Effect of Aeration on the Novobiocin Fermentation

CHARLES G. SMITH

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

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A direct relationship has been shown between the maximum cell concentration and aeration efficiency with aerobic microorganisms (Maxon and Johnson, 1953; Smith and Johnson, 1954). Since the antibiotic titer generally varies directly with the weight of mycelium in streptomycete fermentations, it is imperative to define the dependence of antibiotic and mycelial syntheses on the aeration efficiency in the particular fermentation under study. Relationships among novobiocin synthesis, mycelial production, and aeration level in shake flask fermentations have been investigated and the results are presented in this publication.

In a previous report (Smith, 1956), it was shown that aeration was probably not a limiting factor in the novobiocin fermentation under certain shake flask conditions. It has since been observed that the addition of various nitrogenous materials to a distillers' solubles medium depresses novobiocin synthesis. This depression appears to be due in part to an induced oxygen deficiency, as described in this publication. A simplified ultraviolet (UV) assay procedure for novobiocin is also presented.

MATERIALS AND METHODS

Two experimental procedures were used to vary the effective aeration level: (a) serially diluted media and (b) indented shake flasks (Smith and Johnson, 1954; Dion *et al.*, 1954). In the first procedure, the culture was grown in full, half, or quarter strength medium at a given aeration efficiency in unbaffled shake flasks, resulting in available oxygen to mycelium ratios of approximately 1, 2, and 4. By this procedure, traumatic effects of baffled shake flasks were obviated.

All fermentations were conducted in 500-ml Erlenmeyer flasks containing 100 ml of medium, incubated at 28 C on a Gump[1] rotary shaker (230 rpm). Vegetative seed was grown in a medium containing Cerelose (glucose monohydrate) and Egg Peptone,[2] both at 25 g per L. The seeding rate was 3 to 5 ml per 100-ml shake flask. *Streptomyces niveus* strains 15R and BC-333 were used for this work. Strain 15R was used in all experiments unless noted otherwise in the text.

Sugar was determined by a modification of the anthrone procedure (Morris, 1948). To measure mycelial dry weight, a 5-ml aliquot of whole beer was filtered on paper, washed with water, and dried overnight at 100 C. No correction was made for residual distillers solubles. Optical density was determined in a Lumetron[3] colorimeter at 650 mμ after dilution of the whole beer 1:50 in water. The samples were read against a water blank in 18-mm test tubes. Oxygen uptake was determined by a modification of the sulfite oxidation method of Cooper *et al.* (1944).

Novobiocin concentration was determined by a simplified UV assay. Although a more precise assay was available (Smith *et al.*, 1958), the simplified version was developed to accommodate large numbers of samples. In this procedure, 0.2 ml of whole or clarified beer was pipetted into 2.0 ml of phosphate buffer (KH_2PO_4, 39 g per L plus K_2HPO_4, 37 g per L) in a 14 by 100 mm test tube, and the buffer was used to rinse thick suspensions from the pipette. Five ml of butyl acetate, pretreated as described previously (Smith *et al.*, 1958), was added and the tube was closed with a cork and shaken by hand for 10 to 30 sec to extract the novobiocin. The butyl acetate layer was separated by centrifugation and its optical density determined in the Beckman[4] model DU spectrophotometer at wave lengths of 338 and 290 mμ. The concentration of novobiocin was calculated from the equation novobiocin (μg/ml) = 25 [(22) (OD_{338}) $-$(2) (OD_{290})]. The conversion factors were adapted from Smith *et al.* (1958).

The standard error per determination with the simplified assay was approximately 2 per cent (range: 450 to 490 μg per ml in 15 determinations of thick whole beer).

When beers to which known amounts of novobiocin had been added were assayed as unknowns, the added novobiocin was recovered in 96 per cent yield. The absolute potency of the beers agreed within 3 per cent with the values obtained by the more elaborate procedure of Smith *et al.* (1958). These data show that the simple UV assay for novobiocin is reproducible and accurate. It was used in all studies reported in this publication.

