Action of Substituted Phenylalanines on *Escherichia coli*

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(Received 17 June 1952)

In a previous communication (Volcani, Sicher & Lichtenstein, 1951) it had been shown that *o*-hydroxyphenylalanine (OHPA) is an antagonist of phenylalanine and that it possesses different modes of action for different strains of *Escherichia coli*: it was inactive in a wild type and a tyrosineless mutant, but inhibited competitively a phenylalanineless mutant. It was concluded, therefore, that the compound interferes with the utilization, and not with the synthesis, of phenylalanine.

In the present investigation, other substituted phenylalanines were studied, both as to the point at which they block the pathway of the synthesis and utilization of the aromatic amino-acids and as to the nature of the influence of structural variations on the phenylalanine molecule, such as would permit graded changes in its chemical and physico-chemical properties.

Particular interest attached to substituted phenylalanines because of their similarity to chloramphenicol and its active structural analogues (Bambas, Troutman & Long, 1950; Buu-Hoi, Hoán, Jacquignon & Khoi, 1950; Dann, Ulrich & Möller, 1950; Long & Troutman, 1951; Woolley, 1950; Truhaut, Lambin & Boyer, 1951).

The inhibition of *Esch. coli* by β -phenylserine which has previously been studied by Beerstecher & Shive (1946, 1947*a*) and Marnay (1951), has been shown by Woolley (1950) to apply also to the phenylalanineless mutant.

A recent paper by Maas & Davis (1950) and also the results recorded in the preceding communication (Volcani *et al.* 1951) have made it evident that when the antagonist-metabolite relation only is used in order to elucidate the point at which inhibition blocks biochemical reactions, the results are sometimes ambiguous. A clearer picture of the nature of the inhibition is obtained, as shown in the present study, by the additional use of mutants in which one particular mechanism is blocked genetically (Tatum, 1946).

For an evaluation of the results obtained by these two methods it is necessary to know the biogenetic relationships of the aromatic amino-acids. From the experiments of Beerstecher & Shive (1946, 1947*a, b*), the following 'straight' scheme suggests

itself for the wild type of *Esch. coli*: precursor $\rightarrow$ tryptophan $\rightarrow$ phenylalanine $\rightarrow$ tyrosine $\rightarrow$ metabolic product of tyrosine. On the other hand, a 'branched' scheme of the type

though somewhat more complex, has been proposed by Davis (1950, 1951) for *Esch. coli* and by Atkinson, Melvin & Fox (1951) for *Lactobacillus arabinosus*. In this scheme, the three aromatic amino-acids are formed by parallel but independent routes from a common precursor. A decision between the two alternative schemes seemed possible by the study of 'sparing effects'; according to the straight scheme, and only according to it, phenylalanine and tyrosine would be expected to show a sparing effect in a tryptophanless mutant and tyrosine, but not tryptophan in a phenylalanineless mutant, whilst for a tyrosineless mutant neither tryptophan nor phenylalanine should show sparing effects. These effects have been observed in *Esch. coli*, although Simmonds, Tatum & Fruton (1947) and Simmonds (1950) have failed to detect them in a phenylalanineless and a tyrosineless mutant of the bacterium.

Inhibitors of various steps of the straight scheme have been discovered in *p*-nitrophenylalanine, *p*-fluorophenylalanine and 4-hydroxy-3-nitrophenylalanine (*m*-nitrotyrosine), which were tested on *Esch. coli*, wild type, and its tryptophan-, phenylalanine- and tyrosine-less mutants, as well as on a mutant which required all three aromatic amino-acids. Certain substituted β -phenylserines, whilst having no inhibitory action by themselves, counteract the inhibition caused by the phenylalanine derivatives. From the experimental results it is concluded that *Esch. coli* possesses the ability to circumvent the steps of the suggested straight pathway by alternative pathways, which are not normally utilized by the micro-organism. It has further been shown that, in the mutants inhibited by the above-mentioned phenylalanine derivatives, the sparing effect of the aromatic amino-acids for each other is more pronounced than without such inhibition.

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EXPERIMENTAL

Cultures and media. Cultures used included: *Esch. coli*, parent wild type strain (ATCC 9637), *Esch. coli* tryptophanless mutant (19-2), *Esch. coli* phenylalanineless mutant (M83-5), *Esch. coli* tyrosineless mutant (M83-9) and *Esch. coli* triply deficient mutant (M83-2) (tryptophan + phenylalanine + tyrosine), all kindly supplied by Dr B. D. Davis. They were grown and assayed in the mineral medium of Davis & Mingoli (1950) as described previously. Media and experimental procedures were those employed in a preceding paper (Volcani *et al.* 1951). All experiments were carried out in duplicate.

For inoculum in the 'sparing' experiments, the surface growth from an 8 hr. culture was gently scraped off and suspended in sterile 0.9% NaCl solution to a turbidity reading of 56-60 on the Klett-Summerson photoelectric colorimeter using a no. 64 red filter. From this heavy suspension, 0.3 ml. was diluted into 10 ml. sterile saline; each experimental culture was inoculated with a drop of this dilute suspension. Final volume 5 ml. Cultures were incubated at 37° for 18 hr. and growth turbidity was determined as above.

In the inhibitor experiments, DL-*p*-aminophenylalanine (PAPA), DL-*p*-nitrophenylalanine (PNPA), L-4-hydroxy-3-nitrophenylalanine (MNT), *o*-hydroxyphenylalanine (OHPA), DL- β -phenylserine (PS), and DL-tryptophan, DL-phenylalanine and L-tyrosine were added to a series of test tubes containing 2.5 ml. of the double-strength medium. The cultures were diluted to 5 ml. with distilled water, and autoclaved at 15 lb. pressure for 15 min. DL-*p*-Fluorophenylalanine (PFPA), and the substituted β -phenylserines were added aseptically as aqueous solutions which had been filtered through a glass filter. The final volume used was 2.5 ml.

Materials. DL-*p*-Nitro- β -phenylalanine was prepared according to Erlenmeyer & Lipp (1883), DL-*p*-amino- β -phenylalanine according to Bergmann (1952). The former was recrystallized from *tert*.-butanol, m.p. 240-245° (decomp.) (Burckhalter & Stephens (1951): 222-240°), the latter from boiling water, m.p. 234° (decomp.) (after drying at 140° *in vacuo*) (Burckhalter & Stephens (1951): 235-242°).

DL-*p*-Fluoro- β -phenylalanine, which was obtained by the method of Bergmann (1952), crystallized from a mixture of water and ethanol (3:1) in needles of m.p. 238-242° (decomp.).

For the synthesis of L-4-hydroxy-3-nitro- β -phenylalanine, from water or glacial acetic acid, m.p. 225-230° (decomp.), the nitration of L-tyrosine (Staedeler, 1860; Johnson & Kohmann, 1915), was employed, and for the preparation of DL- β -phenylserine (*threo*) the method of the Gesellschaft für Kohlentechnik (1936) and of Carrara & Weitnauer (1949); from 50% ethanol, m.p. 196° (decomp.).

DL-*p*-Nitro- β -phenylserine, from water, m.p. 186° (decomp.), has been obtained according to Dalglish (1949) by hydrolysis of its esters (Bergmann, Genas & Bendas, 1950; Bergmann, Bendas & Taub, 1951); its hydrogenation (Dalglish, 1949) gave DL-*p*-amino- β -phenylserine, from water, m.p. 220° (decomp.).

DL-*m*-Nitro- β -phenylserine, from water, m.p. 208-210°, has been described before by Bergmann *et al.* (1950, 1951).

