GROWTH RESPONSES OF A SULFONAMIDE-REQUIRING MUTANT STRAIN OF NEUROSPORA[1]

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A mutant strain of *Neurospora crassa* has appeared in which the antagonistic roles of *p*-aminobenzoic acid and the sulfonamides have been reversed to a considerable extent. Optimal growth of this strain occurs only in the presence of sulfonamides. Conversely, *p*-aminobenzoic acid is a potent fungistatic agent for this strain under certain conditions.

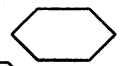
To say that sulfanilamide has become an essential metabolite and *p*-aminobenzoic acid an inhibiting analog would be to oversimplify the altered physiology of this mutant strain. It will be shown that, in this strain, both sulfonamides and *p*-aminobenzoic acid are essential for growth, and that each acts as an inhibiting analog of the other. These interrelations are further complicated by the effect of temperature on the need for sulfonamides, and on the inhibition by *p*-aminobenzoic acid.

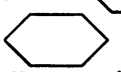
The present report deals exclusively with the growth responses of this mutant strain to sulfonamides, to temperature, and to *p*-aminobenzoic acid. At the present time nothing definite is known of the physiological role of sulfonamides in this strain.

MATERIALS AND METHODS

Methods. The procedures followed are essentially those described in a previous report (Emerson and Cushing, 1946). Growth responses are recorded as growth rates, which were determined by the tube method of Ryan, Beadle, and Tatum (1943).

Symbols. For the sake of clarity and brevity, the following symbols will be used:

PABA—*p*-aminobenzoic acid, H_2N  COOH

SA —sulfanilamide, H_2N  $SOONH_2$

pab —"*p*-aminobenzoicless," a gene interrupting the synthesis of PABA, strain 1633 of Tatum and Beadle (1942).

$+^{pab}$ —the wild-type allele of pab.

sfo —"sulfonamide-requiring," a gene carried by strain E-15172 described in this paper.

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[2] With the technical assistance of Mary R. Emerson and Lydia Hawk.

$+^{sfo}$ —the wild-type allele of sfo.
S-T —"sulfanilamide tolerant," a gene for resistance to SAN, strain C-40 (Emerson and Cushing, 1946).
$+^{S\text{-}T}$ —the wild-type allele of S-T.

Origin of sulfonamide-requiring strain. In a previous communication (Emerson and Cushing, 1946) mention was made of a mutant strain (E-13190) which apparently required sulfonamides for growth. Mutant E-13190 appeared as a segregant in one ascus of a cross between the sulfanilamide-tolerant strain and a wild-type strain [C-40(E-8577)A × E-5297a].

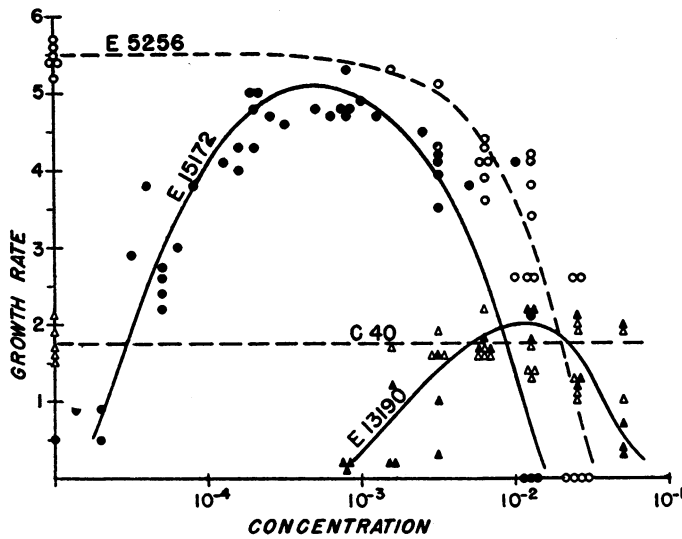

FIG. 1. GROWTH RATES (IN MILLIMETERS PER HOUR) OF FOUR GENETICALLY DIFFERENT STRAINS ON VARYING CONCENTRATIONS OF SULFANILAMIDE AT 35 C

E-5256, wildtype ($+^{sfo}$ $+^{S\text{-}T}$); E-15172, sulfonamide-requiring strain (sfo $+^{S\text{-}T}$); C-40, sulfanilamide-tolerant strain ($+^{sfo}$ S-T); E-13190, double mutant, sulfanilamide-tolerant and sulfonamide-requiring (sfo S-T).

Mutant strain E-13190 proved to be a "double mutant" carrying the gene for sulfanilamide tolerance (S-T) characteristic of strain C-40 as well as the new mutant gene (sfo) for sulfonamide requirement. In an outcross of strain E-13190A to wild type (Abb-12a) these two genes segregated independently. The gene responsible for sulfonamide requirement was isolated from this cross as E-15172A. The four different genetic constitutions resulting from this cross are identified by their responses to varying concentrations of SA (figure 1). The double mutant (sfo S-T) has the maximal growth rate of about 2 mm per hour characteristic of the S-T strain and requires about 50th molar SA for optimal growth at 35 C. By itself sfo has a maximal growth rate of over 5 mm per hour, similar to that of wild-type, and grows optimally on a much higher dilution of SA.

