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Genetically Modified Folic Acid Synthesizing Enzymes of Pneumococcus*

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Cell-free extracts have been prepared from eight genetically related sulfanilamide-resistant mutant strains of pneumococcus. The biosynthesis of folic acid has been studied in these extracts, the growth of *Streptococcus faecalis* being used for the assay of folic acid compounds. This biosynthesis is dependent upon an exogenous supply of *p*-aminobenzoic acid. As with the growing bacteria, the cell-free extracts are subject to competitive inhibition by specific *p*-aminobenzoic acid analogs, the resistance patterns being characteristic of the mutant strain used. Differences in temperature sensitivity of the rate of synthesis were also observed; with extracts of some strains, including the wild type, the reaction was effective at temperatures up to 50°, whereas extracts from all strains containing the particular genetic marker F_d were inactive at 50°, and optimal synthesis occurred below 37°. These differences are all attributable to genetically controlled alterations of affinity in the substrate binding groups of one enzyme in the folic acid synthesizing system.

The now classical work of Beadle and Tatum (1941) described mutations having biochemical effects ultimately attributed to the loss of specific enzymatic capacities of the mutant strains. Many of these seem to be due to essentially complete loss of a specific protein from the organism, but less drastic biochemical changes also occur because of genetic mutations resulting in altered physical properties of the proteins (*e.g.*, Maas and Davis, 1952; Horowitz, 1956; Fincham, 1960). These more subtle changes are of interest from both the biochemical and the genetic points of view. An enzyme which has been altered in several ways in different mutants can be used as a tool to study the mode of action of the catalyst. Geneticists have accomplished fine-structure mapping within single genetic loci governing the presence or absence of specific enzymes, and when different alterations within a single enzyme can be recognized in the phenotype, it becomes possible to map the genetic regions determining these alterations.

A highly sulfonamide-resistant mutant strain of pneumococcus, RF29, isolated in 1955 (Hotchkiss and Evans, 1958), has provided a system in which qualitative phenotypic alterations brought about by mutation could be detected and also correlated on a biochemical basis with a genetic analysis of the strains involved.

The genetic analysis of the mutant was accomplished by transformation experiments and showed that the high resistance of the mutant to sulfanilamide was genetically attributable to a complex locus bearing three closely linked but separate subunits (called *a*, *b*, and *d* for convenience). These subunits could be transferred *via* transformation processes either singly or together in any of seven combinations, thereby conferring on the recipient strains quantitatively distinct levels of resistance to sulfanilamide. Thus, for example, a wild recipient strain, which is barely resistant to 4 μg of sulfanilamide/ml, upon incorporating the *a* or *d* region of a DNA donor molecule becomes resistant to about 20 μg or 80 μg of sulfanilamide/ml, respectively. Incorporation of both the *a* and *d* portions of the donor molecule results in a resistance to approximately 400 μg of sulfanilamide/ml in the transformed cells. This fact suggests that the same cell function is being affected cumulatively by each of the genetic units.

According to the classical system described by Woods (1940), *p*-aminobenzoic acid competitively releases the bacteriostatic action of sulfanilamide. Later it was shown (Lascelles and Woods, 1952) that folic acid synthesis by resting bacterial suspensions required *p*-aminobenzoic acid and was inhibited by sulfathiazole. The same relationships hold for all of our pneumococcal strains. It was therefore suggested that the genetic alterations in the pneumococcal strains, phenotypically expressed as a resistance to sulfanilamide, had altered the affinities of an enzymatic system concerned with the synthesis of folic acid and, more specifically, with the reaction involving *p*-aminobenzoic acid as substrate.