DL-*m*-Amino- β -phenylserine. In the manner prescribed for the *p*-compound (Dalglish, 1949), DL-*m*-nitro- β -phenylserine (1.82 g.) was hydrogenated in aqueous medium. The

filtered solution was evaporated to dryness *in vacuo* and the residue treated with aqueous ammonia solution and a little ethanol. Upon standing, the desired product separated. It was recrystallized from *tert*.-butanol or isopropanol, containing a little water; m.p. 154-157° (decomp.). (Found: C, 55.0; H, 6.1; N, 14.4. $C_9H_{11}O_3N_2$ requires C, 55.1; H, 6.1; N, 14.3%.)

RESULTS

*Sparing experiments**Tryptophanless mutant*

Response to DL-phenylalanine. Whilst the tryptophanless mutant failed to grow on phenylalanine alone, addition of increasing amounts of DL-phenylalanine to various levels of tryptophan caused an increase in growth; the combination of 12 μ g./ml. of DL-tryptophan with 40 μ g./ml. of DL-phenylalanine gave full growth (Fig. 1).

Fig. 1. Sparing effect of phenylalanine on tryptophan requirements of *Esch. coli*, tryptophanless mutant, at various tryptophan levels. Curve A, no tryptophan. Curves B-G, 0.4, 1, 2, 4, 12 and 20 μ g./ml. tryptophan, respectively.

Fig. 2. Sparing effect of tyrosine on tryptophan requirements of *Esch. coli*, tryptophanless mutant, at various tryptophan levels. Curve A, no tryptophan. Curves B-E, 1, 4, 12 and 20 μ g./ml. tryptophan, respectively.

Response to L-tyrosine. Effects are observed analogous to those with DL-phenylalanine. At the level of 12 μ g./ml. of DL-tryptophan, the addition of 20 μ g./ml. of L-tyrosine caused the growth to exceed the maximum growth possible with tryptophan alone (Fig. 2).

Phenylalanineless mutant

Response to DL-tryptophan. The phenylalanineless mutant could not utilize tryptophan in any concentrations up to 1000 $\mu\text{g./ml.}$ Addition of increasing amounts of DL-tryptophan to various levels of DL-phenylalanine had only a negligible effect. At initial growth levels of 38 and 50 %, 600 $\mu\text{g./ml.}$ of tryptophan caused a 7 % increase in growth.

Response to L-tyrosine. As shown in Fig. 3, the phenylalanineless mutant failed to grow in the presence of tyrosine alone (from 0 to 1000 $\mu\text{g./ml.}$). Addition of increasing amounts of L-tyrosine to various levels of phenylalanine, however, caused appreciable increases in growth. The sparing effect of tyrosine was most marked around an initial growth of 20 %, and it decreased with ascending growth levels, showing no effect above 70 % of full growth. At none of the phenylalanine concentrations studied, did addition of tyrosine cause full growth.

Fig. 3. Sparing effect of tyrosine on phenylalanine requirements of *Esch. coli*, phenylalanineless mutant, at various phenylalanine levels. Curve A, no phenylalanine. Curves B-G, 1, 2, 4, 10, 14 and 18 $\mu\text{g./ml.}$ phenylalanine, respectively.

Table 1. *Reversal, by the amino-acids DL-tryptophan, DL-phenylalanine and L-tyrosine, of the inhibition of Esch. coli, wild type, by various substituted phenylalanines*

(Full growth gives galvanometer reading of 98. Antibacterial index = ($\mu\text{g. inhibitor/ml.}$)/($\mu\text{g. amino-acid/ml.}$).)

A. DL-p-Aminophenylalanine (PAPA)				B. DL-p-Fluorophenylalanine (PFPA)			
Inhibitor ($\mu\text{g./ml.}$)	Amino-acid	Quantity required for 50% growth (galv. reading: 49) ($\mu\text{g./ml.}$)	Antibacterial index	Inhibitor ($\mu\text{g./ml.}$)	Amino-acid	Quantity required for 50% growth (galv. reading: 49) ($\mu\text{g./ml.}$)	Antibacterial index
Galv. reading without amino-acids for 1 $\mu\text{g./ml.}$ PAPA: 49 for 4 $\mu\text{g./ml.}$ PAPA: 0				Galv. reading without amino-acids for 3 $\mu\text{g./ml.}$ PFPA: 49 for 60 $\mu\text{g./ml.}$ PFPA: 0			
6	Tyrosine	0.18	33	4	Tyrosine	0.6	6.6
10	Tyrosine	0.32	31	40	Tyrosine	3.0	13.3
20	Tyrosine	0.50	40	4	Phenylalanine	0.7	8.0
6	Phenylalanine	0.72	7.6	40	Phenylalanine	3.9	10.2
10	Phenylalanine	1.30	7.7	4	Tryptophan	1.4	2.8
20	Phenylalanine	2.70	7.4	40	Tryptophan	*	—
6	Tryptophan	2.40	2.5				
10	Tryptophan	4.80	2.1				
20	Tryptophan	40.0	0.5				
C. DL-m-Nitrotyrosine (MNT)				D. β -Phenylserine (PS)			
Galv. reading without amino-acids for 120 $\mu\text{g./ml.}$ MNT: 49 for 400 $\mu\text{g./ml.}$ MNT: 0				Galv. reading without amino-acids for 560 $\mu\text{g./ml.}$ PS: 49 for 1000 $\mu\text{g./ml.}$ PS: 0			
200	Tyrosine	1.4	143	600	Tyrosine	2500	0.2
600	Tyrosine	9.4	64	1000	Tyrosine	1111	0.9
200	Phenylalanine	6	33	600	Phenylalanine	3000	0.2
600	Phenylalanine	460	1.3	1000	Phenylalanine	833	1.2
200	Tryptophan	0.7	285	600	Tryptophan	1500	0.4
600	Tryptophan	0.9	666	1000	Tryptophan	625	1.6

SUMMARY

Ratio antibact. index tyrosine:2 $\times$ antibact. index phenylalanine:2 $\times$ antibact. index tryptophan.† (Antibact. index tyrosine=1.)

Inhibitor	($\mu\text{g./ml.}$)	Ratio	Inhibitor	($\mu\text{g./ml.}$)	Ratio
PAPA	6	1:2:1:6.6	MNT	200	1:2:1:0.25
	10	1:2:0:7.5		600	1:24:4:0.05
	20	1:2:6:40.0		600	1:0:5:0.6
PFPA	4	1:0:4:1.1	PS	1000	1:0:75:0.56
	40	1:0:6:—			

* 200 $\mu\text{g./ml.}$ tryptophan restored growth to 20 % (galvanometer reading 20). Higher concentrations inhibited growth, fully at 2000 $\mu\text{g./ml.}$

† Ratio calculated on the basis of the L-isomer.

Tyrosineless mutant

Response to DL-tryptophan. The tyrosineless mutant did not utilize tryptophan alone in any concentration up to 1000 $\mu\text{g./ml.}$ Addition of graded amounts of DL-tryptophan (up to 1000 $\mu\text{g./ml.}$) at various levels of L-tyrosine (yielding 20 and 50 % of full growth) showed no effect on the growth of the organism.

Response to DL-phenylalanine. Phenylalanine was not utilized in concentrations up to 1000 $\mu\text{g./ml.}$ The addition of 600 $\mu\text{g./ml.}$ at different levels of tyrosine (giving 16, 25, 52 and 90 % of full growth) caused increases of 5–12 % at the lower growth levels only.