RESULTS

Novobiocin synthesis in diluted medium. In a previous publication (Smith, 1956), a medium containing Cerelose, 28 g per L, and distillers' solubles, 40 g per L, was shown to be optimal for the novobiocin fermentation.

[1] B. F. Gump Company, Chicago, Illinois.
[2] Viobin Corporation, Monticello, Illinois.
[3] Will Corporation, Buffalo, New York.
[4] Beckman Instruments, Inc., Fullerton, California.

The effect of varying the oxygen to mycelium ratio in this medium was investigated by growing *S. niveus* in media of full strength, 1:2 and 1:4 dilutions at a constant aeration efficiency (sulfite oxidation number = 0.3 mM O_2 per L-min). Since the concentration of mycelium depended upon the medium concentration (figure 1), the ratio of oxygen available per g of mycelium was approximately 2- and 4-fold greater in the diluted media than in the control (undiluted). The rates of mycelium and novobiocin production under these conditions are shown in figure 1. The production of novobiocin was directly proportional to the synthesis of mycelium.

The efficiency of novobiocin production under these conditions is presented in table 1. These data show that essentially no increase in the efficiency of novobiocin production occurred over a 4-fold increase in the oxygen to mycelium ratio.

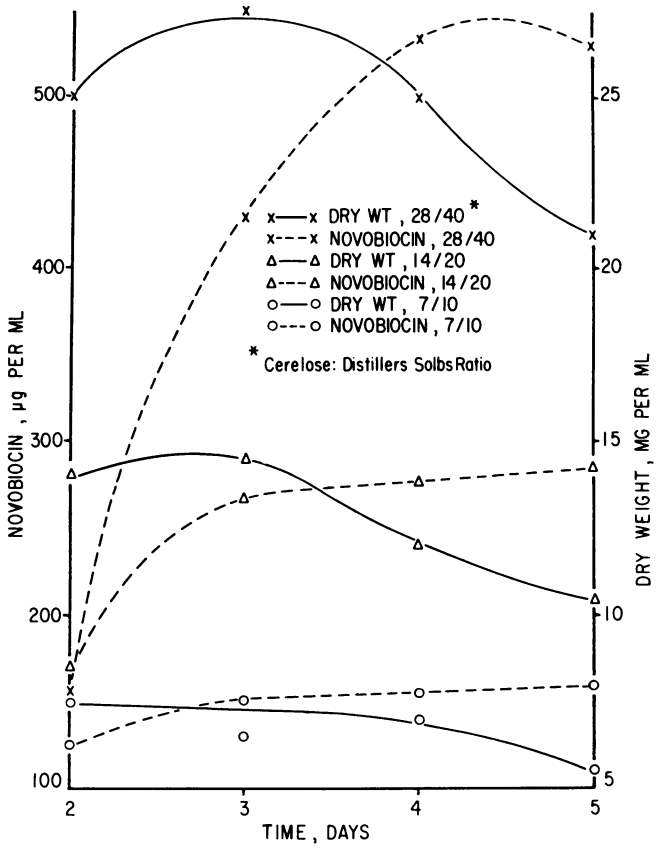

Figure 1. Growth and novobiocin production in diluted media.

TABLE 1

Efficiency of novobiocin production in diluted media

Ratio of Cerelose* to Distillers Solubles	Peak Novobiocin Yield	Peak Dry Wt	Efficiency, µg Novobiocin per mg Cells
g/L	*µg/ml*	*mg/ml*	
28:40	535	27.5	19.4
14:20	285	14.5	19.7
7:10	160	7.5	21.4

* Corn Products Refining Co., Argo, Illinois.