Early biochemical investigations of these mutant strains were directed toward observing effects of specific inhibitory analogs of *p*-aminobenzoic acid upon growing cultures. As in the case with sulfanilamide, other analogs gave characteristic different quantitative growth-inhibition patterns; the seven mutant strains differed in their growth responses depending upon how

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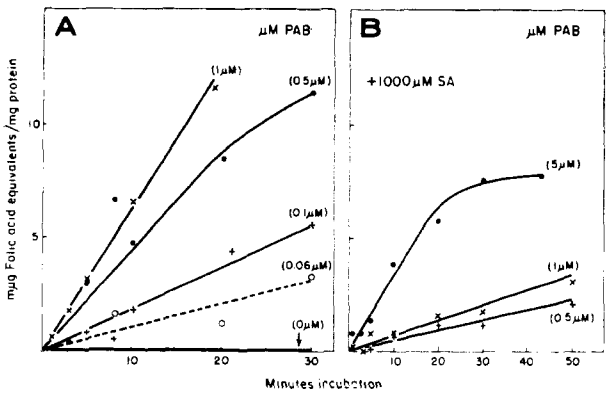

FIG. 1.—*A*, the rate of synthesis of folic acid compounds as a function of *p*-aminobenzoic acid concentration. The reaction mixture contained 0.01 M phosphate buffer, *p*H 8, 0.01 M sodium thiomalate, crude F_{adb} extract (containing 1 mg protein), and micromolar concentration of *p*-aminobenzoic acid as indicated, in a total volume of 1 ml. The mixture was incubated at 0°. The *p*-aminobenzoic acid was added to start the reaction. *B*, the rate of synthesis of folic acid compounds in the presence of 1000 μM sulfanilamide. The *p*-aminobenzoic acid concentrations are indicated, all other conditions being the same as in *A*.

the *p*-aminobenzoic acid molecule was altered.

The independent, yet cumulative, effects of the three genic units, *a*, *b*, and *d*, toward each analog indicated that these units must exert their control over one and the same enzymatic function. For this reason, Hotchkiss and Evans (1960) concluded that a mutation within a complex genetic locus had brought about alteration of a *single enzyme*. The characteristic specific responses to different analogs by strains carrying the different genetic units indicated that fine structural regions within a certain DNA molecule control point for point the fine structure of the protein molecule.

The above-mentioned studies included principally measurements of growth inhibition, and also endogenous folic acid synthesis by growing cultures of pneumococcus. The present study of cell-free extracts was undertaken as the next step toward the isolation and identification of the enzyme system involved. The ultimate and as yet technically unfeasible goal of this work would be the point-for-point correlation on a molecular level of the protein molecule and of that portion of DNA structure which determines it.

MATERIALS AND METHODS

Strains of Pneumococcus.—The origin of the sulfonamide-resistant strain (RF29) and the use of its DNA to transform wild cells to the strains bearing the genetic subunits has been outlined in a previous publication (Hotchkiss and Evans, 1958). For convenience, the symbols F_a, F_d, etc., will be used to signify the strains bearing the genetic units *a*, *d*, etc. The wild strains, R6 and R1, will not grow at concentrations of sulfanilamide above 5 μg/ml (in neopeptone broth), whereas the strains bearing the single genetic units are resistant to low levels of sulfanilamide (F_a, 20 μg/ml; F_b, 15 μg/ml; and F_d, 80 μg/ml). The strains carrying two of the subgenic units have low or intermediate resistance levels (F_{ab}, 70 μg/ml; F_{ad}, 400 μg/ml; and F_{db}, 300 μg/ml); whereas the strain bearing all three of the mutated subunits resists a high level of sulfanilamide (800 μg/ml).

Preparation of Cell-Free Extracts.—It was found that the accumulation of endogenous folic acid compounds was low when pneumococcus was grown in a meat infusion broth containing 1% neopeptone, 0.03% glucose, and approximately 0.005 M K_2HPO_4. Cultures containing about 2×10^8 colony-forming units per ml were harvested by centrifugation and allowed to respire for a total of 2 hours in two changes of a medium containing 0.85% NaCl, 0.03 M K_2HPO_4, 0.18% albumin (Armour, bovine plasma fraction V), and 0.25% glucose. This procedure, rather than dialysis, was used to rid the bacteria of accumulated folic acid products, since the co-factors and substrates needed for the reaction were unknown (see below). At this point the culture was always checked for its sulfanilamide resistance level. The cells were then washed once in 0.01 M phosphate buffer (containing 0.01 M sodium thiomalate), *p*H 8. Acetone powders of the bacterial cells were prepared by the general procedure outlined by Gunsalus (1955), the technique being scaled down to handle small quantities of material. One preparation from 3 liters of culture usually yielded about 500 mg of dried powder. The powders were stored at −10° and retained their activity for at least a month. Portions to be used were suspended in iced 0.01 M phosphate-thiomalate buffer, *p*H 8, for an hour and then centrifuged in the cold at 10,000 rpm for 10 minutes. The cell-free supernatant solution contains the enzyme system that catalyzes the synthesis of folic acid compounds.