Genetic tests show that sfo lies very close to the centromere of a different chromosome from that carrying S-T. Both these genes are independent of pab, which is located some distance from the centromere of an undetermined chromosome.

RESULTS

Substances stimulating growth in strain E-15172. The sulfonamide-requiring strain is able to utilize each of the sulfonamides that have been tested (figure 2) though the concentration necessary for optimal growth is different for different drugs. Growth was also supported by *p*-sulfamido-phenylalanine,[3] but never to maximal extent, perhaps because of inhibition resulting from competition between this analog and phenylalanine (cf. Mitchell and Niemann, 1947).

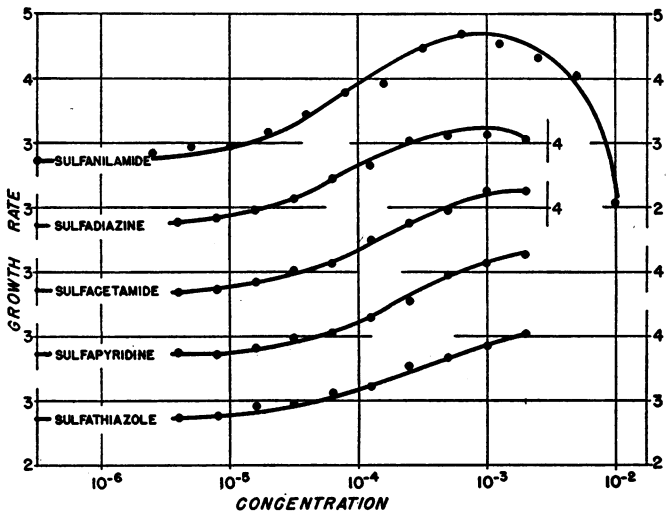

FIG. 2. GROWTH RATES (IN MILLIMETERS PER HOUR) OF SULFONAMIDE-REQUIRING STRAIN SFO, ON VARYING MOLAR CONCENTRATIONS OF DIFFERENT SULFONAMIDES AT 30 C
See discussion on temperature effect.

Methionine, the sulfone and sulfoxide of methionine, and taurine were unable to support growth of this strain, though methionine and its sulfoxide are utilized by certain other strains which require an organic source of sulfur (Horowitz, unpublished).

Effect of temperature on sulfonamide requirement. Although the sulfonamide-requiring strain will not grow at 35 C unless sulfonamides are present, considerable growth occurs at lower temperatures in the absence of sulfonamides. Data from experiments in which SA concentration and temperature were varied simultaneously are summarized in a contour graph in figure 3. In this diagram SA concentration increases from about millionth molar at the left to hundredth molar at the right. Temperatures increase from 25 C at the bottom of the diagram to over 36 C at the top. The contour lines pass through intersections of temperatures and concentrations at which equal growth rates occur.

[3] The *p*-sulfamido-phenylalanine was kindly supplied by Professor Carl G. Niemann.

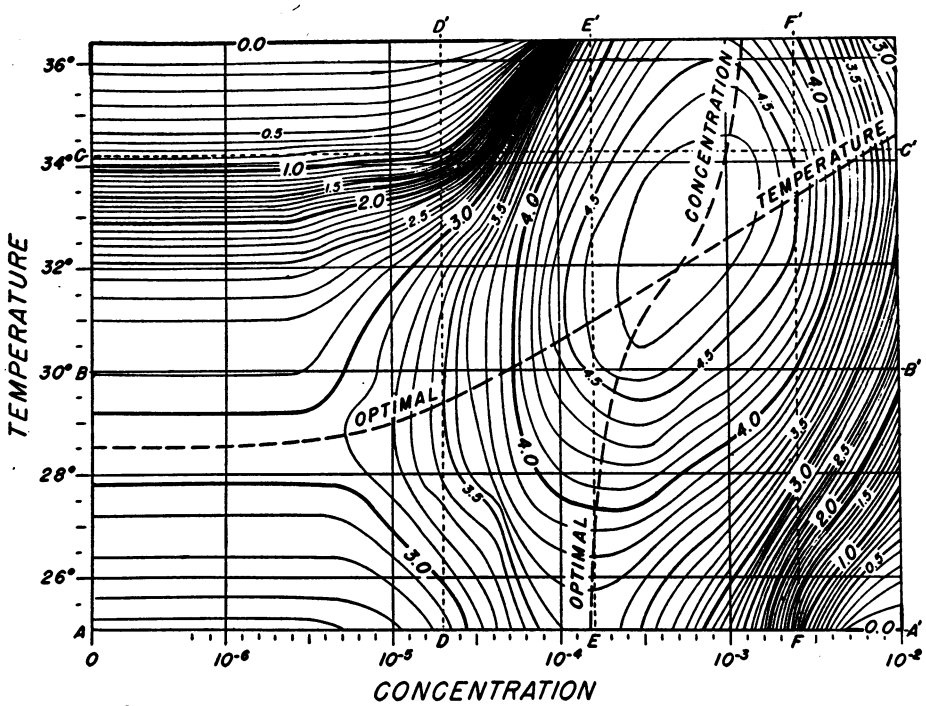

FIG. 3. GROWTH RATES OF SULFONAMIDE-REQUIRING STRAIN, SFO, WITH VARYING TEMPERATURE AND SA CONCENTRATION

Contour lines pass through points having equal growth rates (expressed as millimeters per hour). Concentrations are expressed as moles per liter. Rates were determined at 25, 27.8, 30, 32, 34.2, and 36.4 C, and at twofold dilutions from M/100 to M/1,638,400, for a total of 96 different combinations of temperature and concentration, one quarter of which were run in duplicate.