Novobiocin synthesis in indented shake flasks. Available oxygen in a shake flask fermentation vessel can be increased by indenting the sides of the flask (Smith and Johnson, 1954; Dion *et al.*, 1954). With a medium containing Cerelose, 28 g per L, and distillers solubles, 40 g per L, the novobiocin fermentation course in an indented shake flask (figure 3) (sulfite oxidation number = 1.3 mM O_2/L-min) was almost identical with that in unindented flasks (figure 2). These data confirm the

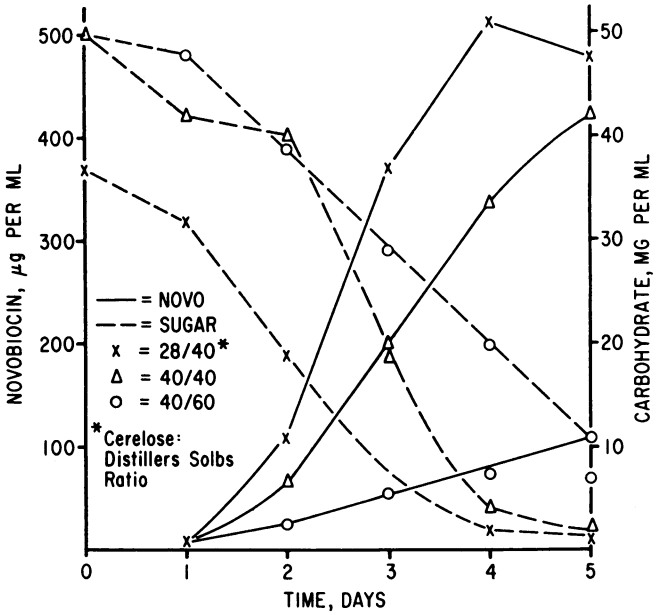

Figure 2. Carbohydrate utilization and novobiocin production at low aeration level (unindented shake flakes).

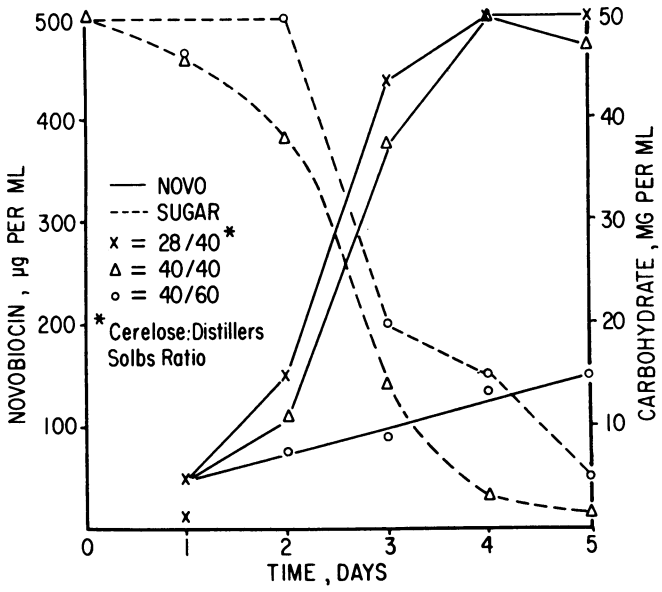

Figure 3. Carbohydrate utilization and novobiocin production at high aeration level (indented shake flakes).

studies reported above, that oxygen was not a limiting factor for novobiocin production in the 28:40 (Cerelose-distillers' solubles) medium.