Suitable conditions for the reaction are presented in the legend to Figure 1A. No precautions had to be taken to keep the system under anaerobic conditions. Although Brown *et al.* (1961) have found this necessary with their *E. coli* enzyme system, anaerobic and aerobic experiments in this laboratory with pneumococcal extracts showed no substantial difference in yield. Since *E. coli* and pneumococcus are relatively dissimilar bacteria, this observed difference need not be surprising. It is also possible that in our system folic acid is being produced and measured in oxidized form. Also, sterile filtration instead of sterilization by heat in our case did not appreciably raise the yield of the product. The reactions therefore were stopped normally by heating the solutions in a boiling water bath for 5 minutes. Aliquots were then removed, placed into folic acid assay medium, autoclaved in the presence of 0.5 mg/ml of sodium ascorbate for 13 minutes at 15 lbs. steam pressure, and assayed with *S. faecalis*. In kinetic studies, aliquots of the reaction mixture were removed at various time intervals, immediately pipetted into pre-heated water, and assayed.

Folic Acid Assay.—*Streptococcus faecalis* was used as the organism for detecting the presence of folic acid compounds (see Jukes, 1955). The bacteria were grown in Difco Folic Acid Assay Medium.[1] Growth of the organism was followed by making turbidity measurements with a Coleman Model 9 nephelometer. Throughout the text "folic acid compound" refers to any compound of the folic acid family which stimulates the growth of *S. faecalis*, and would include folic acid itself as well as pteroic acid, dihydropteroic acid, etc. Because of the relative stability of folic acid, a standard curve employing this compound was used with each assay and all results are reported in terms of equivalents of folic acid.

Protein Assay.—The protein concentration of the crude extracts was determined by the method of Lowry *et al.* (1951), employing the procedure suggested by Oyama and Eagle (1956).

RESULTS

Initial experiments with crude cell-free pneumococcal extracts indicated that *p*-aminobenzoic acid

[1] Difco Products, Detroit, Mich.

was the only supplement required for formation of product active in the folic acid bioassay. Figure 1A shows the effect of increasing the concentration of this substrate upon the rate of synthesis of folic acid compounds. The reaction occurring was obviously limited by the endogenous concentration of one or more unknown co-factors or substrates, as can be seen by the early cessation of synthesis (compare below, Figures 4 and 5). It is interesting to note that the only compound actually limiting the initial synthesis was *p*-aminobenzoic acid; the addition of this material was essential for any reaction to occur. Glutamate or glutamine did not enhance the synthesis, nor did 2-amino-4-hydroxy-6-carboxaldehyde pteridine[2] (in the presence or absence of added adenosine triphosphate). Incidentally, this pteridine in 0.1 μM or higher concentration is sufficiently inhibitory to the growth of *S. faecalis* to interfere with the bioassay.

p-Aminobenzoylglutamate in some cases could replace *p*-aminobenzoic acid as substrate at a comparable concentration (2 μM) for extracts of strains not containing the *d* marker. No extract of cells containing the *d* marker used *p*-aminobenzoylglutamate at this concentration (Fig. 2). However, when 200 times that amount was supplied, some synthesis did occur. The *p*-aminobenzoylglutamate may be used directly by the enzymes, or it is possible that it is hydrolyzed to *p*-aminobenzoic acid by the crude extract.

Our immediate aim was to investigate whether the various synthesizing extracts showed any drug responses comparable to the resistance of the whole cells from which they came. Since a limited reaction could be measured with unsupplemented cell-free extracts, no concerted attempt was made to elucidate any other substrate or co-factor required for more prolonged synthesis.