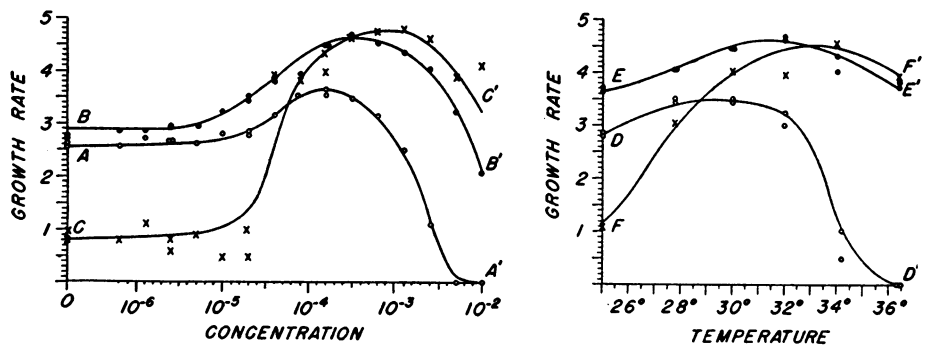

FIG. 4. GROWTH RATES OF THE SULFONAMIDE-REQUIRING MUTANT, SFO, ON VARYING CONCENTRATIONS OF SA (AT LEFT), AND AT VARYING TEMPERATURE (AT RIGHT)

Curve A-A', 25 C; B-B', 30 C; C-C', 34.2 C. Curve D-D', M/51,200; E-E', M/6,400; F-F', M/400. The curves represent sections through the graph in figure 3 along the lines A-A', B-B', E-E', etc. The points represent observed values.

Sections through this graph parallel to the base give curves showing the variations in growth rates with changing SA concentration at constant temperatures. Three such sections are reproduced in figure 4. Sections parallel to the sides of

the graph (figure 3) result in curves showing variations in growth rates with changing temperature at particular concentrations of SA. Three such sections are reproduced in figure 4.

Growth rates are fairly constant from experiment to experiment throughout most of the range covered by the graph in figure 3. However, when growth is retarded by high concentration or by high temperature, the rates are much less constant and reproducible (note points on curve C-C′ in figure 4).

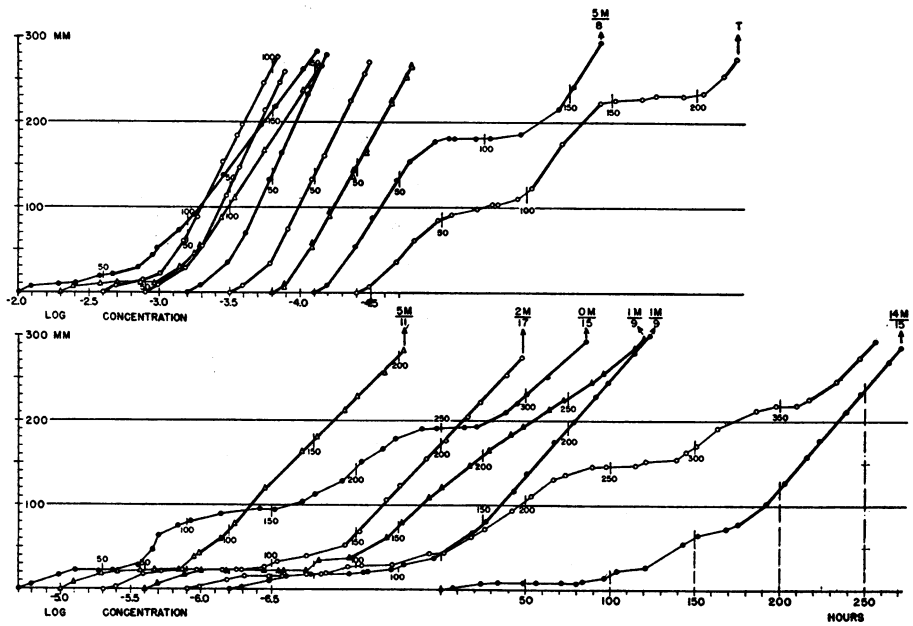

FIG. 5. GROWTH CURVES OF THE SULFONAMIDE-REQUIRING STRAIN, sfo, ON DIFFERENT CONCENTRATIONS OF SA AT 36.4 C

The position at which a curve arises along the base line indicates the molar concentration of SA for that curve. Over the remainder of the curve, horiozontal distance represents elapsed time in hours, vertical distance represents total growth in millimeters. Heavy lines indicate that growth was luxuriant, with well-defined frontiers, lighter lines that growth was "feathery". The arrows indicate transfers to fresh growth tubes lacking SA. T, transient, or nonpersistent reversion as shown by such transfers; the fractions show the number of mutant nuclei among the total nuclei tested in outcrosses following such transfers.