*Inhibition experiments**Response of Esch. coli, wild type*

The amounts of each of the phenylalanine derivatives and of β -phenylserine needed to reduce growth of *Esch. coli*, wild type, to 50 % of the maximum, or to cause complete inhibition, were determined from a dose-response curve. The reversal of growth inhibition by tryptophan, phenylalanine and tyrosine was tested at various inhibitory levels, and the antagonist ratio obtained from the corresponding growth curves. The antagonist ratios of the inhibiting compounds to the respective amino-acids ('antibacterial index') which permit half maximum growth, and the ratios of the concentrations of the aromatic amino-acids which also give 50 % growth, are summarized in Table 1.

p-Aminophenylalanine (PAPA) inhibits the wild strain of *Esch. coli*. All three aromatic amino-acids reverse the inhibition, but the amounts required for this purpose vary significantly, tyrosine being the most, tryptophan the least effective. This ratio points to the utilization of tyrosine as the step which is blocked by the inhibitor. In the case of

p-fluorophenylalanine (PFPA) and *m*-nitrotyrosine (MNT), which also have an inhibiting effect (PFPA of approximately the same order of magnitude as PAPA, MNT very much less), the ratio of the reversing activities of the three aromatic amino-acids is different; in the former case, phenylalanine, in the latter tryptophan proved the most effective. PFPA, undoubtedly, interferes with the utilization of phenylalanine, whilst MNT appears to block the synthesis of tryptophan. However, it will be shown later that this is not the only effect exerted by the anti-metabolite. Phenylserine (PS) also shows a significant inhibiting action only in concentrations of 500–1000 $\mu\text{g./ml.}$, and in the reversal of this effect, phenylalanine seems to be the most active of the three aromatic amino-acids.

None of the compounds *p*-nitrophenylalanine (PNPA), *p*-amino- β -phenylserine (PAPS), *p*-nitro- β -phenylserine (PNPS), *m*-amino- β -phenylserine (MAPS) and *m*-nitro- β -phenylserine (MNPS) affected growth, when added in quantities from 0 to 2000 $\mu\text{g./ml.}$

In this connexion it seemed interesting to test the claim of Beerstecher & Shive (1947*b*) that arginine, glutamic acid and cystine are precursors of a metabolite which is involved in the synthesis of phenylalanine. L-Arginine hydrochloride, L-glutamic acid and L-cystine, added in quantities up to 100 $\mu\text{g./ml.}$ to an inhibiting concentration of 10 $\mu\text{g./ml.}$ of *p*-aminophenylalanine, were completely inactive.

Response of the tryptophanless mutant

The action of PAPA, PFPA and β -phenylserine (PS) on this mutant and the antagonist:tryptophan ratios are summarized in Table 2. PFPA had a much stronger inhibiting effect than PAPA, and the inhibiting action of PS is weak. The other substances investigated showed no effect on the tryptophanless mutant.

Table 2. *Inhibition of Esch. coli, tryptophanless mutant, by substituted phenylalanines, and its reversal by DL-tryptophan*

(Maximum growth, with tryptophan alone, gives galv. reading of 85. Antibacterial index = ($\mu\text{g. inhibitor/ml.}$) / ($\mu\text{g. amino-acid/ml.}$)).

Inhibitor	($\mu\text{g./ml.}$)	Tryptophan ($\mu\text{g./ml.}$)	Galvanometer reading	Antibacterial index at galv. reading 42.5 (50 % of full growth)
None	—	1.2	28	—
PAPA	20	1.2	14	—
	16	3.2	42.5	5
	40	20	42.5	2
	400	No reversion	—	—
PFPA	4	1.2	14	—
	4	40	42.5	0.1
	20	No reversion	—	—
PS	1200	1.2	14	—
	400	1.0	42.5	400
	1200	3.0	42.5	400
	2000	10.0	42.5	200

Response of the phenylalanineless mutant

The mutant could not utilize *p*-aminophenylalanine (PAPA) in amounts up to 600 $\mu\text{g./ml.}$ instead of phenylalanine.

Fig. 4. Effect of phenylalanine and *p*-aminophenylalanine (PAPA) on growth of *Esch. coli*, phenylalanineless mutant. Curve A, phenylalanine alone. Curves B-E obtained with 400, 600, 800 and 1000 $\mu\text{g./ml.}$ PAPA, respectively. Incubated 18 hr. at 37°.

Fig. 4 shows the effect of increasing amounts of DL-phenylalanine on various inhibiting levels of PAPA. At 400 $\mu\text{g./ml.}$ PAPA, 2 $\mu\text{g./ml.}$ of phenylalanine restored growth to the level caused by phenylalanine alone. Above that concentration, phenylalanine was synergic in increasing the inhibition. This effect was enhanced with increasing

concentrations of phenylalanine. For inhibiting concentrations of PAPA, PFPA, and PS and the antagonist:phenylalanine ratios see Table 3. No effect was observed when *p*-nitrophenylalanine (PNPA) and *m*-nitrotyrosine (MNT) were added at 30 % of full growth (4 $\mu\text{g./ml.}$ DL-phenylalanine).

Response of the tyrosineless mutant

The actions of PAPA, PNPA, PFPA and MNT are summarized in Table 4. The organism could not effectively utilize PAPA (up to 2000 $\mu\text{g./ml.}$) instead of tyrosine. In the presence of tyrosine, however, growth of the mutant was enhanced by low concentrations of PAPA, while higher ones caused inhibition.

In contradistinction to *Esch. coli*, wild type, and its phenylalanineless mutant, the growth of the tyrosineless mutant was stimulated by low concentrations, and inhibited by high concentrations, of PNPA. The mutant could not, however, utilize effectively PNPA (up to 800 $\mu\text{g./ml.}$) alone in place of tyrosine.

It should be noted that for equal inhibition, much larger quantities of PAPA are required by the tyrosineless mutant than by the phenylalanineless one, the tryptophanless one and the wild type. This fact gives a lead as to the point of attack of the inhibitor.

None of the four β -phenylserine derivatives affected growth of the mutant.

Response of the triply deficient mutant

In the triply deficient mutant strain M83-2 the relationship between the individual aromatic amino-acids and the antagonists could be studied

Table 3. *Inhibition of Esch. coli, phenylalanineless mutant, by substituted phenylalanines, and its reversal by DL-phenylalanine*

(Maximum growth, with phenylalanine alone, gives galv. reading of 92. Antibacterial index = ($\mu\text{g. inhibitor/ml.}$) / ($\mu\text{g. amino-acid/ml.}$)).

Inhibitor	($\mu\text{g./ml.}$)	Phenylalanine ($\mu\text{g./ml.}$)	Galvanometer reading	Antibacterial index at galv. reading 46 (50 % of full growth)
None	—	2	17	—
	—	4	24	—
	—	6	46	—
	—	8	51	—
PAPA	110	8	26	—
	600	8	0	—
	400	3.0	46	133
	600	3.2	46	187
	800	3.2	46	250
	1000	3.2	46	312
PFPA	36	6	23	—
	1000	6	0	—
	60	9.6	46	6.3
	100	16.0	46	6.3
	200	25.6	46	7.8
PS	600	4	12	—
	600	11.0	46	55
	2000	13.0	46	150

Table 4. *Inhibition of Esch. coli tyrosineless mutant, by substituted phenylalanines, and its reversal by L-tyrosine*

(Maximum growth, with tyrosine alone, gives galv. reading of 92. Antibacterial index = ($\mu\text{g. inhibitor/ml.}$)/($\mu\text{g. amino-acid/ml.}$)).