The initial velocity of the reaction is approximately proportional to the amount of dialyzed extract added over the range from 0.9 to 9 mg total protein per ml with a concentration of 1 μM *p*-aminobenzoic acid.

Synthesis was rapid at either 24° or 37°; for kinetic experiments a suitable slow and regular reaction occurred at 0° with extracts of the wild type strain R1 and strain F_{adb}. For some of the other strains synthesis was erratic or non-linear at 0°.

Figure 1 illustrates the type of results obtained when F_{adb} extracts are incubated with *p*-aminobenzoic acid in the absence or presence of sulfanilamide. From such velocity data, Lineweaver-Burk kinetic plots were obtained for the F_d, F_{adb}, and wild type extracts (Fig. 3). They obviously indicate differences that exist between the strains. One of the plots shows unexplained curvature at low substrate concentration. The fact that the maximum velocities (V_{max}, at the ordinate intercept) are the same in the presence and absence of drug demonstrates that in this cell-free form this inhibition is of a competitive nature.

The calculated values for K_m, the concentration of substrate giving half-maximal velocity, and those for the apparent dissociation constant (K_i) of the enzyme for the inhibitor sulfanilamide, are shown in the figures. These constants are approximate values only, for precise measurements could not be obtained with the microbiological assay employed. The wild R1 extract requires $^1/_4$ to $^1/_5$ as much *p*-aminobenzoic acid to reach half-maximal velocity as do the two mutant extracts with which it is compared. A further striking difference between the wild strain and the two mutant strains is reflected in the apparent dissociation constants of the enzymes for sulfanilamide. The wild type strain binds sulfanilamide 600–1000 times more efficiently than do the F_{adb} and F_d strains. The V_{max} values as determined from the plots of Figure 3 are: wild type 1.3, *d* 0.4, and *adb* 0.85 (mμg equivalents/mg protein/min.).

[2] Kindly provided by Dr. C. Stock of the Sloan-Kettering Institute, New York.

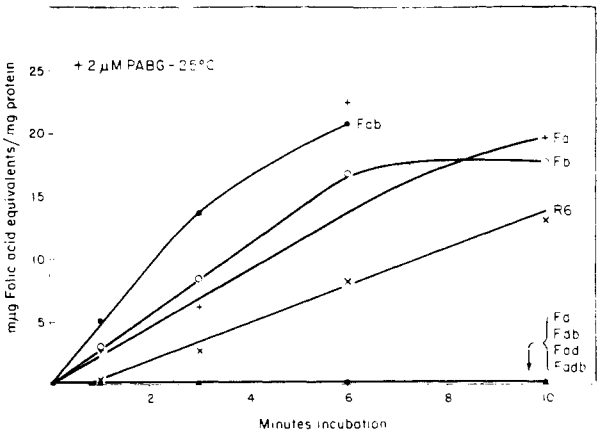

FIG. 2.—The rate of synthesis of folic acid compounds with *p*-aminobenzoylglutamate as substrate. The reaction mixtures contained from 1.5–2 mg protein/ml; 2 μM *p*-aminobenzoylglutamate replaced *p*-aminobenzoic acid as substrate. The reaction was carried out at 25°.

Detailed kinetic studies of the other strains and with other drugs were postponed until the requirements of the synthesis are more fully known. As mentioned previously, the *a* and *b* extracts could not be used at 0°, and synthesis was extremely rapid at higher temperatures, making kinetic measurements difficult.

