firmed on intestinal strips obtained from guinea pigs, rats, dogs, and cats. On the basis of these observations a revaluation of certain data in the literature concerning the sites of action of several intestinal stimulants is needed.

Evidence that a substance stimulates the intestine of the rabbit and that this action is "atropine-fast" indicates only that the substance is not "muscarinic" and does not differentiate between "musculotropic" and "nicotinic" modes of action. On the other hand, a stimulating action on the guinea pig's intestine which is inhibited by atropine indicates musculotropic action but does not distinguish between actions which are either muscarinic or nicotinic.

For examples of different types of intestinal stimulants we have reinvestigated 3 compounds, whose sites of actions on the intestine do not appear sufficiently clear from the data available. Furfuryltrimethylammonium iodide (Furmethide) was reported (*3*) to have nicotinic action on the blood pressure of atropinized animals and to have a stimulant action on the rabbit's intestine which could be inhibited by atropine. Piperidine has been reported to stimulate the intestine (*4*). Von Euler considered the actions of piperidine similar to, but less potent than, those of nicotine. On the rabbit's intestine atropine did not inhibit the action of piperidine. Lockett (*5*) described the action of piperidine on the guinea pig intestine as muscarinic, based upon the ability of atropine to interfere with its stimulating action on this tissue. A recent abstract (*6*) indicated that a purified extract of Veratrum Viride (Veriloid®) increased the tone of the isolated rabbit's intestine. This action was not prevented by previous treatment with atropine.

The actions of Furmethide, piperidine, and veratridine[2] were compared with the actions of acetylcholine and nicotine on the isolated, atropinized intestines of rabbits and guinea pigs in order to determine the classification of these stimulants of the intestine. The data are summarized in Table 1.

TABLE 1

ACTION OF INTESTINAL STIMULANTS IN THE PRESENCE OF ATROPINE (1: 1,000,000)

Drug	Conc.	Intestine of	
		Rabbit	Guinea pig
Acetylcholine	$1:10^6$	No action	No action
Nicotine	$1:10^6$	Stimulates	"
Furmethide	$1:10^6$	No action	"
Piperidine	$2:10^5$	Stimulates	"
Veratridine	$1:10^6$	"	Stimulates

Since Furmethide is unable to stimulate the atropinized intestine of either the rabbit or the guinea pig, it is justifiable to call its effect muscarinic. Piperidine then appears as a nicotinic agent. Further evidence favoring a nicotinelike action for piperidine is that large amounts of nicotine prevent the stimulating action of piperidine (but not that stimulation due to acetylcholine nor to Furmethide).

Veratridine stimulates the intestine in a manner that can be classified as neither muscarinic nor nicotinic. Its stimulating action is not blocked by atropine on intestinal strips from either the rabbit or the guinea pig. Furthermore, high concentrations of nicotine which produce ganglionic blockade do not prevent the action of veratridine. Amounts of D-tubocurarine sufficient to prevent the action of stimulating amounts of nicotine, do not prevent the action of veratridine.

The mechanism of action of an agent which stimulates the isolated intestine can be analyzed quite simply as muscarinic, nicotinic, or musculotropic by the expedient of using atropinized intestines of both the rabbit and the guinea pig. Results obtained with the intestine of only one of these species are misleading.

[2] A pure sample of veratridine was obtained through the kindness of Otto Krayer, of the Harvard Medical School.

References

1. ELLIS, S., and RASMUSSEN, H. *Federation Proc.*, **10**, 292 (1951).
2. VON EULER, U. S. *Acta Physiol. Scand.*, **7**, 285 (1944).
3. FELLOWS, E. J., and LIVINGSTON, A. E. *J. Pharmacol. Exptl. Therap.*, **68**, 231 (1940).
4. VON EULER, U. S. *Acta Pharmacol. Toxicol.*, **1**, 29 (1945).
5. LOCKETT, M. F. *Brit. J. Pharmacol.*, **4**, 111 (1949).
6. COURZIE, J. T., and BAUER, R. O. *J. Pharmacol. Exptl. Therap.* (abst.), **101**, 14 (1951).