Inhibitor	($\mu\text{g./ml.}$)	Tyrosine ($\mu\text{g./ml.}$)	Galvanometer reading	Antibacterial index at galv. reading 46 (50% of full growth)
None	—	2	23	—
PAPA	900	2	11.5	—
	1600	2	0	—
	1000	3.8	46	263
	1600	9.8	46	163
	2000	11.6	46	172
PNPA	1300	2	11.5	—
	2000	2	0	—
	1000	3.2	46	312
	2000	5.6	46	357
	4000	12.5	46	320
MNT	490	2	11.5	—
	600	2	0	—
	600	6.2	46	97
	1000	52.0	46	17
	1600	480.0	46	3.5

more specifically, by keeping constant two of the required acids and varying the third together with the given antagonist.

p-Aminophenylalanine. Low concentrations of PAPA decrease only slightly the growth obtained with 20 $\mu\text{g./ml.}$ each of DL-tryptophan and DL-phenylalanine and 1 $\mu\text{g./ml.}$ of L-tyrosine. The effect becomes marked above 400 $\mu\text{g./ml.}$ PAPA and inhibition is complete at 2000 $\mu\text{g./ml.}$ of the antagonist; the inhibition is reversed by tyrosine (at 2000 $\mu\text{g./ml.}$ PAPA by 40 $\mu\text{g./ml.}$).

When the organism is grown in the presence of 20 $\mu\text{g./ml.}$ each of DL-tryptophan and L-tyrosine and of 2 $\mu\text{g./ml.}$ of DL-phenylalanine, the inhibiting effect of PAPA becomes marked at 120 $\mu\text{g./ml.}$ and is complete at 200 $\mu\text{g./ml.}$ Relatively small doses of phenylalanine (8 $\mu\text{g./ml.}$ at 120 $\mu\text{g./ml.}$ PAPA) bring the level back to about 70% of full growth; for complete reversal much larger quantities (40 $\mu\text{g./ml.}$) of phenylalanine are necessary.

p-Fluorophenylalanine. In the presence of 20 $\mu\text{g./ml.}$ of DL-tryptophan, 20 $\mu\text{g./ml.}$ L-tyrosine and 2 $\mu\text{g./ml.}$ DL-phenylalanine, already low concentrations of PFPA (8 $\mu\text{g./ml.}$) bring about complete inhibition. This is fully reversed by 20 $\mu\text{g./ml.}$ of phenylalanine. As in the phenylalanineless mutant, the antagonism is competitive and indicates, thus, that the inhibitor affects the utilization of phenylalanine.

o-Hydroxyphenylalanine. This compound was about half as active an inhibitor of PFPA under the same conditions of growth.

m-Nitrotyrosine. Whilst 120 $\mu\text{g./ml.}$ of MNT caused a 50% reduction in growth rate in the presence of 20 $\mu\text{g./ml.}$ of tryptophan, 20 $\mu\text{g./ml.}$ of phenylalanine and 1 $\mu\text{g./ml.}$ of tyrosine, for 75 and

100% inhibition, 400 and 1200 $\mu\text{g./ml.}$, respectively, were required. This inhibition is reversed by L-tyrosine. The behaviour of the triply deficient mutant towards *m*-nitrotyrosine thus resembles that of the tyrosineless mutant of *Esch. coli*.

Reversal of PAPA, PFPA, MNT and PS inhibition of Esch. coli, wild type, by PAPS, PNPS, MAPS and MNPS

The various compounds (PAPS, PNPS, MAPS and MNPS) were added in amounts varying from 0 to 1000 $\mu\text{g./ml.}$ to the basal medium, containing quantities of the antagonists which gave inhibition levels of 40–70% ('lower') and 90–100% ('higher'), respectively. Growth response curves were drawn; the maximal effective quantities of 'the anti-antagonist' that reversed the inhibition at both levels are summarized in Table 5.

It was shown that both *m*-amino- and *m*-nitro-phenylserine counteract the inhibition of the wild type *Esch. coli*, which is caused by PAPA, PFPA, MNT and PS, whilst *p*-aminophenylserine was less effective and the corresponding *p*-nitro compound entirely devoid of any activity.

In a number of systems studied, involving the inhibited phenylalanineless and tyrosineless mutants of *Esch. coli*, practically no reversal of the above type was observed.

Table 6, which summarizes the experimental results and includes also the previously (Volcani *et al.* 1951) reported data for *o*-hydroxyphenylalanine, illustrates the fact that a seemingly slight variation in the structure of the substituted phenylalanines investigated, changes the point of attack of these compounds; it gives examples of almost every possible combination of effects.

Table 5. Comparative effectiveness of p-aminophenylserine (PAPS) p-nitrophenylserine (PNPS), m-aminophenylserine (MAPS) and m-nitrophenylserine (MNPS), in overcoming the inhibition of *Esch. coli*, wild type, by p-aminophenylalanine (PAPA), p-fluorophenylalanine (PFPA), m-nitrotyrosine (MNT) and β -phenylserine (PS)

A1. PAPS v. PAPA			A2. PNPS v. PAPA		
PAPA ($\mu\text{g./ml.}$)	PAPS ($\mu\text{g./ml.}$)	Galv. reading	PAPA ($\mu\text{g./ml.}$)	PNPS ($\mu\text{g./ml.}$)	Galv. reading
8	—	60	8	—	53
60	—	16	60	—	15
8	600	72	8	200-1000	60
8	1000	65	60	0-1000	15
60	200-600	22			
60	1000	16			
A3. MAPS v. PAPA			A4. MNPS v. PAPA		
PAPA ($\mu\text{g./ml.}$)	MAPS ($\mu\text{g./ml.}$)	Galv. reading	PAPA ($\mu\text{g./ml.}$)	MNPS ($\mu\text{g./ml.}$)	Galv. reading
8	—	50	10	—	46
40	—	12	40	—	18
8	60	81	10	1000	91
8	100	86	40	1000	40
40	1000	70			
B1. PAPS v. PFPA			B2. PNPS v. PFPA		
PFPA ($\mu\text{g./ml.}$)	PAPS ($\mu\text{g./ml.}$)	Galv. reading	PFPA ($\mu\text{g./ml.}$)	PNPS ($\mu\text{g./ml.}$)	Galv. reading
4	—	34	4	—	36
40	—	3	40	—	0
4	1000	86	4	0-1000	36
40	0-1000	3	40	0-1000	6
B3. MAPS v. PFPA			B4. MNPS v. PFPA		
PFPA ($\mu\text{g./ml.}$)	MAPS ($\mu\text{g./ml.}$)	Galv. reading	PFPA ($\mu\text{g./ml.}$)	MNPS ($\mu\text{g./ml.}$)	Galv. reading
4	—	43	4	—	42
40	—	0	40	—	0
4	1000	88	4	1000	90
40	60-1000	10-14	40	0-1000	0
C1. PAPS v. MNT			C2. PNPS v. MNT		
MNT ($\mu\text{g./ml.}$)	PAPS ($\mu\text{g./ml.}$)	Galv. reading	MNT ($\mu\text{g./ml.}$)	PNPS ($\mu\text{g./ml.}$)	Galv. reading
100	—	58	100	—	53
200	—	10	200	—	7
100	1000	72	100	1000	7
200	10-1000	16	200	0-1000	7
C3. MAPS v. MNT			C4. MNPS v. MNT		
MNT ($\mu\text{g./ml.}$)	MAPS ($\mu\text{g./ml.}$)	Galv. reading	MNT ($\mu\text{g./ml.}$)	MNPS ($\mu\text{g./ml.}$)	Galv. reading
100	—	53	100	—	56
200	—	7	200	—	9
100	1000	92	100	1000	85
200	1000	83	200	1000	62
D1. PAPS v. PS			D2. PNPS v. PS		
PS ($\mu\text{g./ml.}$)	PAPS ($\mu\text{g./ml.}$)	Galv. reading	PS ($\mu\text{g./ml.}$)	PNPS ($\mu\text{g./ml.}$)	Galv. reading
600	—	43	600	—	37
1000	—	0	1000	—	0
600	600-1000	90	600	0-1000	37
1000	1000	25	1000	0-1000	0
D3. MAPS v. PS			D4. MNPS v. PS		
PS ($\mu\text{g./ml.}$)	MAPS ($\mu\text{g./ml.}$)	Galv. reading	PS ($\mu\text{g./ml.}$)	MNPS ($\mu\text{g./ml.}$)	Galv. reading
600	—	33	600	—	32
1000	—	0	1000	—	0
600	600-1000	91	600	200-1000	98
1000	0-40	0	1000	0-100	0
1000	1000	90	1000	600-1000	84