Accordingly, synthesis was compared in extracts of the various strains at 25° and the reaction was stopped in each case at a suitably early time to measure not more than 75–85% of the maximum amount of synthesis. These measurements were used in place of more precise initial rates for the purpose of comparing the effects of selected inhibitory *p*-aminobenzoic acid analogs on the extracts of the various strains. The data (Table I) are compared with those found in studies of growing cells (Hotchkiss and Evans, 1960). The two systems can be compared only qualitatively, as the numbers expressed in the table refer to different measurements at different temperatures. In the case of cell-free extracts, the values refer to the relative quantity of antimetabolite required to reduce folic acid synthesis to a certain diminished level, on the principle that in intact cells this should have corresponded to a constant reduced growth capacity. This involved adjustment of the 50% inhibition values (on the relative basis, mutant/wild type) for the actual relative rates of synthesis at 25° in these extracts (which are a function of the K_m and V_{max} values and the concentration of enzyme). The whole-cell studies are expressed in terms of the amount of analog causing half normal growth in a meat-infusion neopeptone medium at *p*H 7.5 at 37°. The data in Table I show that the cell-free enzyme systems from the different strains do indeed exhibit different sensitivities to the specific inhibitors, reasonably parallel to the whole-cell growth inhibition.

The various extracts of the wild-type strain and the sulfonamide-resistant mutants were examined at different temperatures (Fig. 4 and 5). It can be seen that, in four strains not carrying the *d* marker, the enzymatic reaction rate was comparable whether the reaction was carried out at 37° or 50°. A striking difference exists in those enzyme extracts isolated from the four strains

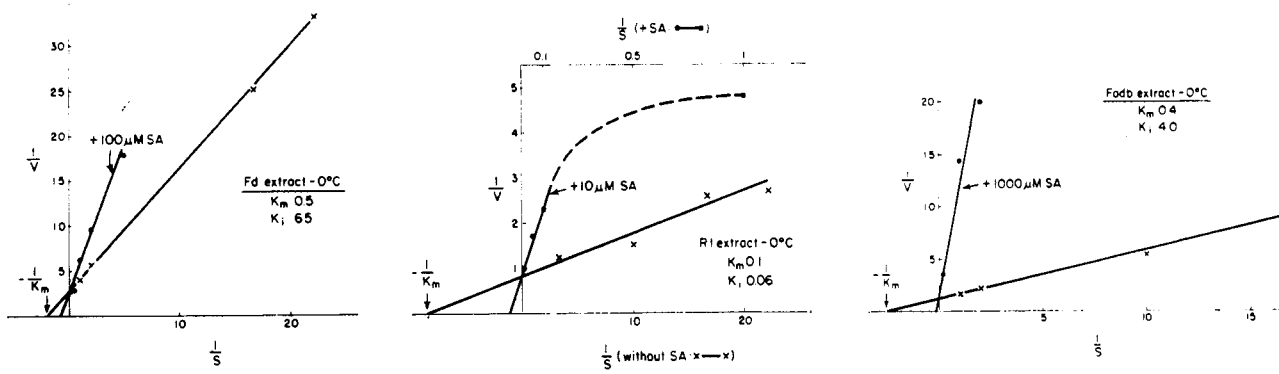

FIG. 3.—F_{adb}, F_d, and R1 extracts: Lineweaver-Burk kinetic plots. V = velocity of reaction, mμg folic acid equivalent/mg protein/minute, S (substrate) = *p*-aminobenzoic acid, μM concentrations.

TABLE I

RELATIVE RESISTANCE OF PNEUMOCOCCAL STRAINS (MUTANT/WILD TYPE)[a]

	Cell-Free Extracts[b] (25°)			Whole Cells[c] (37°)		
Strain	SA	pAS	pNA	SA	pAS	pNA
Wild	1	1	1	1	1	1
F_a	4	7.5	5.5	4	8	3
F_d	7	0.1	1	16	0.7	3
F_{ad}	8	0.05	1	80	2	7
F_{adb}	15	0.35	5.5	240	—	10
	Absolute Inhibitory Concentrations (μM)					
Wild[d]	10	3	75	30	13	720

[a] Abbreviations: SA, sulfanilamide; pAS, *p*-aminosalicylate; pNA, *p*-nitroaniline. [b] Drug concentrations required to reduce the synthesis of folic acid in each extract to a fixed quantity (relative to wild type). (25°, *p*H 8) [c] Drug levels giving half-normal growth (relative to wild type). (37°, *p*H 7.5) (Hotchkiss and Evans, 1960). [d] *p*-Aminobenzoic acid concentration added to cell-free extracts, 0.5 μM. The endogenous *p*-aminobenzoic acid in whole cells was estimated at 0.1 μM (*loc. cit.*).

bearing the *d* subgenic factor. In each of these cases, there is a decrease in the rate of the reaction at temperatures above 25°; by 50° the reaction is markedly inhibited (in all extracts the reaction does not occur at 60°).

