARY

Preservative treatments of wood up to the present have been based almost entirely on the application of materials that are toxic to wood-destroying organisms. While the older preservatives still account for a high percentage of the total volume, there has been a decided increase in the use of several of the newer preservatives. The search for effective preservatives continues, especially for chemicals that may be used to produce a clean, odorless, paintable wood product.

A two-stage diffusion process called double diffusion was developed at the Forest Products Laboratory, and is giving promising results in field tests. The process consists of soaking green wood in two separate solutions of chemicals that diffuse into the wood and then react within the wood to deposit a toxic precipitate. An interesting application is in the treatment of cooling towers that have begun to decay.

Fermentation Studies with *Streptomyces niveus*

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Novobiocin[1] (streptonivicin) is a new antibiotic discovered in the laboratories of The Upjohn Company. The announcement of the antibiotic (Smith *et al.*, 1956), chemical properties (Hoeksema *et al.*, 1956), structure (Hinman *et al.*, 1956; Hoeksema *et al.*, 1955), therapeutic activities (Wilkins *et al.*, 1956), tissue assay method (Taylor *et al.*, 1956a), pharmacologic properties (Taylor *et al.*, 1956b and Larson *et al.*, 1956), and clinical studies (Lin *et al.*, 1956 and Martin *et al.*, 1955) have been reported previously. This publication describes fermentation studies with *Streptomyces niveus*.

MATERIALS AND METHODS

Streptomyces niveus was maintained on maltose-tryptone agar slants prepared from soil or freeze-dried stocks as described previously (Smith *et al.*, 1956). All fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium. The flasks were sterilized at 120 C for 20 minutes and incubated on a Gump shaker rotating at 250 rpm with a 2-inch throw. Vegetative seed was prepared by inoculating a medium containing glucose monohydrate, 25 g per L, and cottonseed meal, 40 g per L, with a loopful of spores. One hundred ml of the seed medium was incubated in a 500-ml Erlenmeyer flask as described above. The fermentation samples were prepared for assay by diluting the fermentation broth in 0.1 M phosphate buffer of pH 7.85 after removing the solids by centrifugation. The disc plate assay procedure for novobiocin using *Klebsiella pneumoniae* as test organism was described in a previous publication (Smith *et al.*, 1956). The standard error of the assay was about 20 per cent.

Cell dry weight was determined by washing the cells three times with distilled water and drying the residue at 110 C for 24 hours. Nitrogen was determined by a modified Kjeldahl procedure. Carbohydrate was determined with the anthrone reagent (Morris, 1948). Two ml of the sugar solution plus 4 ml of anthrone reagent (2 g per L in 95 per cent H_2SO_4) were heated on a steam bath for 10 minutes to develop the color, which was determined at a wave length of 620 mμ in a Bausch and Lomb "Spectronic 20" photocolorimeter. Protein nitrogen in the cells was determined on the trichloracetic acid precipitate obtained by suspending the centrifuged cells, previously washed twice with water, in 12 per cent trichloracetic acid. The precipitated cell protein was washed three times with trichloracetic acid solution prior to analysis to remove TCA soluble peptides and amino acids. Total cellular nitrogen was determined on the intact cells after washing three times with water.

RESULTS AND DISCUSSION

The effect of composition of the medium on antibiotic production. Early media studies were reported in a

[1] The trademark of The Upjohn Company for novobiocin is Albamycin.

previous publication (Smith *et al.*, 1956). Extended media studies were carried out with various carbohydrate and nitrogen sources. The pH of the various media was adjusted to a value which would give a pH of approximately 6.5 after sterilizing. Media were run generally in two or more flasks and the samples pooled prior to assaying. The standard deviation among runs was approximately 160 μg per ml determined on 17 runs which averaged 475 μg per ml. The results of the media studies are shown in tables 1, 2, and 3. The effect of single nitrogen sources on novobiocin production is shown in table 1.

The data in table 1 show distillers solubles to be the best single nitrogen source tested for novobiocin production. The effect of combining various nitrogen sources with distillers solubles in an effort to stimulate production was investigated and the results are presented in table 2.

TABLE 1. *The effect of single nitrogen sources on the production of novobiocin*

Nitrogen Source	Concentration	Peak Yield of Novobiocin
	g/L.	*μg/ml*
Distillers solubles (Brown-Forman Co.)	40	475
Distillers solubles	20	380
Lykanut (Traders Oil Mill Co.)	30	125
Soy bean meal	40	45
Meat and bone scaps (Darling Co.)	35	75
Soya protein (The Glidden Co.)	20	30
Wheat germ meal (General Mills)	40	105
Guar seed meal (Stein-Hall Co.)	40	90
Guar seed meal	20	150

In addition to the nitrogen, the media contained glucose, 25 g per L as the carbon source. The nitrogen sources were added at levels to furnish approximately 2 g per L total nitrogen to the medium. Fermentation temperature was 28 C.