Table 6. *Effect of various substituted phenylalanines and phenylserine on growth of Esch. coli and its mutants*

Compound	Wild type	Tryptophanless mutant	Phenylalanineless mutant	Tyrosineless mutant	Triply deficient mutant		
					Tryptophan	Phenylalanine	Tyrosine
<i>p</i> -Aminophenylalanine (PAPA)	+	+	+	+	+	+	+
<i>p</i> -Nitrophenylalanine (PNPA)	-	-	+	+	-	-	+
<i>p</i> -Fluorophenylalanine (PFPA)	+	+	+	-	+	+	-
<i>m</i> -Nitrotyrosine (MNT)	+	-	-	+	-	-	+
<i>o</i> -Hydroxyphenylalanine (OHPA)	-	-	+	-	-	+	-
β -Phenylserine (PS)	+	+	+	-	+	+	-

-, no effect; +, inhibition; * growth enhancement at low concentrations of the compound in the presence of tyrosine.

'Sparing effects' in the inhibited mutants†

Tryptophanless mutant, inhibited by p-aminophenylalanine (PAPA)

Sparing effect of phenylalanine on tryptophan. The organism was grown in the presence of 4 μ g./ml. of tryptophan and inhibited by 40 and 200 μ g./ml.,

Fig. 5. Reversal of *p*-aminophenylalanine-inhibited phenylalanineless *Esch. coli* by DL-tryptophan. Curve A, growth in the presence of phenylalanine. Curve B, growth in the presence of phenylalanine and 400 μ g./ml. PAPA. Curve C, addition of 100-600 μ g./ml. of tryptophan to 400 μ g./ml. PAPA at 8 μ g./ml. of phenylalanine. Curve D, addition of 600 μ g./ml. of tryptophan to 400 μ g./ml. PAPA at 14 μ g./ml. of phenylalanine. Incubated 18 hr. at 37°.

respectively, of PAPA (70 and 100% inhibition, respectively). 20 and 200 μ g./ml., respectively, of phenylalanine reversed the inhibition to 75% of full growth.

† Percentages of growth given in this section relate to maximum growth obtained with designated quantities of substrates.

Sparing effect of tyrosine on tryptophan. When the organism was grown under the same conditions as in the previous paragraph, addition of 4 μ g./ml. of tyrosine at 80, and of 8 μ g./ml. of tyrosine at 200 μ g./ml. PAPA restored 75% of the full growth.

Fig. 6. Reversal of *p*-aminophenylalanine-inhibited phenylalanineless *Esch. coli* by L-tyrosine. Curve A, growth in the presence of phenylalanine. Curves B and C, growth in the presence of phenylalanine with 400 and 1000 μ g./ml. PAPA, respectively. Curve D, addition of 8 μ g./ml. of tyrosine to 400 μ g./ml. PAPA at 8 μ g./ml. of phenylalanine. Curves E, E' and E'', addition of 1, 2 and 20 μ g./ml. of tyrosine, respectively, to 400 μ g./ml. PAPA at 14 μ g./ml. of phenylalanine. Curve F, addition of 0.6 μ g./ml. of tyrosine to 1000 μ g./ml. PAPA at 8 μ g./ml. of phenylalanine. Curve G, addition of 6 μ g./ml. tyrosine to 1000 μ g./ml. PAPA at 20 μ g./ml. of phenylalanine. Incubated 18 hr. at 37°.

Phenylalanineless mutant, inhibited by p-aminophenylalanine (PAPA)

Sparing effect of tryptophan on phenylalanine. In the presence of 8 and 14 μ g./ml. phenylalanine, addition of 400 μ g./ml. PAPA decreased the growth

by 70 and 86 %, respectively. Addition of increasing amounts of DL-tryptophan gradually reversed the inhibition to 90 and 60 %, respectively, of full growth. The effects are schematically presented in Fig. 5.

Sparing effect of L-tyrosine on phenylalanine. The mutant was grown in the presence of 8 and 14 $\mu\text{g./ml.}$ phenylalanine and 400 $\mu\text{g./ml.}$ PAPA, which permitted only 10 and 27 % growth, respectively. When increasing amounts of L-tyrosine were added at these two levels of inhibition, growth was restored from 27 to 100 % by 8 $\mu\text{g./ml.}$ tyrosine, and from 10 to 95 % by 2 $\mu\text{g./ml.}$ tyrosine. Fig. 6 summarizes all the data obtained.

Tyrosineless mutant, inhibited by PAPA

Sparing effect of tryptophan on tyrosine. In the presence of 8 and 12 $\mu\text{g./ml.}$ tyrosine and 1600 $\mu\text{g./ml.}$ PAPA, DL-tryptophan, added in a wide range of concentrations (up to 1000 $\mu\text{g./ml.}$), was without effect.

Fig. 7. Reversal of *p*-aminophenylalanine-inhibited tyrosineless *Esch. coli* by DL-phenylalanine. Curve A, growth in the presence of tyrosine. Curve B, growth in the presence of tyrosine with 1600 $\mu\text{g./ml.}$ PAPA. Curves C and C', addition of 12 and 20 $\mu\text{g./ml.}$ of phenylalanine, respectively, to 1600 $\mu\text{g./ml.}$ PAPA at 2 $\mu\text{g./ml.}$ of tyrosine. Curves D and D', addition of 0.8 and 2-20 $\mu\text{g./ml.}$ of phenylalanine, respectively, to 1600 $\mu\text{g./ml.}$ PAPA at 7 $\mu\text{g./ml.}$ of tyrosine. Curve E addition of 0.6-20 $\mu\text{g./ml.}$ of phenylalanine to 1600 $\mu\text{g./ml.}$ PAPA at 12 $\mu\text{g./ml.}$ of tyrosine. Incubated 18 hr. at 37°.

Sparing effect of phenylalanine on tyrosine. The organism was grown in the presence of 2, 7 and 12 $\mu\text{g./ml.}$ tyrosine and inhibited with 1600 $\mu\text{g./ml.}$ PAPA. As schematically shown in Fig. 7, the inhibition was completely reversed by phenylalanine in all cases, the quantities required being 12, 0.8 and 0.6 $\mu\text{g./ml.}$ respectively. Addition of phenylalanine above the concentrations required for complete reversal increased the growth response at low

inhibiting levels still further. The effect is most noticeable at 7 $\mu\text{g./ml.}$ tyrosine, where addition of 2 $\mu\text{g./ml.}$ phenylalanine stimulated growth by 20 % above maximum growth at that tyrosine level—a level which could be reached only by addition of 10 $\mu\text{g./ml.}$ tyrosine.

Other inhibitors

In view of the above results, it seemed interesting to study the effect of known inhibitors on the utilization of phenylalanine and tyrosine by the tryptophanless mutant, of tryptophan and tyrosine by the phenylalanineless mutant, and of phenylalanine by the tyrosineless mutant. Experiments with the latter mutant and tryptophan were not carried out, as this amino-acid had proved inactive in the above experiments with PAPA.