The Bacterial Oxidation of Tryptophan: A Study in Comparative Biochemistry[1]

R. Y. Stanier and O. Hayaishi[2]

Department of Bacteriology, University of California, Berkeley

As discovered independently by several workers (*1–3*), the analysis of adaptive patterns is of much value in the study of microbial metabolism. This technique (sometimes referred to as "simultaneous adaptation" or as "successive adaptation") is essentially an extension and refinement of the technique of kinetic analysis, and may be used to study the course of any microbial metabolic process that is under adaptive enzymatic control (*4*).

One of the specific problems that has been investigated primarily by the analysis of adaptive patterns is the pathway for the complete oxidation of tryptophan by bacteria belonging to the *Pseudomonas* group. Suda, Hayaishi, and Oda (*2*) found that an unidentified *Pseudomonas* sp.[3] adapted to oxidize tryptophan was also fully adapted to oxidize kynurenine, anthranilic acid, and catechol, but not to

[1] This work was supported by grants-in-aid from the Rockefeller Foundation, and from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. We are indebted to R. E. Kallio, W. C. Evans, D. Bonner, T. Sakan, and the Research Division of Merck & Co., Inc., for gifts of chemicals, and to S. H. Hutner for the provision of biological material. In addition, we wish to express our thanks to Martha Tsuchida for help with many experiments.

[2] Present address: National Institutes of Health, Bethesda, Md.

[3] This organism has been subsequently identified by us as a typical member of the *P. fluorescens* species group.

TABLE 1

ADAPTIVE PATTERNS SHOWN BY 29 STRAINS BELONGING TO THE *Pseudomonas* GROUP AFTER GROWTH IN THE PRESENCE OF DL-TRYPTOPHAN

Strains	Cells adapted (+) or unadapted (−) to oxidize				
	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid	Kynurenic acid
P. fluorescens (strain of Suda, Hayaishi, and Oda)	+	−	+	+	−
Pseudomonas sp. (strain of Stanier and Tsuchida)	+	+	+	−	+
Pseudomonas spp., 20 additional strains (10 belonging to the *P. fluorescens* group)	+	±(1) or −	+	+ or ±(2)	−
Pseudomonas spp., 5 additional strains (none belonging to the *P. fluorescens* group)	+	+	+	−	+
Pseudomonas sp., strain Tr-14	+	−	+	−	−
P. fluorescens, strain Tr-13	+	−	−	−	−

(1) A few strains of this group can oxidize D-tryptophan at a low rate (20% or less of the rate at which they oxidize L-tryptophan).

(2) Two strains of this group show greatly impaired ability to oxidize anthranilic acid, which accumulates when they are supplied with tryptophan or kynurenine.

oxidize other, theoretically possible intermediates. The organism attacked only the L-isomer of tryptophan. Accordingly, the following pathway was proposed for the initial steps in the oxidation:

L-tryptophan → L-kynurenine → anthranilic acid → catechol → ?

Independently, Stanier and Tsuchida (*5*) conducted a similar investigation upon another unidentified *Pseudomonas* sp. This organism attacked both isomers of tryptophan, and tryptophan-adapted cells were also adapted to oxidize kynurenine and kynurenic acid, but none of a wide variety of other compounds tested, including anthranilic acid. The observed adaptive patterns implied the existence of distinct mechanisms for attack on the two stereoisomers of tryptophan and kynurenine, and hence the following initial steps were postulated:

L-tryptophan → L-kynurenine ↘
kynurenic acid → ?
D-tryptophan → D-kynurenine ↗

In the ensuing discussion, the oxidation of tryptophan through anthranilic acid and catechol will be referred to as *the aromatic pathway*, and that through kynurenic acid as *the quinoline pathway*.