TABLE 2. *The effect of combined nitrogen sources on antibiotic production*

Nitrogen Source Added to Basal*	Concentration	Peak Yield of Novobiocin
	g/L	*μg/ml*
None	—	380
Distillers solubles	20	475
Yeast (Pabst)	12	270
Animal stick liquor (Armour and Co.)	15	315
KNO_3	5	225
Nutrient 22 (A. E. Staley)	20 (wet)	375
Ammonium sulfate	7.5	<100
N-Z amine B	12	355
Beef extract (Difco)	10	395
Nutrient L-1 (Sheffield)	10	425
Thio peptone (Wilson and Co.)	10	410
Soya protein	10	145
Pharmamedia (Traders Oil Mill Co.)	12	365

* Basal medium was glucose·H_2O, 25 g per L; distillers solubles, 20 g per L; presterilization pH = 7.8; fermentation temperature 28 C.

TABLE 3. *The effect of carbohydrate sources on antibiotic production*

Carbohydrate Added to Basal* at 25 g/L	Peak Yield of Novobiocin
	μ/ml
Glucose·H_2O (Cerelose)	475
Glucose·H_2O, 40 g/L	405
Sucrose	555
Sucrose, 40 g/L	560
Molasses, blackstrap (dry weight)	245
Molasses, high test (dry weight)	150
Maltose	325
Lactose	140
Starch	405
Glycerol	405

* Basal medium was distillers solubles, 40 g per L; presterilization pH = 7.8; fermentation temperature 28 C.

The data in tables 1 and 2 show that no single or combined nitrogen sources investigated could give a significantly better yield of novobiocin than distillers solubles, and most of the nitrogen sources, when combined with distillers solubles, decreased the yield of antibiotic obtained on the latter. The reason for the superiority of distillers solubles is unknown, and simple fractionation procedures have afforded no fractions with stimulatory activity.

The effect of various carbohydrate sources on novobiocin production was investigated with distillers solubles as the nitrogen source. The results are shown in table 3.

The data in table 3 show sucrose was the best carbohydrate source investigated when distillers solubles were the nitrogen source. Antibiotic titer was not significantly affected when the concentration of glucose or sucrose was increased from 25 to 40 g per L. In a previous publication (Smith *et al.*, 1956), molasses was reported to be the best carbohydrate sources for novobiocin production, and sucrose was found to give only traces of activity when the nitrogen source was egg peptone plus beef extract. Sucrose and molasses were compared in the same run with egg peptone-beef extract and distillers solubles as nitrogen sources and the results, as described previously, were confirmed. Sucrose was superior to glucose for novobiocin production in shaken flasks when the nitrogen source was distillers solubles, but it gave only traces of activity when the nitrogen source was egg peptone plus beef extract.

The yields of novobiocin varied from 400 to 500 μg per ml and no increase in production over that obtained with sucrose alone was realized with a carbohydrate source of glucose 12.5 g per L combined with one of the following: starch, glycerol, molasses, sucrose, dextrin, or lactose at 12.5 g per L.

The effect of slant and seed media on antibiotic production. The effect of slant media was investigated by

TABLE 4. *The effect of slant media and storage on production*

Composition of Slant Medium (g/L)	Peak Yield	
	One week	Four months
	μg/ml*	
Glucose·H_2O, 20; N-Z Amine B, 5; soy peptone, 20; agar 20	350	300
Maltose, 10; tryptone, 5; K_2HPO_4, 2; Mg, 20 μg/ml; Mn, Fe and Zn, 1 μg/ml; agar, 15	465	425
Glucose·H_2O, 10; soy peptone, 8; yeast extract, 4; agar, 15	500	295
Glucose·H_2O, 10; ammonium sulfate, 2.5; salts as above; agar, 15	365	310

* Fermentation medium was glucose·H_2O, 28; distillers solubles, 40 g per L; fermentation temperature 28 C.

TABLE 5. *The effect of seed media on antibiotic production*

Composition of Seed Medium (g/L)	Peak Yield of Novobiocin
	μ/ml*
Glucose monohydrate, 25; cottonseed meal, 40	500
Glucose monohydrate, 25; distillers solubles, 40	560
Glucose monohydrate, 25; egg peptone, 26	400
Glucose monohydrate, 25; Pharmamedia, 25	450

* Fermentation medium was glucose 25 g per L; distillers solubles, 40 g per L; fermentation temperature 28 C.

planting vegetative seed of *S. niveus* onto four different agar slant media, and checking the production of the culture after one week and again after four months storage of these slants at 5 C. The results of the investigation are shown in table 4.

Although the effect of slant media on the yield of antibiotic was not striking, maltose-tryptone slants were used for future work since the culture appeared most stable when stored on this medium.