The effect of aeration efficiency on novobiocin production when the Cerelose to distillers solubles ratio was varied was also investigated. The media used in this study were (per L): (1) Cerelose, 28 g, and distillers' solubles, 40 g (28:40); (2) Cerelose, 40 g, and distillers' solubles, 40 g (40:40); and (3) Cerelose, 40 g, and distillers solubles, 60 g (40:60). Biochemical changes during growth of *S. niveus* in these media were determined in unindented and indented shake flasks (sulfite oxidation numbers of 0.3 and 1.3 mM O_2 per L-min, respectively). The data for novobiocin production and sugar utilization in unindented shake flasks are shown in figure 2 and for indented shake flasks in figure 3. The dry weight changes for both types of flasks are shown in table 2. The pH course was similar in all fermentations, rising from approximately 7 at 24 hr to 8 at 96 hr. Comparison of figures 2 and 3 shows that the production of novobiocin was independent of the aeration efficiency in a medium containing 28 g per L of Cerelose and 40 g per L of distillers' solubles (as noted above). When the distillers' solubles concentration was raised to 60 g per L, novobiocin synthesis (and possibly glucose utilization) was inhibited and increasing the aeration efficiency had little effect (figures 2 and 3). In the medium containing 40 g per L Cerelose and 40 g per L distillers' solubles, novobiocin production was inhibited at the lower aeration level (figure 2), but returned to normal in the indented flask (figure 3). Increasing aeration did not markedly affect the mycelial dry weight in these media (table 2). It should be noted that the dry weight determination included residual distillers' solubles.

Inspection of figures 2 and 3 shows that substrates furnished by the distillers solubles were used in preference to glucose by *S. niveus* since the rate of sugar utilization was retarded for 24 to 48 hr in all three media, although growth was luxurious within 24 hr. A very marked difference in rate of production and maximum titer of novobiocin in medium 3 is obvious.

TABLE 2

Dry weight changes of Streptomyces niveus in various media

Medium	Sulfite Oxidation, Number mM O_2/L-min	Dry Wt, mg/ml per Day:				
		1	2	3	4	5
28:40*	0.3	23	23	27	23	21
28:40	1.3	—	24.5	27	23	21
40:40	0.3	21.5	22	24	24	22
40:40	1.3	21	23.5	25.5	23	23
40:60	0.3	31.5	30	29	23	22
40:60	1.3	31.5	33	28	28	25

* Cerelose to distillers solubles ratio, g/L.

Inspection of table 2 shows that the weight of mycelium was greater in medium 3 than in media 1 or 2 (uncorrected for residual distillers solubles). Perhaps increased cell density and a higher level of readily oxidizable metabolites caused a relative oxygen deficiency which resulted in decreased novobiocin synthesis.

Effect of nitrogen source and aeration on novobiocin and mycelium synthesis. The effects of various nitrogen sources on growth and novobiocin production were investigated, and the results are shown in table 3 (mycelial growth was estimated by turbidimetric measurement). The addition of nitrogenous materials to 40 g per L of distillers' solubles (basal 1) inhibited novobiocin production as reported previously (Smith, 1956). On the contrary, the same nitrogen sources (with the exception of soy protein) had no adverse effect or stimulated novobiocin production when added to media containing 20 g per L of distillers' solubles (basal 2).

The turbidity changes in these media are particularly

TABLE 3

Effect of nitrogen sources on mycelial growth and novobiocin production

N-Source Added at 10 g/L	Peak Novobiocin Titer, μg/ml		Peak Optical Density*	
	Basal 1†	Basal 2‡	Basal 1	Basal 2
None	600	310	0.30	0.15
Pharmamedia§	105	360	0.42	0.23
Soy protein‖	110	190	0.37	0.23
N-Z-Amine B¶	150	350	0.44	0.24
Nutrient L-1¶	170	365	0.43	0.25
Casamino acids**	140	440	0.39	0.24

* At 650 mμ, 1:50 dilution.
† Basal 1 = Cerelose, 28 g/L; distillers solubles, 40 g/L.
‡ Basal 2 = Cerelose, 28 g/L; distillers solubles, 20 g/L.
§ Traders Oil Mill Company, Fort Worth, Texas.
‖ Glidden Company, Chicago, Illinois.
¶ Sheffield Chemical Company, Norwich, New York.
** Difco Laboratories, Detroit, Michigan.