Effect of p-fluorophenylalanine (PFPA) on the utilization of phenylalanine and tyrosine by the tryptophanless mutant. Both at 10 % of full growth and complete inhibition (8 and 20 $\mu\text{g./ml.}$ of PFPA, in presence of 4 $\mu\text{g./ml.}$ of tryptophan), DL-phenylalanine in quantities of 4 and 8 $\mu\text{g./ml.}$ respectively, gives 85 % of full growth. Even quantities of 2000 $\mu\text{g./ml.}$ do not restore full growth. Very similar effects were observed in parallel experiments with L-tyrosine: 2 and 20 $\mu\text{g./ml.}$ of tyrosine restored full growth at the two levels, respectively.

Effect of PFPA on the utilization of tryptophan and tyrosine by the phenylalanineless mutant. When the growth of the mutant (with 8 $\mu\text{g./ml.}$ phenylalanine) was reduced to 35 % of full growth by 80 $\mu\text{g./ml.}$ PFPA, addition of 200 and 600 $\mu\text{g./ml.}$ of DL-tryptophan increased growth very slightly, i.e. by 7 and 12 %, respectively (as compared to 3 and 7 % increase obtained with tryptophan alone in the above concentrations). Addition of up to 60 $\mu\text{g./ml.}$ tyrosine showed no effect on the growth of the organism; 100-200 $\mu\text{g./ml.}$ tyrosine increased growth by 9 %; at 600 $\mu\text{g./ml.}$ this effect dropped slightly (to 5 %).

Effect of β -phenylserine (PS) on the utilization of tryptophan and tyrosine by the phenylalanineless mutant. In these experiments, too, at 35 % of full growth (10 $\mu\text{g./ml.}$ phenylalanine and 1000 $\mu\text{g./ml.}$ PS), addition of DL-tryptophan or L-tyrosine in a wide range of concentrations (up to 600 $\mu\text{g./ml.}$), did not affect the growth of the organism.

Effect of m-nitrotyrosine (MNT) on the utilization of phenylalanine by the tyrosineless mutant. In the presence of 8 $\mu\text{g./ml.}$ L-tyrosine and 600 $\mu\text{g./ml.}$ MNT (52 % of full growth), addition of increasing amounts of DL-phenylalanine (up to 100 $\mu\text{g./ml.}$) caused no response; at phenylalanine levels of 200-1000 $\mu\text{g./ml.}$, a 10 % increase of growth was observed. Similar stimulation is caused by 60-1000 $\mu\text{g./ml.}$ phenylalanine in the uninhibited mutant.

DISCUSSION

In the tyrosineless mutant, the addition of tryptophan was without effect, and that of phenylalanine was practically negligible. In the phenylalanineless mutant, however, a sparing effect of tyrosine, and in the tryptophanless mutant a pronounced synergism, if not a sparing effect of both phenylalanine and tyrosine has been demonstrated. It may be emphasized that these effects are evident only under certain well-specified conditions, and this may be the reason why Simmonds *et al.* (1947) failed to observe them in their experiments. They are compatible only with a straight scheme, not, at least for the mutants used, with a branched one (see p. 1). In this connexion, it may be recalled that in higher animals the conversion of phenylalanine to tyrosine has been demonstrated beyond doubt (Moss & Schoenheimer, 1940; Womack & Rose, 1946; Jervis, 1947; Grau, 1947; Lerner, 1949; Udenfriend, Cooper & Clark, 1951).

It may well be possible that, in general, the relationship between the aromatic amino-acids cannot be described by a single scheme. The following discussion, however, is based on the straight scheme; it is assumed that a compound which inhibits the 'A'-requiring mutant in a competitive manner, does so because it affects the utilization of the metabolite 'A'.

p-Aminophenylalanine (PAPA). PAPA inhibits competitively the utilization of tyrosine; replacement of the hydroxyl group of tyrosine by the amino group evidently brings about an increased affinity of the phenylalanine molecule for the enzyme concerned. One may also conclude that tryptophan and phenylalanine reverse the effect, in the wild strain, due to their conversion into tyrosine; the difference in the effectiveness of the three amino-acids is, then, understandable from their increasing distance from the point of inhibition. The parent strain is much more sensitive to the inhibitor than the mutants. This is to be expected, as the inhibition is dependent upon the ratio of metabolite to anti-metabolite. In the parent strain, the amino-acids are probably utilized about as quickly as they are formed, and do not accumulate; in the mutant, on the other hand, one has to administer the preformed amino-acid and this increases the metabolite: anti-metabolite ratio. Similar observations have been made by Volcani & Snell (1948) in the case of the canavanine inhibition of arginine-requiring micro-organisms.

In accordance with this picture, the quantity of PAPA necessary for an equal inhibition of the tyrosineless mutant is greater than that needed for the phenylalanineless mutant, the tryptophanless mutant, or the wild type.

The results agree with those of Burckhalter &

Stephens (1951) and of Frieden, Stansel & Dittmer (1951), but not with the earlier claim of Elliot, Fuller & Harington (1948) that PAPA inhibits *Esch. coli* only in concentrations of 200 $\mu\text{g./ml.}$

In contrast to the aromatic amino-acids, L-arginine, L-cystine and L-glutamic acid do not relieve the inhibition of *Esch. coli* by PAPA. It is thus not possible to assume, as Beerstecher & Shive (1947*b*) have done, that these amino-acids are precursors of a metabolite which functions in the biosynthesis of phenylalanine, unless one wishes to assume that PAPA, in addition to the effect discussed here, also interferes with the conversion of these aliphatic amino-acids into phenylalanine.

p-Nitrophenylalanine (PNPA). The competitive inhibition of the tyrosineless mutant by this substance proves that the point of attack is the utilization of tyrosine. The lack of response to PNPA, shown by the wild type of *Esch. coli* and by its other mutants, must then be explained by the assumption that these strains are capable of synthesizing the metabolic product of tyrosine by a pathway which circumvents tyrosine, e.g. directly from phenylalanine.

The experiments permit the further conclusion that the wild strain, the phenylalanineless, the tryptophanless and the triply deficient mutant, at least, do not reduce PNPA to PAPA (as the latter inhibits these strains). This difference between the two substituted phenylalanines is interesting in connexion with the observation (Lampen & Peterson, 1943; Housewright & Koser, 1944) that in *Clostridium acetobutylicum*, *p*-nitrobenzoic acid replaces *p*-aminobenzoic acid as a growth factor. Experiments with PNPA also have been reported by Woolley (1950), by Burckhalter & Stephens (1951) and by Frieden *et al.* (1951).

p-Fluorophenylalanine (PFPA). This compound acts on the utilization of phenylalanine, as indicated by the fact that these two substances show a competitive antagonism in the phenylalanineless and the triply deficient mutants and that, in the wild strain, the reversal of the PFPA inhibition is most effective with phenylalanine. Similarly, recent experiments with PFPA have shown it to interfere with phenylalanine metabolism in rats (Armstrong & Lewis, 1951*a*), *Lactobacillus arabinosus* (Atkinson *et al.* 1951), *Pseudomonas aeruginosa* (Martin & Mon, 1949) and *Saccharomyces cerevisiae* (Armstrong & Lewis, 1951*b*).