Reversions. When the growth of the sulfonamide-requiring strain (sfo) is depressed by simultaneous high temperature and low SA concentration (cf. figures 3 and 4) the additional complication of reversion is encountered. By "reversion" is meant a fairly abrupt change in growth rate and habit from those characteristic of the mutant strain to those closely resembling wild-type.

Growth curves illustrating the character of the growth before and after reversion are reproduced in figure 5. At low SA concentrations (M/10,000 or less) and high temperatures (34 C or over), growth is characteristically light and "feathery," with no well-defined frontier. Such growth is represented by the lighter lines in figure 5. After reversion occurs, the growth of the fungus is

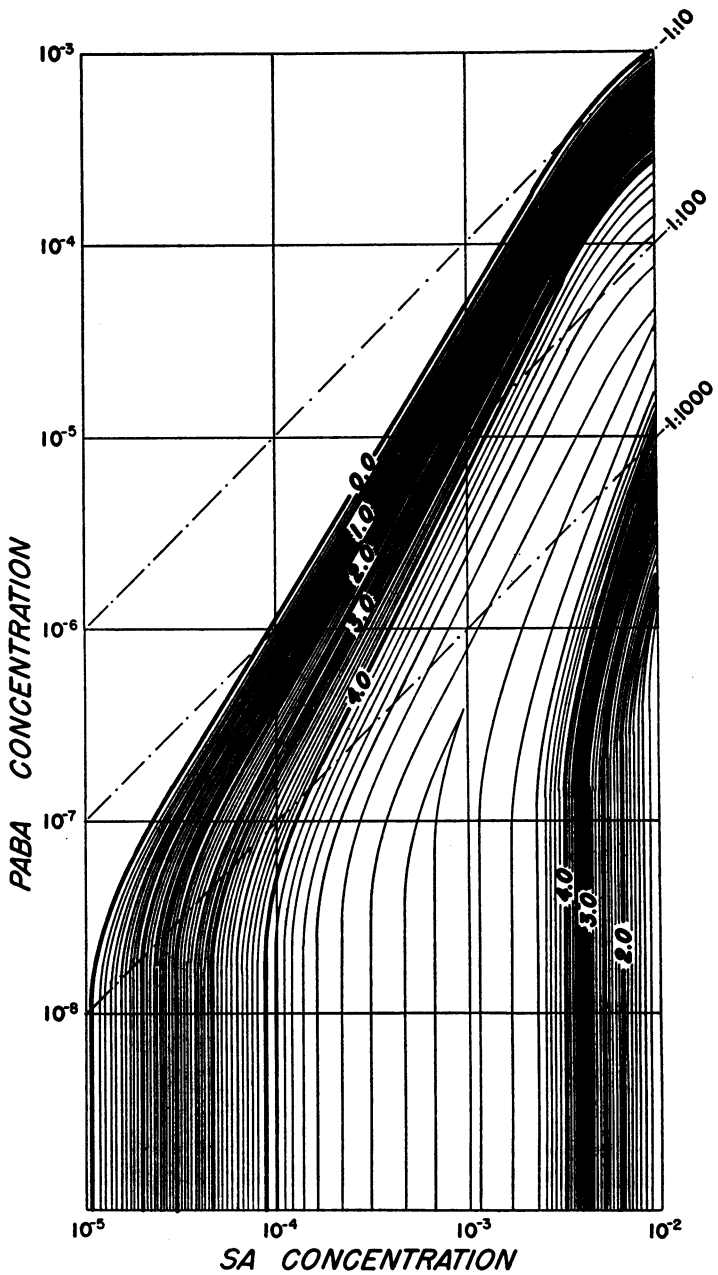

FIG. 6. CONTOUR GRAPH SHOWING GROWTH RATES OF THE SULFONAMIDE-REQUIRING STRAIN, SFO, AT 35 C ON VARYING MOLAR CONCENTRATIONS OF SA AND PABA

Growth rates are indicated by the contour lines. The ratios in the upper right margin are the molar ratios of PABA to SA. Based on determinations at 60 different combinations of SA and PABA concentrations.

luxuriant, and has a sharply defined frontier; such growth is represented by the heavier lines in figure 5.