TABLE 4

Effects of aeration and nitrogen levels on novobiocin production with Streptomyces niveus strain BC-333

Medium	Aeration Efficiency mM O_2/L-min	Novobiocin μg/ml
Basal 1*	0.3 (unindented)	590
+ Soy peptone (10 g/L)	0.3	350
Basal 2†	0.3	420
+ Soy peptone	0.3	585
Basal 1	1.3 (indented)	400
+ Soy peptone	1.3	425
Basal 2	1.3	380
+ Soy peptone	1.3	415

* Cerelose, 28 g/L + distillers solubles, 40 g/L.
† Cerelose, 28 g/L + distillers solubles, 20 g/L.

interesting. Optical density increased when nitrogen sources were added to basal 1 or 2, which indicated increased synthesis of mycelium (table 3). It should be noted that the optical density of basal 2 plus supplements approached that of basal 1 minus supplements, but was always lower. These data suggest that the inhibition of novobiocin production by the nitrogen sources added to basal 1 (table 3) may have been caused by a stimulation of mycelial synthesis and metabolism to the point of induced oxygen deficiency. The addition of the same nitrogen sources to basal 2 may not have raised the mycelial mass above the hypothetical threshold level, and thus did not depress novobiocin production (with the exception of soy protein).

If the inhibition of novobiocin synthesis (table 3) is indeed due to an induced oxygen deficiency, it should be reversed by providing more air during the fermentation. Experiments were set up in which two strains of *S. niveus* were grown in media of varying nitrogen contents and aeration levels, and dry weights and novobiocin titers were determined. The results varied with the individual cultures. The inhibition of novobiocin production by strain BC-333 in nitrogen-rich media was reversed in indented shake flasks as illustrated in table 4. With strain 15R (used in all experiments reported above), reversal was not always observed.

Reversal experiments did not substantiate completely the hypothesis of an induced oxygen deficiency. However, it must be remembered that an indented shake flask may result in injury to filamentous organisms when they strike the baffles as has been described previously (Dion *et al.*, 1954; Vondrackova, 1957). With certain strains of *S. niveus*, the use of an indented shake flask actually decreased novobiocin production in the media investigated (table 4).

Discussion

The data presented in this paper indicate a close relationship between the level of nitrogenous material in the fermentation medium and the aeration level required for efficient novobiocin production by *S. niveus*. Novobiocin synthesis was inhibited 80 per cent when the nitrogenous components were increased in a medium already sufficient in nitrogen (distillers solubles, 40 g per L). The inhibition was not due to a direct toxic effect of additives on the organism, since most of the nitrogen sources were noninhibitory (or stimulatory) to novobiocin production when added to a basal medium deficient in nitrogen (distillers' solubles, 20 g per L). It was also noted by turbidimetric measurement that increasing the nitrogen source generally gave rise to increased synthesis of mycelium. Increasing the level of distillers' solubles above 40 g per L resulted in a markedly decreased synthesis of novobiocin and perhaps a decreased rate of sugar utilization.

These data can be interpreted as the result of an induced oxygen deficiency caused by stimulating (a) mycelial synthesis and (b) oxygen uptake by furnishing preferred substrates. Experiments designed to demonstrate reversal of the oxygen deficiency by increased aeration were only partly successful when available oxygen was increased by indenting the shake flask, which inhibited novobiocin synthesis with some strains of *S. niveus*. The inhibition of penicillin synthesis under conditions of high agitation has also been described (Dion *et al.*, 1954; Vondrackova, 1957). In media containing distillers' solubles, 40 g per L, and Cerelose, 28 g per L, the efficiency of novobiocin production was shown not to be influenced by the aeration level in unindented shake flasks, since there was no change in efficiency of synthesis upon serial dilution of the medium. The use of unindented shake flasks with nitrogen-rich media and an oxygen-enriched gas phase might provide more definitive answers to the question of an induced oxygen deficiency.

Acknowledgments

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Summary

The efficiency of novobiocin production by *Streptomyces niveus* was shown to be independent of aeration level in media of full strength (Cerelose, 28 g per L; distillers' solubles, 40 g per L), half strength and quarter strength, under certain shake flask conditions.