That the heat sensitivity is not due to an unstable substrate or co-factor being destroyed at 50° was shown in two ways. An F_{ad} extract was dialyzed, and the diffusible material ("dialysate") was heated to boiling for 10 minutes and then recombined with the dialyzed enzyme extract. When *p*-aminobenzoic acid was added, the reaction proceeded at 37° as usual. Furthermore, when an F_b extract was inactivated by dialysis and then replenished with *p*-aminobenzoic acid, it could be reactivated at 50° by an F_d extract (itself inactive at 50°). There was no appreciable difference between the amount of substrates (or co-factors) present in the F_b and F_d strains.

In the studies just described the enzymatic reaction was tested at the elevated temperature. To determine whether the enzyme is itself unstable, we preincubated the various extracts at 50° for specified times up to 30 minutes and then measured the amount of activity remaining for a reaction carried out at 25°. With moderately high concentration of extract, any mild inactivation observed was always too little to account for the lowered activity of extracts of the *d*-containing strains at a reaction temperature of 50°.

The relatively high values of K_m for the reactions carried out by *d*-containing enzymes suggest a possible basis for their heat sensitivity. The K_m values for the F_d and F_{adb} strains as measured at 0° are in the range 0.4–0.5 μM, whereas the K_m for R1 wild type is 0.1 μM (Fig. 3). However, at 25°, preliminary determinations gave K_m values of 1.2 μM and 0.4 μM for the F_d and wild-type extracts respectively. If at still higher temperatures the substrate affinity of the F_d enzymes continues to decrease, it may be that when tested at 50° they did not have sufficient substrate to express their lowered capacity to react with it. Preliminary experiments also indicate that at a high concentration of *p*-

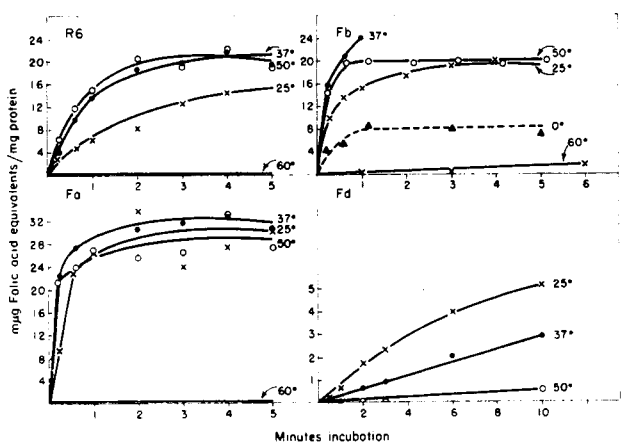

FIG. 4.—The effect of incubation temperature on the rate of reaction. All samples contained 0.5 μM *p*-aminobenzoic acid and 1.5–3 mg protein in a volume of 1 ml.

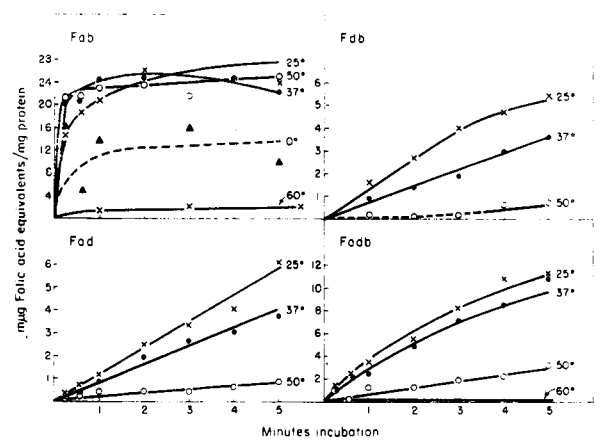

FIG. 5.—The effect of incubation temperature on the rate of reaction. All samples contained 0.5 μM *p*-aminobenzoic acid and 1.5–3 mg protein in a volume of 1 ml.

aminobenzoic acid (2 μM) some synthesis by the F_d enzyme did occur at 50°. The K_m constants for the other mutant strains have not yet been determined.