The effect of seed media on antibiotic production was investigated by growing the vegetative seed of *S. niveus* in four different seed media. The results are shown in table 5.

The data in table 5 show that a seed medium containing distillers solubles may have been superior to the other seed media tested, although the differences were not significant.

The effect of temperature and aeration rate on antibiotic production. Fermentations with *S. niveus* were carried out in the glucose-distillers solubles medium described earlier at 24, 28, and 32 C. Antibiotic titer was not significantly different when fermentations were carried out at 24 or 28 C, but the yields were depressed at 32 C.

In several fermentations, the flasks were incubated at 28 C for three days when they were transferred to 24 or 32 C. No significant change in titer was observed as a result of these temperature changes. Temperature of seed incubation has been reported to influence subsequent growth and fermentation by *B. subtilis* (Wellerson and Tetrault, 1955). The effect of temperature of incubation of *S. niveus* seed on subsequent fermentation was investigated by cultivating the seed at 28 and 32 C and incubating the fermentation flasks inoculated with these seeds at 28 C. A decrease in titer was observed when the seed was grown at 32 C. In general, *S. niveus* did not grow well at 32 C, although the flasks that were transferred from 28 C remained viscous at 32 C.

The effective aeration rates of the shake flasks employed were varied from approximately 0.3 to 2.6 by indenting the sides of the flasks as described by Smith and Johnson (1954). No change in titer was observed when *S. niveus* was incubated at various effective aeration rates in baffled flasks. With some actinomycetes, it has been observed that in a baffled shake flask the dry cell yields are not increased, and may be decreased because the cells are pounded continually against the baffle. With bacteria, the cell yields obtained in shake flasks have been shown to vary with the increased aeration rate when the flasks were indented (Smith *et al.*, 1956). With filamentous organisms, the cells often are broken by striking against the baffles in the flask. Although the baffle increases the effective aeration rate to the medium within the flask, it may cause extensive disruption of filamentous cells and actually do more harm than good. In an effort to circumvent injury to the cells from striking the baffle, the aeration efficiency was increased by decreasing the volume of medium in the flask. No change in titer was observed under these conditions of aeration.

The effect of inhibitors and precursors on antibiotic production. The following metabolic inhibitors were added to the glucose-distillers solubles medium in an effort to stimulate antibiotic production: arsenite, fluoride, dinitrophenol, cycloheximide, iodoacetate, sulfadiazine, bromopyruvic acid, and malonic acid. The concentrations used varied from 10^{-3} M to 0.1 g/L depending upon the substance. No increase in antibiotic titer was observed in the presence of these inhibitors, although the yields were depressed in some instances.

The following substances were added to the basal medium as possible precursors of novobiocin: *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, benzoic acid, ethyl acetoacetate, acetone powder of *S. niveus* mycelium, extracts of distillers solubles, α-aminobutyric acid, phenylacetaldehyde, quinic acid, shikimic acid, and 4-acetoxy-3-(3-methyl-2-butenyl)-benzoic acid. The concentrations used varied from 0.1 to 1.0 g per L. The last-mentioned compound is a crystalline degradation product of novobiocin. This compound inhibited growth of *S. niveus* when added at zero time, and was added after the mycelium had reached full growth. No increase in antibiotic titer was observed with any of the compounds added as precursors. In the presence of shikimic acid, a dark green pigment was produced

which was not observed with quinic acid, tyrosine, or phenylalanine.

The effect of slow feeding on production. The following substances were added to fermentations once a day after the third day of growth: glucose, sucrose, fructose, ammonium salts, urea, vitamin mixture, distillers solubles, gluconic acid, shikimic acid, sedoheptulosan, cottonseed oil, citrate, and methionine. No stimulation of production was observed, although ammonia consistently depressed the yields.

Biochemical changes in the culture medium during production of novobiocin. A correlation between the protein metabolism of *Streptomyces aureofaciens* and chlortetracycline production has been suggested by the work of Biffi and co-workers (1954). These investigators observed the total cell weight to be approximately equal on media of high or low phosphate content, although the antibiotic production varied by a factor of two and the mycelial protein by a factor of five. The soluble nitrogen in the medium in both high and low phosphate conditions was approximately the same. The results suggest a possible correlation between protein breakdown to trichloracetic acid soluble, nondiffusible peptides, and chlortetracycline synthesis in *S. aureofaciens*.

The protein metabolism of *S. niveus* was investigated in an effort to determine whether the protein breakdown reported for *S. aureofaciens* during antibiotic production was occurring in *S. niveus* also.