Biochemical changes during the fermentation of novobiocin under various conditions of medium and aeration efficiency were described.

Increasing the nitrogen content of the medium beyond a threshold level depressed novobiocin production markedly. However, addition of the same nitrogen sources to media of low initial nitrogen content generally resulted in stimulatory or noninhibitory responses. This effect was due perhaps to an induced oxygen deficiency in the nitrogen-rich media.

A simplified ultraviolet assay for novobiocin was described.

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Production of Tetracycline by *Streptomyces aureofaciens* in Synthetic Media

MARJORIE A. DARKEN, HERMAN BERENSON, RICHARD J. SHIRK, AND NEWELL O. SJOLANDER

Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

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The production of tetracycline[1] by fermentation was disclosed by Minieri *et al.* (1953). Details of this fermentation process in synthetic media of low chloride content are presented, and the culture isolation program which was carried out in conjunction with this study is discussed.

In this fermentation the composition of the medium and the strain of streptomycete are both important factors, since *Streptomyces aureofaciens* is capable of producing at least two antibiotic substances as pointed out by Backus *et al.* (1954). In the presence of chloride, which is incorporated within the chlortetracycline[2] molecule (Broschard *et al.*, 1949) and which is therefore essential for its production as disclosed by Petty and Matrishin (1950), the antibiotic formed was predominately chlortetracycline. In media low in chloride tetracycline predominates and the chlortetracycline fraction diminishes since 1 ppm of available chloride ion can produce at most 14 μg/ml chlortetracycline.

The simultaneous production of two or more antibiotics in a fermentation is well known, and the substances formed may be either closely related on a chemical or a biological basis or widely separated. Typical examples of closely related compounds produced simultaneously by the same organism are the penicillins (Clarke, 1949), streptomycins (Waksman, 1949), polymyxins (Brownlee, 1949), bacitracins (Newton and Abraham, 1950), cephalosporins (Crawford *et al.*, 1952), nisins (Berridge *et al.*, 1952), neomycins (Waksman, 1953), rhodomycins (Brockmann *et al.*, 1951), and candicidins (Lechevalier *et al.*, 1953). Compounds which differ in their structure and in their biological activity and are produced simultaneously by the same organism are illustrated by spinulosin, fumigatin, and gliotoxin (Menzel *et al.*, 1944); actidione, grisein, and streptomycin (Waksman *et al.*, 1948; Whiffen, 1948); rimocidin and oxytetracycline (Davisson *et al.*, 1951); fradicin and neomycin (Waksman, 1953); chlortetracycline and an antifungal compound (Duggar *et al.*, 1954); and fungicidin and an actidione-like antibiotic (Hazen and Brown, 1951).

The effect of medium and strain upon the concurrent production of these antibacterial and antifungal agents is well established. Calam and Levi (1944) found different types of penicillin produced in synthetic and natural media, and Smith and Bide (1944) established the phenylacetyl grouping as a necessary component of the medium for the production of penicillin G in contrast to penicillin F, which was formed in its absence. That different isolates derived from the same parent culture were capable of producing different types of penicillin was demonstrated by Calam and Levi (1944). Changes in medium were found by Whiffen (1948) to alter the ratio between streptomycin and actidione, and strain selection led to the sole production of either component. Perlman (1949) showed that substrains could be chosen which produced more of the desired streptomycin and less of mannosidostreptomycin than the parent. Mayer *et al.* (1951) reported on two antibacterial substances produced by *Actinomyces vinaceous* which were markedly influenced by the medium. Waksman (1953) pointed out that *Streptomyces fradiae* produced fradicin, neomycin A, and a subtilis factor along with neomycin, and that medium and strain influenced the proportion of the individual components of the neomycin complex. Backus *et al.* (1954) disclosed that different strains of *S. aureofaciens* possess the capacity to produce the antibiotics chlortetracycline and tetra-

[1] The trade-mark of American Cyanamid Company for the antibiotic tetracycline is Achromycin.

[2] The trade-mark of American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.