Discussion

Several conclusions are suggested by the results with cell extracts: (1) First of all, the cell-free syntheses do show characteristic drug resistances as do the cells. (2) Altered permeability does not seem to play a primary role in the resistance to *p*-aminobenzoic acid antimetabolites of the growing resistant cultures. (3) The three genetic alterations studied appear to cause modifications within a *single enzyme*. (4) These changes appear to manifest themselves in altered affinities for substrates and drugs, corroborating the conclusions based on whole-cell studies (Hotchkiss and Evans, 1960).

Although resistance of the growing cells to some of the *p*-aminobenzoic acid analogs tested may be secondarily influenced by the ability of the compound to penetrate into the cell, altered permeability or permease does not seem to be the primary basis for resistance, since the cell-free extracts of the various mutants are also resistant to the antimetabolites.

The present work brings additional experimental support for the conclusion reached from the experiments with the growing sulfonamide-resistant cultures (Hotchkiss and Evans, 1960) that these eight closely related strains all have changes occurring in a single enzyme involved in folic acid synthesis. Alternative hypotheses might designate three distinct enzymes, A, B, and D, affected by the mutant genic sites *a*, *b*, and *d*. These *a priori* possibilities would involve (1) each enzyme catalyzing the formation of different, essential end-products, (2) each enzyme producing the same product or interconvertible products (a form of folic acid) *via* separate pathways, and (3) the enzymes being involved sequentially in a reaction chain, the end-product of which is folic acid.

The fact that it is folic acid production which is limited by drug concentration in each of the various extracts eliminates alternative (1). The qualitative equivalence of each of the mutations for both pneumococcal growth and *S. faecalis* bioassay indicates that any separate products are interconvertible.

The reaction temperature sensitivity of the extracts of strains bearing the *d* marker negates alternative (2). In these extracts little or no folic acid synthesis occurs at a reaction temperature of 50°, yet at this temperature any hypothetical A and B enzymes must function normally under the conditions employed. Because of the low efficiency of the D enzyme, if separate A and B enzymes were also present in the *ad*, *bd*, and *adb* extracts, these extracts would synthesize almost as much folic acid at 50° as at 37°. Figure 5 shows that the total synthesizing capacity of these extracts is heat sensitive.

Principal alternatives of type (3) can be eliminated by consideration of Table I, insofar as they relate to A and D. The case for B must await further data but can also be argued from the findings with growth inhibition of whole cells. The unmutated forms of enzymes A and D must each be limiting factors in the wild-type extract when folic acid synthesis is inhibited by sulfanilamide, since the altered form of either one permits increased synthesis (4 or 7 fold) in the presence of sulfanilamide. If the A and D enzymes were in a sequential relationship, then the unmutated A enzyme, unable to function at the 4-fold level, would still remain limiting in the reaction chain of the F_d extract. Correspondingly, the unmutated D enzyme should limit any synthetic sequence of which it is a part in the F_a extract in the presence of sulfanilamide. The potentiation observed virtually requires that the mutations of A and D affect the same entity, that is, the single enzyme combining properties A and D. It will be clear that several other combinations in Table I lend themselves to similar argument; these have already been presented for the case of growth inhibition (Hotchkiss and Evans, 1960).

This study was undertaken to test our hypothesis that whole cell responses of the mutant strains to *p*-aminobenzoic acid analogs could be explained on the basis of changed affinities in a series of genetically altered *p*-aminobenzoic acid–utilizing enzymes. Davis and Maas (1952) had earlier reasoned that several unrelated sulfanilamide-resistant mutants of *E. coli* which they investigated might bear enzymes with decreased relative affinities for individual *p*-aminobenzoic acid analogs.