The marked differences in dissimilatory patterns of closely related bacteria revealed by these reports made a more extensive investigation desirable, and this we have recently undertaken. A large collection of tryptophan-oxidizing pseudomonads has been subjected to systematic comparative study. Adaptive patterns were determined by growing each strain on a medium containing DL-tryptophan as the energy source, harvesting the cells in phosphate buffer, and testing manometrically their ability to oxidize the following 5 compounds: D-tryptophan, L-tryptophan, L-kynurenine, anthranilic acid, and kynurenic acid. Limiting amounts of the substrates (2 μM) were used in the manometric tests to permit detection of any anomalies in total oxygen consumption. More extensive confirmatory tests, employing a wider range of compounds, were subsequently conducted with a few representative strains and gave results in accord with expectations. Table 1 summarizes the general findings, which provide confirmation of the existence of the two pathways diverging from kynurenine that had been proposed earlier by Suda, Hayaishi, and Oda and by Stanier and Tsuchida. Of 27 new strains tested, 20 followed the aromatic pathway and 5 the quinoline pathway. The difference between the two biochemical groups with respect to the oxidation of D-tryptophan has also been confirmed: every strain using the quinoline pathway oxidizes the D-isomer at a high rate, whereas strains using the aromatic pathway either do not attack it at all, or oxidize it at a rate that is low in comparison to the rate of oxidation of the L-isomer.

Two strains that decompose tryptophan through the aromatic pathway are characterized by an interesting metabolic abnormality, which proivdes additional evidence for the existence of this pathway. Their ability to oxidize anthranilic acid is severely impaired, and consequently a substantial accumulation of this compound occurs when they are fed either tryptophan or kynurenine. The severity of the metabolic block between anthranilic acid and catechol in these two strains varies somewhat, depending on the conditions of prior cultivation and the age of the cells, but in some experiments accumulations of anthranilic acid approaching 90% of the theoretical yield on a molar basis have been obtained following the addition of tryptophan or kynurenine to cell suspensions.

Two of the 29 strains tested displayed adaptive patterns after growth on tryptophan that were incompatible with an oxidation through either the aromatic or the quinoline pathway, and in both cases the total oxygen uptake per mole of tryptophan de-

composed was exceptionally low, indicating an early and absolute metabolic block. The first of these strains, Tr-14, could oxidize only L-tryptophan and L-kynurenine of the substrates tested (Table 1). Spectrophotometric analysis of the supernatant liquid from a reaction vessel in which tryptophan had undergone oxidation suggested the accumulation of a mixture of anthranilic and kynurenic acids, an inference subsequently confirmed by isolation and characterization of the two compounds in an experiment conducted on a larger scale. The oxidation of either tryptophan or kynurenine by Tr-14 always results in the formation of these acids, the sum of the amounts of the two products being equal on a molar basis to the amount of substrate decomposed. Tr-14 is the only strain so far investigated in which a "mixed" dissimilation, involving the production of both benzene and quinoline derivatives, has been shown to occur; and its metabolism of tryptophan is so defective that the result could be described better as a "branched cul-de-sac" than as a "mixed pathway."

The second strain showing anomalous adaptive patterns (strain Tr-13) could metabolize only L-tryptophan of the compounds tested (Table 1); spectrophotometric analysis, followed by isolation and characterization of the product, showed that it converted tryptophan quantitatively to indole. We have not further investigated the mechanism of oxidation in this exceptional strain, which belongs to the *Pseudomonas fluorescens* species group. Ten other fluorescent pseudomonads tested all dissimilated tryptophan by the aromatic pathway.

Guided by these findings with intact cells, we then turned our attention to the enzymatic aspects of the problem. One previous report on an enzyme operative in tryptophan oxidation by the aromatic pathway had been made by Hayaishi and Hashimoto (*6*), who worked with the strain of *P. fluorescens* studied by Suda *et al.* (*2*). By extraction of acetone-dried cells grown at the expense of anthranilic acid, Hayaishi and Hashimoto obtained a catechol-oxidizing enzyme which they named pyrocatechase. After extensive purification, pyrocatechase was found to catalyze an oxidation of catechol with an uptake of 2 atoms of oxygen and with the formation of an acid which appeared to be identical with *cis-cis*-muconic acid, on the basis of melting point and elementary analysis. A crude enzyme system of very similar properties was isolated independently by Stanier *et al.* (*7*) from dried cells of another strain of *P. fluorescens,* grown on phenol, benzoic acid, or mandelic acid. It was shown to catalyze a conversion of catechol, again with an oxygen uptake of 2 atoms, to β-ketoadipic acid. Since it appeared improbable that two distinct enzymes catalyzing an opening of the catechol ring would be formed by *P. fluorescens,* we decided to reinvestigate catechol oxidation, using extracts from tryptophan-grown cells. A crude cell-free system was readily obtained from vacuum-dried cells, and was found to catalyze the reaction described by Stanier *et al.*—namely, an oxidation of catechol to β-ketoadipic acid. This indicated that the enzymatic degradation of catechol might involve an initial oxidative and a subsequent nonoxidative step:

$$\text{catechol} + O_2 \xrightarrow[\text{(pyrocatechase)}]{} \textit{cis-cis}\text{-muconic acid} \quad (1)$$

$$\textit{cis-cis}\text{-muconic acid} + H_2O \xrightarrow[\text{(unknown enzyme)}]{} \beta\text{-ketoadipic acid} \quad (2)$$

$$\text{catechol} + O_2 + H_2O \xrightarrow[\text{(crude extracts)}]{} \beta\text{-ketoadipic acid} \quad (1) + (2)$$

It was easy to test this hypothesis, since a small amount of the alleged *cis-cis*-muconic acid, isolated by Hayaishi and Hashimoto after the action of pyrocatechase on catechol, was still available. An attempt to demonstrate the conversion of this material to β-ketoadipic acid by our crude catechol-oxidizing extract gave *completely negative results.* The work of others has recently clarified this very puzzling situation. Elvidge *et al.* (*8*) have discovered the third geometrical isomer of muconic acid, which possesses the *cis-trans* configuration, and have further shown that the *cis-cis* isomer is unstable in aqueous solution, undergoing ready isomerization to the *cis-trans* form, from which it cannot be readily distinguished, since the physical and chemical properties of the two substances (including their melting points) are very similar. It is thus probable that most earlier chemical data reported for the *cis-cis* isomer were obtained with the *cis-trans* isomer, or with a mixture of the two. Following these chemical studies, Evans and Smith (*9*) examined the biological behavior of the 3 isomers of muconic acid, and found that the *cis-cis* isomer is an intermediate in the bacterial degradation of benzoic acid and phenol to β-ketoadipic acid, whereas the *cis-trans* and *trans-trans* isomers are inactive. Tests made by us on samples of the *cis-cis* and *cis-trans*-muconic acids have shown that our crude catechol-oxidizing enzyme system from tryptophan-grown cells smoothly converts the former isomer in a nonoxidative reaction to β-ketoadipic acid, but does not attack the latter isomer, thus fully confirming the conclusions of Evans and Smith. In the light of these discoveries, it is now evident that Hayaishi and Hashimoto had purified pyrocatechase to the point at which the second, nonoxidative step was eliminated; but that the *cis-cis* muconic acid produced enzymatically underwent isomerization during the the isolation procedure, which explains its inactivity when subsequently tested with the crude enzyme by us.

Althqugh extracts from tryptophan-adapted, vacuum-dried cells of strains that follow the aromatic pathway show strong activity against catechol, they are inactive against tryptophan itself. In a search for better methods of making cell-free preparations, we found that the technique of grinding wet cells with alumina, discovered by McIlwain (*10*), yielded extracts containing many additional enzymes. Fresh, concentrated extracts prepared in this manner from tryptophan-grown cells catalyze an oxidation of tryptophan which results in the consumption of 8 atoms of oxygen per mole of substrate and yields β-keto-