Conidial transfers from the ends of growth tubes showing reverted growth (designated by arrows in figure 5) indicated that the reversions were persistant for the most part (see discussion of persistant "adaptive" changes in Emerson

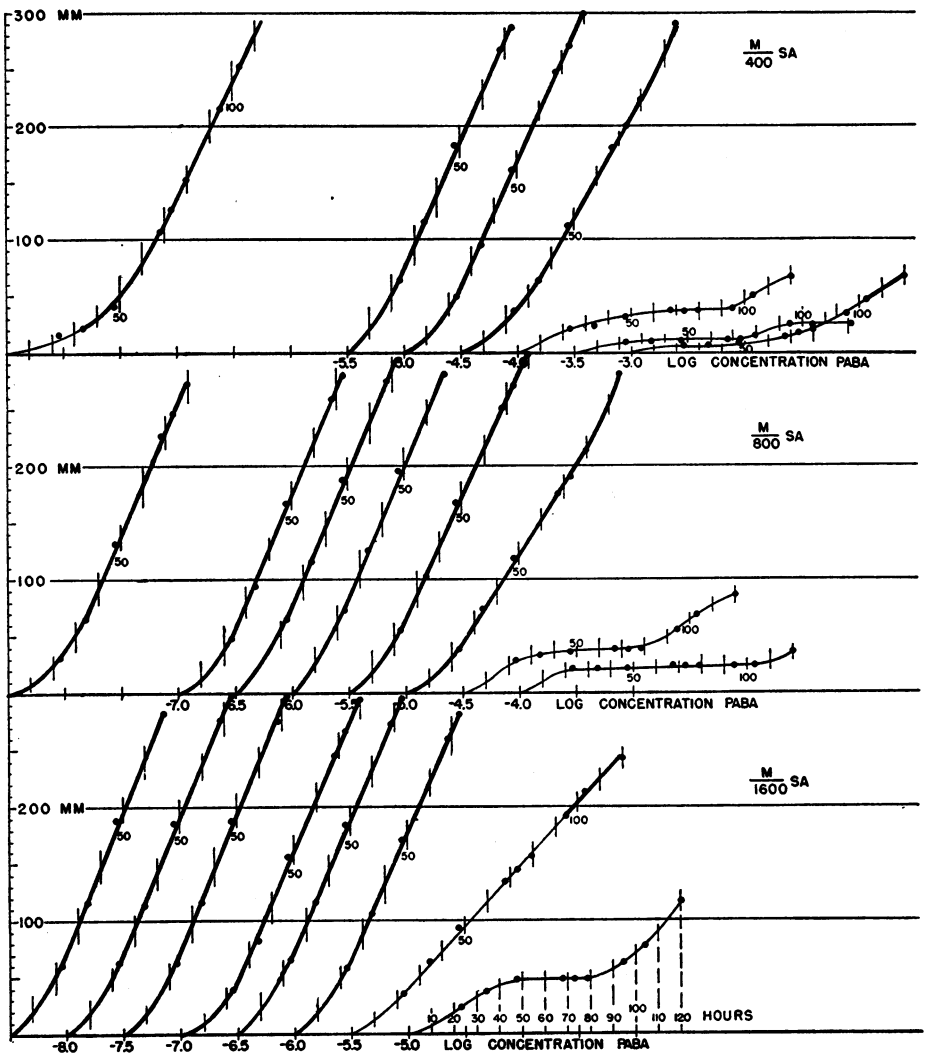

FIG. 7. FAMILIES OF GROWTH CURVES OF sfo STRAIN ON VARYING CONCENTRATIONS OF PABA

Upper set of curves in the presence of M/400 SA; middle set, M/800; lower set, M/1,600. Positions of the origins of the curves along the base line indicate the concentrations of PABA for each. In each curve, horizontal distance represents elapsed time in hours, vertical distance represents total growth in millimeters. Heavy lines represent mycelial growth possessing definite frontiers, lighter lines represent "feathery" growth, with no well-defined frontier.

and Cushing, 1946). Transfers from such tubes to fresh tubes containing no SA generally resulted in growth resembling that of wild-type without any preliminary "feathery" stage such as is characteristic of sfo. Furthermore, crosses

from such reverted cultures generally showed that reversion had been accompanied by mutation at a locus distinct from that responsible for sulfonamide requirement. The fractions at the tops of growth curves in figure 5 show the num-