Two sampling procedures were employed in this study. Replicate shake flasks were run on each medium investigated, and the flasks sampled either by removing an aliquot from each flask and pooling the aliquots prior to analyzing, or by removing and pooling three flasks at each sampling. In the former procedure, each flask remained on the shaker for the entire experiment. The latter sampling procedure was much more convenient, and gave similar results. The medium chosen for the study of biochemical changes in the culture had the following composition: glucose·H_2O, 25 g per L; egg peptone, 20 g per L; beef extract, 10 g per L. This medium was almost clear and the difficulties encountered with suspended solids were obviated. The biochemical changes in the egg peptone-beef extract medium are shown in figures 1 and 2.

Figure 1 shows the nitrogen and cell weight changes during growth and figure 2 shows the carbohydrate, pH, antibiotic, and cell weight relationships. Inspection of figure 1 shows that the total and protein nitrogen contents of the mycelium were approximately equal during the entire fermentation. The sum of mycelial nitrogen and soluble nitrogen as calculated at various times was approximately equal to the total nitrogen present at the time of inoculation. Calculations of mycelial dry weights show that the cells must be formed equally from amino acids and sugar (figures 1 and 2) since the cell yields based on sugar utilized approach

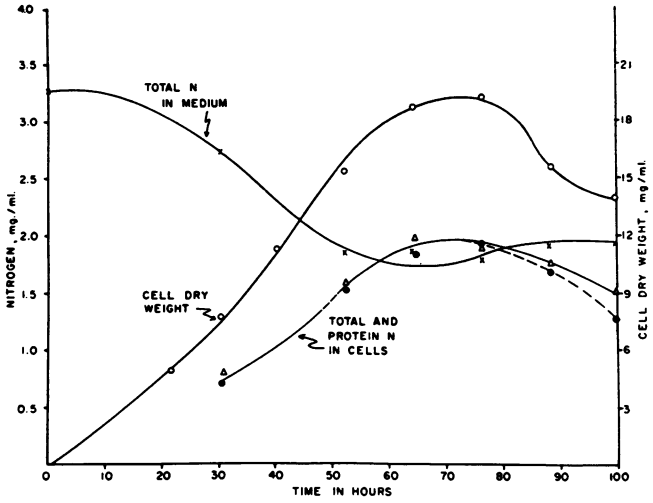

FIG. 1. Nitrogen and cell weight changes during growth of *Streptomyces niveus* on egg peptone-beef extract medium.

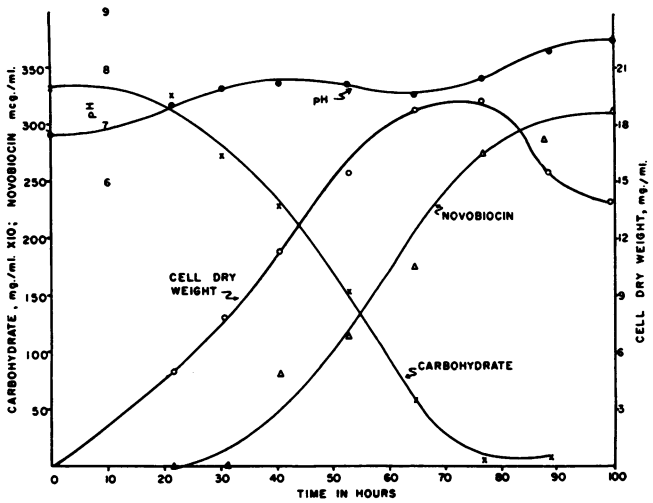

FIG. 2. Cell weight, carbohydrate changes, pH, and antibiotic courses during growth of *Streptomyces niveus* on egg peptone-beef extract medium.

100 per cent, whereas yields based on sugar plus amino acid approximate 50 per cent.

Phosphate has been reported to markedly influence fermentations (Perlman *et al.*, 1952 and 1954, Biffi *et al.*, 1954). The addition of 2 g per L potassium phosphate to the egg peptone-beef extract medium described above after autoclaving had no effect on the fermentation of *S. niveus* under the experimental conditions imposed, and the curves obtained with and without added phosphate were superimposable.

The biochemical changes occurring in the culture when *S. niveus* was grown on a medium containing glucose·H_2O, 25 g per L, egg peptone, 15 g per L, soy peptone, 15 g per L, were qualitatively similar to those shown in the above figures, and, although antibiotic titer was lower than that obtained on the egg peptone-beef extract medium, there were no significant differences in the nitrogen metabolism.

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SUMMARY

Novobiocin has been produced in yields of 550 micrograms per milliliter on a medium containing 25 to 40 g per L of sucrose and 40 g per L of distillers solubles. No increase in antibiotic production by *Streptomyces niveus* was observed when the composition of the slant and seed media, the fermentation temperature, and the effective aeration rate were varied. The addition to the fermentation medium of certain metabolic inhibitors and possible precursors or stimulators did not increase the antibiotic yield. The biochemical changes in the culture medium during growth of *S. niveus* have been determined.

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