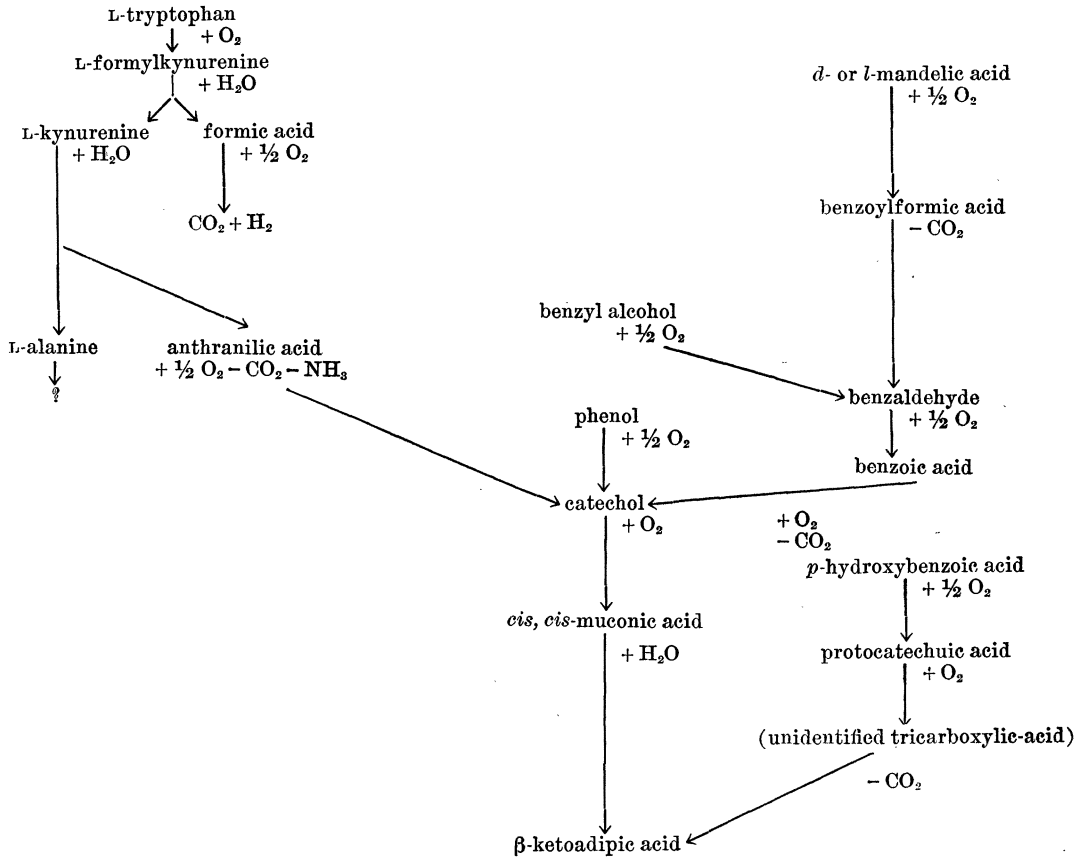


FIG. 1. A comprehensive diagram of the known bacterial metabolic pathways that converge with the formation of β-ketoadipic acid as an intermediary metabolite. Other divergent pathways from tryptophan are not shown.

adipic acid as one of the end products, demonstrating that a complete destruction of the indole nucleus must have taken place. Aging or simple dilution of such extracts results in the elimination of several oxidative steps, the total oxygen uptake per mole of tryptophan falling to 5, 3, or even 2 atoms. The oxidation of tryptophan catalyzed by such aged or diluted preparations involves the steps discovered recently by Knox and Mehler (*11*) for the dissimilation of tryptophan to kynurenine via formyl-kynurenine in mammalian liver extracts. With the bacterial preparations, however, kynurenine does not accumulate, since a powerful kynureninase is also present, which causes a nonoxidative cleavage of L-kynurenine to anthranilic acid and L-alanine. This enzyme, which is the only one that we have so far studied in detail, coincides in all essential properties with the mammalian kynureninase recently described by Wiss (*12*) and by Braunshstein, Goryachenkova, and Paskhina (*13*).