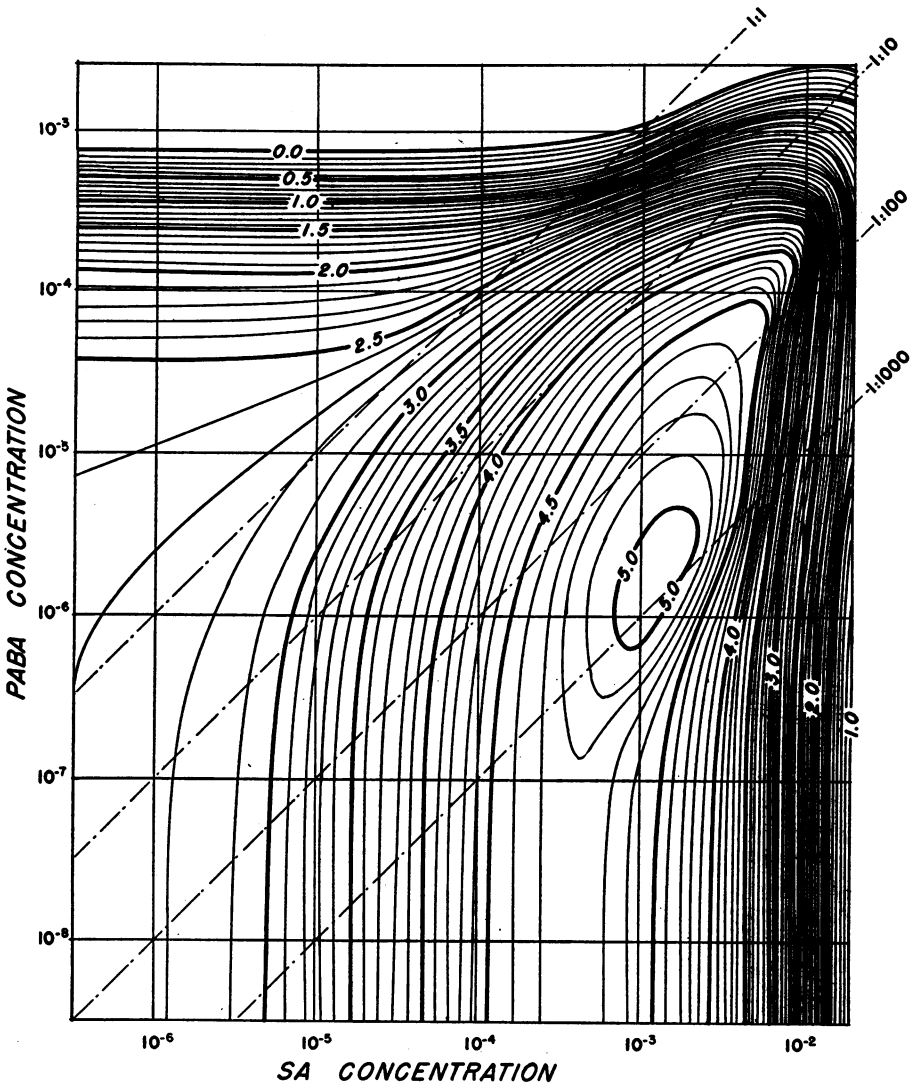

FIG. 8. CONTOUR GRAPH SHOWING GROWTH RATES OF sfo STRAIN AT 30 C IN THE PRESENCE OF VARYING AMOUNTS OF PABA AND SA
See legend to figure 6. Based on 144 determinations at 96 different combinations of SA and PABA concentrations.

ber of nuclei carrying such mutations in the total nuclei tested from each culture. It follows that these mutations are not strictly reversions, but rather suppressions of the effects of sfo by another gene.

Since the more rapid "reverted" growth is presumably always the result of an

altered genetic constitution, the rates obtaining before reversions occur are taken as characteristic of sfo.

Competitive inhibition of growth by p-aminobenzoic acid. Under conditions which make sulfonamides essential for growth of the sulfonamide-requiring strain, PABA inhibits growth in very low concentrations. When grown at 35 C in the presence of optimal or suboptimal concentrations of SA, PABA will inhibit growth at concentrations as low as millionth molar (figure 6). In the presence of an excess of SA, however, relatively small amounts of PABA are beneficial, though inhibition still occurs at higher concentrations. The stimulating effect of PABA at high SA concentration is principally in shortening the lag phase resulting from the toxicity of SA, as illustrated in figure 7, though the final growth rate may also be increased.

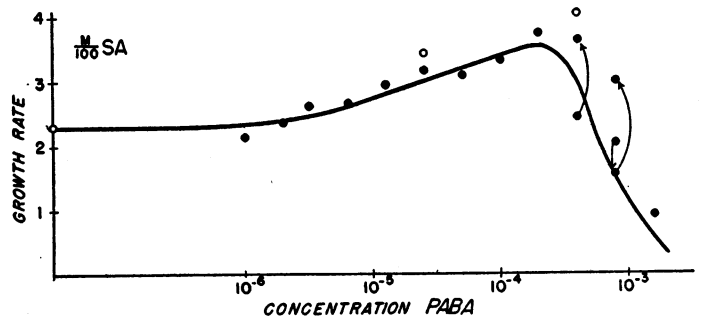

FIG. 9. GROWTH RATES OF SFO STRAIN AT 30 C IN THE PRESENCE OF M/100 SA AND VARYING MOLAR CONCENTRATIONS OF PABA
Growth rates expressed as millimeters per hour. Open circles represent data from one experiment, solid circles from another. Dots connected by arrows represent growth rates which changed during the course of growth down the tube (cf. figures 5 and 7).

At lower temperatures, at which sulfonamides stimulate growth but are not essential for growth, the inhibitory effect of PABA is very much less (figure 8). Relatively high concentrations of PABA are necessary for growth inhibition, and the inhibiting concentration is relatively independent of the amount of SA present. On the other hand, PABA does interfere with growth stimulation by SA. Maximum response to SA occurs only when the molar ratio of PABA to SA is less than 1 to 100. Here again in the presence of excessive amounts of SA, PABA partially overcomes the inhibition caused by the SA (figure 9).

The simultaneous requirement of sulfanilamide and p-aminobenzoic acid by a double mutant. The sulfonamide-requiring strain was crossed to the pab strain of Tatum and Beadle (1942), which requires PABA for growth, and the double mutant (sfo pab) isolated.[4] At 35 C this double mutant requires both PABA and SA (figure 10). Over most of the range of concentrations supporting growth

[4] Tatum and Beadle's pab strain 1633A was first crossed to wild-type strain E-5297a and the pab gene isolated free from an undesirable gene (temperature sensitive on lactose, etc., see Emerson and Cushing, 1946) as strain E-15835a, which was then crossed to sfo E-15172A. The double mutant, sfo pab, was isolated from this cross in strains E-16608A and E-16613a.

of the double mutant, a molar ratio of about 1 PABA to 1000 SA is most favorable. An excess of either analog is inhibitory in a competitive manner.