The admittedly incomplete kinetic data show indications that the mutant strains have characteristic K_m and V_{max} values for *p*-aminobenzoic acid and widely varying characteristic K_i values for the various inhibitors. The general trend of the effective production of folic acid compounds by the extracts under drug inhibition (Table I) seems clearly to follow the general pattern of the cell growth in the same drugs. Some of the deviations which appear can be explained with reference to the temperature dependencies and other features of the kinetic data.

Data for extracts in the table reflect actual comparative rates of synthesis per milligram (or per cell) by the various strains. They do not therefore distinguish between the possibilities that increased rates may be attributable in part to increased enzyme content as well as to increased efficiency (higher V_{max} or lower K_m) or to reduced affinity (higher K_i) for an inhibitor. Nevertheless, the evidence though only partially quantitative seems to point to certain generalizations.

The high uninhibited rate of synthesis of folic acid compounds by the F_a and F_b enzymes seems to indicate primarily a high V_{max} for these enzymes together with a lesser change in K_m for *p*-aminobenzoic acid. It is noteworthy that these enzymes, even more than that from wild type, are efficient at the higher temperatures (25–50°) but relatively sluggish at lower temperatures. These efficiencies at higher temperatures seem to be reflected in the ability of genic factors *a* and *b* to improve the efficiency of *ad*, *db*, and *adb* enzymes slightly over that of the temperature-sensitive *d* enzyme (Fig. 4 and 5).

Although drug inhibition patterns have not been worked out for the F_b extracts, genic factor *a* brings about the expected resistance pattern in several cases.

The temperature sensitivity of synthesis consistently shown by extracts of the four *d*-containing strains and none of the four others suggests that a distinct modification of enzyme protein structure is brought about by the *d*-subgenic region.

The preliminary kinetic data and the results of Table I indicate that the *d* mutation results in an enzyme not only with a lowered affinity for *p*-aminobenzoic acid (high K_m) but also with a remarkably altered capability of binding inhibitor (much lower for sulfanilamide and higher for *p*-aminosalicylate). The low rate of production of folic acid is also mirrored in the V_{max} constant for the F_d strain.

It is interesting to note that, in parallel experiments with growing cultures of the various strains (all containing a similar amount of endogenous *p*-aminobenzoic acid) (Hotchkiss and Evans, 1960), the *d*-containing

mutants accumulated smaller amounts of folic acid than did the other strains. These findings fit the possibility that this enzyme system is the one limiting folic acid synthesis in the living cell.

The availability of these cell-free systems has revealed two unsuspected properties of all of the *d* enzyme systems—the relative heat sensitivity of their enzymatic reaction, and the incapability of the same systems to convert the substrate *p*-aminobenzoylglutamate to active product. This latter is an interesting example of an altered affinity involving a presumably normal substrate. These findings fit nicely with the hypothesis set forth by Hotchkiss and Evans (1960) concerning the way in which the subgenic region *d* modifies the *p*-aminobenzoic acid-utilizing enzyme. They inferred that this modification of the enzyme is not primarily responsive to changes in dissociation, electron distribution, or exact chemical nature of substituents at the site of the carboxyl group on the *p*-aminobenzoic acid molecule, but the *d* enzyme is considerably less able than others to react with—and therefore resistant toward—drugs bearing bulky constituents at this site. Apparently this restriction operates also toward the natural substrate bearing the bulky glutamyl moiety as an amide substituent upon the carboxyl group.

In conjunction with the previous studies upon whole cells, this investigation points to the wealth of information yet to be gained from this pneumococcal system. From the former studies, it was possible to draw certain rather precise conclusions concerning the specific reactivities of the various mutant forms of the *p*-aminobenzoic acid-utilizing enzyme toward spatially and chemically modified substrates, and some of these conclusions have already been corroborated with crude cell-free extracts. Further purification and eventual direct structural study of this enzyme would make possible a correlation of these specific affinities with molecular structure. The availability of eight closely related but different forms of the same enzyme makes this system particularly well suited for studying the mode of action of a protein catalyst.

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