Substitution of a hydrogen atom by fluorine produces an inhibitor of the reaction through which this hydrogen atom is replaced by hydroxyl. In this connection, it is recalled that *p*-fluorobenzoic acid also inhibits *Esch. coli* and that this inhibition is reversed by tyrosine (Sveinbjornsson & Vander Werf, 1951). The effect of fluorine substitution has

been studied extensively in the case of fluoroacetic acid (Chenoweth, 1949) and fluorocitric acid (Martius, 1949; Liebecq & Peters, 1949; Buffa & Peters, 1949; Buffa, Peters & Wakelin, 1951), and also in that of 4-amino-2-fluorobenzoic acid (Wyss, Rubin & Strandskov, 1943); it has been shown that these compounds are incorporated by the living cell instead of the corresponding fluorine-free substances. One may, therefore, well assume that *p*-fluorophenylalanine, too, might be incorporated by *Esch. coli* in metabolites which are normally built up from phenylalanine.

m-Nitrotyrosine (MNT). In the tyrosineless mutant, this substance affects the utilization of tyrosine; this is also evident from the inhibition of the triply deficient mutant and its reversal by tyrosine. This inhibition may also occur in the wild strain, but there it cannot be the only effect of the antagonist, since tryptophan is the most efficient of the aromatic amino-acids in reversing the inhibition. One must, therefore, conclude that the synthesis of tryptophan also is affected by MNT, an effect which can, of course, not be detected in the mutants which require preformed tryptophan, phenylalanine or tyrosine, respectively. The fact that the tryptophanless and the phenylalanineless mutants of *Esch. coli* remain unaffected by MNT, then tends to show that they can produce the metabolic product of tyrosine by a pathway which circumvents tyrosine and is not blocked by MNT.

o-Hydroxyphenylalanine (OHPA). The point of attack which the experiments with this substance have revealed (Volcani *et al.* 1951) is the utilization of phenylalanine, i.e. the synthesis of tyrosine from that amino-acid. The fact that the wild strain and the tryptophanless mutant do not respond to OHPA proves that these organisms possess a way of producing tyrosine by a route which by-passes the phenylalanine stage, e.g. directly from tryptophan. The triply deficient mutant, on the other hand, is inhibited, and addition of phenylalanine reverses the effect.

DL- β -Phenylserine (PS). Beerstecher & Shive (1946) have found that β -phenylserine inhibits *Esch. coli*, wild type, and that this inhibition is counteracted competitively by phenylalanine and one-tenth as effectively by tryptophan, whilst tyrosine was reported to be completely inactive (Beerstecher & Shive, 1947a). With the strain used in the present investigation (see Volcani *et al.* 1951), the position is somewhat different. The ratios of tryptophan:phenylalanine:tyrosine tend to indicate that the point of attack of β -phenylserine lies at the phenylalanine-tyrosine reaction.

Woolley (1950) has also observed that the phenylalanineless mutant (the same as used in the present investigation) is competitively inhibited by PS. His claim, that, at certain concentrations, the sub-

stance stimulated the growth of the mutant, could not be confirmed.

PS resembles *p*-fluorophenylalanine also in its inertness towards the tyrosineless mutant; the point of attack appears, in both cases, to be phenylalanine. Chemically speaking, the substitution at the β position of phenylalanine by the hydroxyl group has an effect analogous to the *p*-substitution by a fluorine atom.

Nitro- and amino-substituted β -phenylserine. These have no effect on either the wild strain of *Esch. coli* or its mutants. With regard to the *p*-nitro compound, the results disagree with those reported by Woolley (1950); this may be due to the fact that Woolley's compound possesses the *threo*-, the one used here the *erythro*-configuration (see Kopp, Larramona & Welvert, 1951; Holland & Nayler, 1952).

This discussion can be summarized in the following extension of the straight scheme, in which the crossed arrows indicate the point of attack of the inhibitor, and the dotted lines the alternative pathways of synthesis which the present investigation has suggested:

The experiments reported here cannot be fitted into any 'branched' scheme, in which the aromatic amino-acids derive independently from a common precursor.

It is noteworthy that the phenylserine derivatives studied, in spite of their inactivity towards *Esch. coli*, are not completely devoid of biochemical potentialities. Table 5 shows very strikingly for the wild type that both *m*-amino- and *m*-nitro- β -phenylserine counteract the inhibition of growth caused by PAPA, PFPA, MNT and PS. *p*-Aminophenylserine was less and *p*-nitrophenylserine not at all effective. Divatia, Gardner, Dufrenoy & Pratt (1951) reported a similar antagonistic relation between *p*-aminosalicylic acid and streptomycin in a streptomycin-resistant strain of *Esch. coli*.

As yet, any attempt to explain the effects observed in these already fairly complex systems must be speculative. Formally, either of two

mechanisms could be responsible for these effects (making the reasonable assumption that the inhibitory substances act by occupying the active spot on the surface of an enzyme involved): (a) the anti-antagonist withdraws the antagonist, and thus makes the active centres available for the natural substrate (one may think of formation of molecular compounds between antagonist and anti-antagonist), (b) the anti-antagonist lays bare masked active centres and thus creates new points of attack for the substrate.

The picture would be much clearer if it were possible to correlate the biological relationships observed with the chemical structures of the compounds concerned. An attempt at doing this seems still premature. However, the study of the sparing effects in the inhibited mutants shed some further light on the intricate situation which appears to exist.

The PFP- and PS-inhibited phenylalanineless mutant hardly responded to tryptophan or tyrosine; on the contrary, PFP- and PS suppress even the sparing effect of tyrosine on phenylalanine, which had been observed in the uninhibited mutant. Whilst MNT did not stimulate the utilization of phenylalanine by the tyrosineless mutant, the sparing effect was quite pronounced in the PFP-inhibited tryptophanless mutant. *p*-Aminophenylalanine (PAPA), on the other hand, stimulates the utilization, in the phenylalanineless mutant, of tryptophan and increases the sparing effect of tyrosine; in the tyrosineless mutant, it causes utilization of phenylalanine exceeding that of tyrosine. Similar effects were observed in the tryptophanless mutant.

This stimulating effect may be related to the fact, previously established, that PAPA, in certain concentrations, stimulates the growth of the tyrosineless mutant. On the other hand, the unusually high utilization of phenylalanine by the inhibited tyrosineless mutant may be due to the opening of a pathway in which phenylalanine is converted directly, not via tyrosine, into the tyrosine metabolite. The possibility of such a detour has been concluded above (p. 10) from experiments with *p*-nitrophenylalanine and *Esch. coli*, wild type, and its phenylalanineless mutant.

This hypothesis is not contradicted by the observation that tryptophan is more effective in the PAPA-inhibited phenylalanineless, than in the PAPA-inhibited tyrosineless mutant. The difference may be simply due to the fact that the way from tryptophan to phenylalanine is shorter than that to tyrosine.

The specific stimulating effect of PAPA, described above, adds a further feature to the picture of this compound, of which two properties have been reported previously: its ability to replace *p*-aminobenzoic acid completely in *Clostridium acetobuty-*

licum (Weizmann, 1949) and partially in the PABA-requiring mutant of *Esch. coli*, in *Lb. arabinosus*, and *Acetobacter suboxydans* (Volcani, Ben-Ishai & Bergmann, 1952), and its ability to inhibit both *Esch. coli*, wild type, and its phenylalanineless and tyrosineless mutants (see p. 10).

The results reported can be described as activation of the cellular faculties by inhibitors. The observation of Aoki, Tanemori & Noda (1951) that traces of antibiotics and drugs enhance the colony growth, the methylene-blue reducing capacity, and the virulence of certain pathogenic bacteria, represents another example of this principle.

SUMMARY

1. A synergic effect of phenylalanine and tyrosine for tryptophan has been demonstrated in the case of a tryptophanless mutant of *Escherichia coli*. For a phenylalanineless mutant, tyrosine, but not tryptophan, showed a sparing effect. For a tyrosineless mutant, neither tryptophan nor phenylalanine showed a sparing effect.

2. These results are best explained by a 'straight' scheme for the biogenetic relationships of the three aromatic amino-acids.