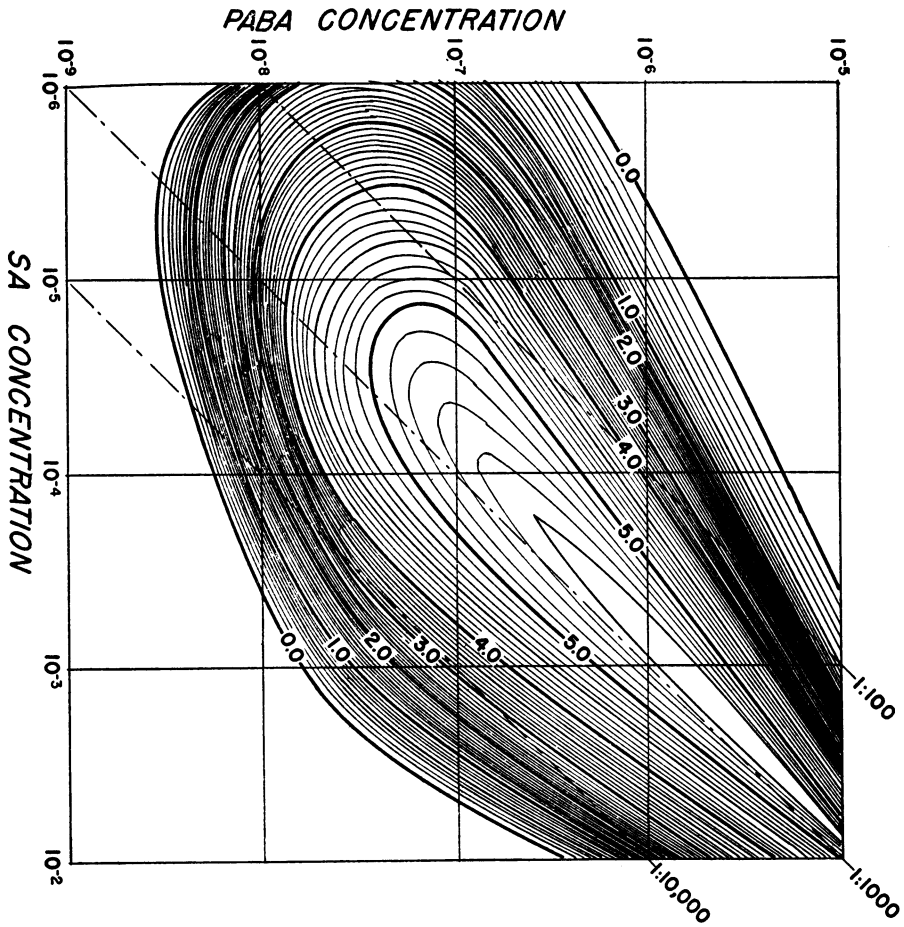

FIG. 10. CONTOUR GRAPH SHOWING VARIATION IN GROWTH RATE WITH CHANGING CONCENTRATIONS OF PABA AND SA OF THE DOUBLE MUTANT (SFO PAB) AT 35 C

See legend to figure 6. Based on 84 determinations at 52 combinations of SA and PABA concentrations.

DISCUSSION

Sulfanilamide as a metabolite. Although growth responses by themselves do not prove that a substance found to be necessary for growth is actually used as a metabolite, the data just reported make it seem highly probable that sulfanilamide is so utilized by strain E-15172 (sfo). In the first place, of the substances tested only sulfonamides were capable of supporting growth of the sfo strain at 35 C. Secondly, this strain does not produce excessive amounts of PABA and thus require sulfonamides as antagonists because: (1) Inhibition of wild-type *Neurospora* by PABA is not antagonized by SA (Emerson and Cushing, 1946).

(2) The double mutant sfo pab cannot synthesize PABA and needs both SA and PABA for growth at 35 C. In the double mutant there can be no question of an overproduction of PABA, yet sulfonamides are still required. Thirdly, the competitive inhibition of growth of the sfo strain by PABA suggests that the structurally similar SA is actually used as a metabolite.

Sulfanilamide-p-aminobenzoic acid ratios. In most instances of competitive growth inhibition the molar ratio of inhibiting analog to metabolite is rather large. The competitive inhibition of wild-type *Neurospora* by SA is of this sort (Tatum and Beadle, 1942; Emerson and Cushing, 1946). It is all the more striking, therefore, that in the PABA inhibition of the sulfonamide-requiring strain the ratio of inhibiting analog to "metabolite" is just reversed, being about 1 PABA to 100 SA.