3. The effect of nuclear-substituted phenylalanines (*p*-amino-, *p*-nitro-, *p*-fluoro- and 4-hydroxy-3-nitrophenylalanine) as well as of β -phenylserine on *Escherichia coli*, wild type, and on its aromatic amino-acid-requiring mutants has been studied: *p*-aminophenylalanine, *p*-nitrophenylalanine and *m*-nitrotyrosine interfere with the utilization of tyrosine; *m*-nitrotyrosine also disturbs the synthesis of tryptophan; *p*-fluorophenylalanine and β -phenylserine inhibit the conversion of phenylalanine into tyrosine.

4. Alternative pathways have been suggested which lead directly (a) from tryptophan to tyrosine, and (b) from phenylalanine to the product into which the cell normally transforms tyrosine.

5. None of the strains studied is affected by *m*-nitro-, *m*-amino-, *p*-nitro- or *p*-amino- β -phenylserine. However, the *m*-amino and *m*-nitro compounds counteract the inhibition caused by *p*-amino- and *p*-fluoro-phenylalanine, *m*-nitrotyrosine and β -phenylserine in *Esch. coli*, wild type.

6. The following sparing effects have been observed in the mutants, inhibited by *p*-aminophenylalanine. (a) Tryptophan shows a much stronger sparing effect in the phenylalanineless mutant, when the latter is inhibited than without such inhibition. (b) Tyrosine shows a strong sparing effect on phenylalanine in the inhibited phenylalanine mutant. (c) Phenylalanine is more active than tyrosine in restoring the growth of the inhibited tyrosineless mutant of *Esch. coli*.

The authors are greatly indebted to Dr B. D. Davis for the cultures of *Esch. coli* and its mutants, used in these studies.

REFERENCES

- Aoki, Y., Tanemori, Y. & Noda, T. (1951). Cited in *Chem. Abstr.* **45**, 712.
- Armstrong, M. D. & Lewis, J. D. (1951a). *J. biol. Chem.* **188**, 91.
- Armstrong, M. D. & Lewis, J. D. (1951b). *J. biol. Chem.* **190**, 461.
- Atkinson, D. E., Melvin, S. & Fox, S. W. (1951). *Arch. Biochem.* **31**, 205.
- Bambas, L. L., Troutman, H. D. & Long, L. M. (1950). *J. Amer. chem. Soc.* **72**, 4445.
- Beerstecher, E. jun. & Shive, W. (1946). *J. biol. Chem.* **164**, 53.
- Beerstecher, E. jun. & Shive, W. (1947a). *J. biol. Chem.* **167**, 49.
- Beerstecher, E. jun. & Shive, W. (1947b). *J. biol. Chem.* **167**, 527.
- Bergmann, E. D. (1952). *J. Amer. chem. Soc.* **74** (in the Press).
- Bergmann, E. D., Genas, M. & Bendas, H. (1950). *C.R. Acad. Sci., Paris*, **231**, 201.
- Bergmann, E. D., Bendas, H. & Taub, W. (1951). *J. chem. Soc.* p. 2673.
- Buffa, P. & Peters, R. A. (1949). *Nature, Lond.*, **163**, 914.
- Buffa, P., Peters, R. A. & Wakelin, R. W. (1951). *Biochem. J.* **48**, 467.
- Burckhalter, J. H. & Stephens, V. C. (1951). *J. Amer. chem. Soc.* **73**, 56.
- Buu-Hoï, Ng. Ph., Hoán, Ng., Jacquignon, P. & Khoi, Ng. H. (1950). *J. chem. Soc.* p. 2766.
- Carrara, G. & Weitnauer, G. (1949). *Gazz. chim. ital.* **79**, 856.
- Chenoweth, M. R. (1949). *Pharmacol. Rev.* **1**, 383.
- Dalgliesh, C. E. (1949). *J. chem. Soc.* p. 90.
- Dann, O., Ulrich, H. & Möller, E. F. (1950). *Z. Naturf.* **5b**, 446.
- Davis, B. D. (1950). *Experientia*, **6**, 41.
- Davis, B. D. (1951). *J. biol. Chem.* **191**, 315.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Divatia, K. J., Gardner, G., Dufrenoy, J. & Pratt, R. (1951). *Experientia*, **3**, 141.
- Elliot, D. F., Fuller, A. T. & Harington, C. R. (1948). *J. chem. Soc.* p. 85.
- Erlenmeyer, E. & Lipp, A. (1883). *Liebigs Ann.* **219**, 179.
- Frieden, E., Stansel, H. C. & Dittmer, K. (1951). *Fed. Proc.* **10**, 184.
- Gesellschaft für Kohlentechnik (1936). D.R.P. 632, 424. (Cited in *Chem. Abstr.* **30**, 6766.)
- Grau, C. R. (1947). *J. biol. Chem.* **170**, 661.
- Holland, D. O. & Nayler, J. H. C. (1952). *Chem. & Ind.*, p. 518.
- Housewright, R. D. & Koser, S. A. (1944). *J. Infect. Dis.* **75**, 113.
- Jervis, G. A. (1947). *J. biol. Chem.* **169**, 651.
- Johnson, T. B. & Kohmann, E. F. (1915). *J. Amer. chem. Soc.* **37**, 2164.
- Kopp, M., Larramona, H. & Welvart, Z. (1951). *C.R. Acad. Sci., Paris*, **233**, 527.
- Lampen, J. O. & Peterson, W. H. (1943). *Arch. Biochem.* **2**, 443.
- Lerner, A. B. (1949). *J. biol. Chem.* **181**, 281.
- Liebecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
- Long, L. M. & Troutman, H. D. (1951). *J. Amer. chem. Soc.* **73**, 542.
- Maas, W. K. & Davis, B. D. (1950). *J. Bact.* **60**, 733.
- Marnay, C. (1951). *Bull. Soc. chim. Biol., Paris*, **33**, 174.
- Martin, G. I. & Mon, I. N. (1949). *Amer. J. Pharm.* **121**, 169.
- Martius, C. (1949). *Liebigs Ann.* **561**, 227.
- Moss, A. R. & Schoenheimer, R. (1940). *J. biol. Chem.* **135**, 415.
- Simmonds, S. (1950). *J. biol. Chem.* **185**, 755.
- Simmonds, S., Tatum, E. L. & Fruton, J. S. (1947). *J. biol. Chem.* **169**, 91.
- Staedeler, G. (1860). *Liebigs Ann.* **116**, 57.
- Sveinbjornsson, A. & Vander Werf, C. A. (1951). *J. Amer. chem. Soc.* **73**, 869.
- Tatum, E. L. (1946). *Cold Spr. Harb. Symp. quant. Biol.* **11**, 278.
- Truhaut, R., Lambin, S. & Boyer, M. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 387.
- Udenfriend, S., Cooper, L. & Clark, C. T. (1951). *Fed. Proc.* **10**, 262.
- Volcani, B. E., Ben-Ishai, R. & Bergmann, E. D. (1952). *Bull. Res. Council, Israel*, **1**, no. 4, 92.
- Volcani, B. E., Sicher, S. & Lichtenstein, N. (1951). *J. biol. Chem.* **192**, 543.
- Volcani, B. E. & Snell, E. E. (1948). *J. biol. Chem.* **174**, 893.
- Weizmann, C. (1949). British Patent no. 573,216 (cited in *Chem. Abstr.* **43**, 5902.)
- Womack, M. & Rose, W. C. (1946). *J. biol. Chem.* **166**, 429.
- Woolley, D. W. (1950). *J. biol. Chem.* **185**, 293.
- Wyss, O., Rubin, M. & Strandkov, F. B. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 155.