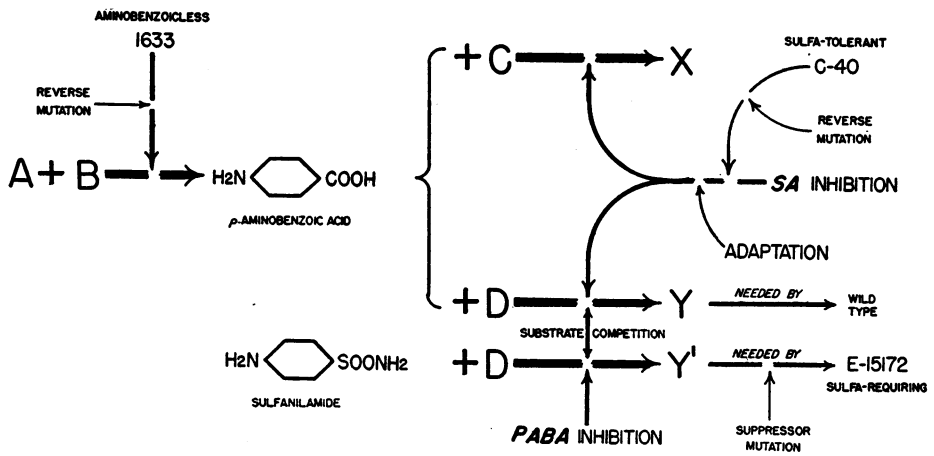

FIG. 11. FORMAL SCHEME TO SUMMARIZE THE INTERPLAY OF MUTATIONS EFFECTING SA-PABA RELATIONSHIPS

Heavy arrows represent enzymatic reactions; breaks in arrows represent interruptions due to genetic blocks or to substrate inhibitions.

Since the molar ratio of PABA to SA is the same regardless of which is the inhibiting analog, one is tempted to suggest that the same enzymatic reaction is involved in both cases. If two substrates (PABA and SA) compete for the same enzyme, and if both are transformed by that enzyme, the relative amounts of the two products resulting will depend upon the relative amounts of the two substrates. Then if wild-type requires one of these end products (Y in figure 11), and the sulfonamide-requiring mutant the other (Y'), the ratios of inhibiting analog to metabolite should be reversed as one metabolite is replaced by the other, just as reported above.[5]

A formal scheme giving a pictorial summary of the interplay of the various

[5] Nearly everyone with whom I have discussed this case has suggested that the sulfonamide requirement of strain E-15172 might be accounted for by some such scheme. I believe that the particulars just outlined were first suggested by my collaborator Dr. Marko Zalokar.

mutations studied is given in figure 11. The aminobenzoicless mutant (pab) is known to interrupt the synthesis of PABA (Tatum and Beadle, 1942). In the absence of PABA the gene pab is sometimes changed to $+^{pab}$ by reverse mutation, restoring the wild-type condition in which the synthesis of PABA continues normally.

It is supposed that PABA takes part in more than one essential reaction (e.g., with substances C and D in the diagram). This would be in agreement with the observations of Lampen *et al.* (1946), which suggest that PABA is concerned with three different sorts of syntheses. The inhibition of growth by SA is supposed to be due to substrate competition with PABA in one or more of these reactions. Such SA inhibition can be lessened by nongenetic adaptation (Emerson and Cushing, 1946), or largely overcome by mutation to sulfanilamide tolerance (S-T). Especially in the presence of PABA or sulfathiazole, reverse mutation changes S-T back to wild-type ($+^{S-T}$).

The sulfonamide-requiring mutant, sfo, is shown as differing from wild-type by needing the end product Y′ in place of Y. As illustrated, the double mutant pab sfo, requiring both PABA and SA for growth, needs X as well as Y′. On this supposition, SA would interfere with the production of X, PABA with the production of Y′. It is also possible that in place of X and Y′ the double mutant needs Y and Y′, say in approximately equal amounts. Again a balance between SA and PABA would be essential as the production of Y is inhibited by excess SA, of Y′ by excess PABA.

"Reversions" of the sfo mutant to growth resembling wild-type are due to "suppressor" mutations. These are mutations of a gene distinct from sfo which suppress the sulfonamide requirement characteristic of sfo.

The scheme illustrated is meant simply as a convenient summary. Direct evidence of the role of SA in the metabolism of the sulfonamide-requiring mutant must await the chemical determination of the fate of SA in the organism.

SUMMARY

Mutant strain E-15172 requires sulfonamides for growth at 35 C. At 30 C or lower sulfonamides are not strictly essential, but growth rates are depressed without them.

At high temperatures (34 C or over) *p*-aminobenzoic acid inhibits growth of this strain at high dilutions (10^{-6} molar). Growth inhibition by *p*-aminobenzoic acid is competitively antagonized by sulfanilamide. The ratio of *p*-aminobenzoic acid to sulfanilamide giving 50 per cent growth inhibition is about 1:100.

A double mutant, carrying the gene for sulfonamide requirement and a gene for the failure of synthesis of *p*-aminobenzoic acid, requires both sulfonamides and *p*-aminobenzoic acid for growth. The molar ratio giving maximum growth at 35 C is about 1,000 sulfanilamide to 1 *p*-aminobenzoic acid.

The possibility that sulfanilamide is utilized by strain E-15172 as a metabolite is discussed.

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