The work reported here permits the construction of a broad conceptual scheme that describes the oxidation of tryptophan by many bacteria, and that links up one of the possible alternate pathways for the metabolism of tryptophan with a series of other primary oxidative sequences previously known to occur in bacteria. It is now evident that the initial attack on tryptophan most commonly involves elimination of the α carbon atom of the indole nucleus, resulting in the formation of kynurenine as an intermediary metabolite. Below kynurenine, alternate metabolic pathways are open; some bacteria degrade this compound to kynurenic acid (presumably by a specific deamination or transamination, followed by secondary ring closure), whereas others split it to alanine and anthranilic acid through the action of kynureninase. At present we do not know the nature of the later intermediates formed on the pathway through kynurenic acid; enzymatic studies have not yet been attempted, and although many benzene derivatives have been tested by analysis of the adaptive patterns of tryptophan-grown cells, the results have been uniformly negative. The outcome of these analyses suggests that the benzene moiety of the quinoline nucleus in kynurenic acid may be first destroyed, with resultant eventual oxidation via pyridine derivatives. Several of the later steps on the aromatic pathway are now firmly established. This pathway merges, near the point of final ring rupture, with oxidations of nonnitrogenous primary aromatic substrates, such as mandelic acid, phenol, and *p*-hydroxybenzoic acid (*4*); as shown in Fig. 1, the common central intermediary metabolite for all these oxidations is β-keto-

adipic acid. Since the present work has also shown that the initial enzymatic steps in the breakdown of tryptophan are common to bacteria and mammals, it would not be surprising if β-ketoadipic acid proves to be an intermediate in the mammalian oxidation of tryptophan and other aromatic compounds. Full experimental details will be published shortly.

References

1. STANIER, R. Y. *J. Bact.*, **54**, 339 (1947).
2. SUDA, M., HAYAISHI, O., and ODA, Y. *Symposium on Enzyme Chemistry* (Tokyo), **1**, 79 (1949); *Med. J. Osaka University*, **2**, 21 (1950).
3. KARLSSON, J. L., and BARKER, H. A. *J. Biol. Chem.*, **175**, 913 (1948).
4. STANIER, R. Y. *Bact. Revs.*, **14**, 179 (1950).
5. STANIER, R. Y., and TSUCHIDA, M. *J. Bact.*, **58**, 45 (1949).
6. HAYAISHI, O., and HASHIMOTO, K. *Med. J. Osaka University*, **2**, 33 (1950).
7. STANIER, R. Y., *et al. J. Bact.*, **59**, 137 (1950).
8. ELVIDGE, J. A., *et al. J. Chem. Soc.*, 2235 (1950).
9. EVANS, W. C., and SMITH, B. S. W. *Biochem. J.*, **49**, Proc. Biochem. Soc., x (1951).
10. MCILWAIN, H. *J. Gen. Microbiol.*, **2**, 288 (1948).
11. KNOX, W. E., and MEHLER, A. H. *J. Biol. Chem.*, **187**, 419 (1950); MEHLER, A. H., and KNOX, W. E. *Ibid.*, 431.
12. WISS, O. *Helv. Chim. Acta*, **32**, 1694 (1949).
13. BRAUNSHSTEIN, A. E. GORYACHENKOVA, E. V., and PASKHINA, T. S. *Biokhimiya*, **14**, 163 (1949).

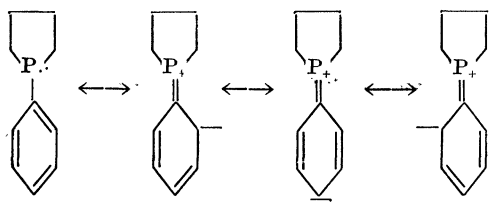
The Ultraviolet Absorption Spectra of Some Heterocyclic Phosphorous Compounds[1]

Arthur Furst and Robert J. Horvat[2]

Department of Chemistry,
University of San Francisco, San Francisco, California

It is becoming increasingly evident that the phenomenon of resonance is not an exclusive property of any group of atoms in the periodic chart. For the ε max value of 1-phenylcyclotetramethylenephosphine (I), its 2-methyl (II), or its 2,5-dimethyl (III) homologues can best be explained in either of two ways; both explanations involve the concept of resonance.

As 1-phenylcyclotetramethylenephosphine (I) is an isologue of a substituted aniline, an analogous postulate can be made:



This hypothesis (A) should predict the following: (*a*) The ε max should have a lower value if the compound is measured in hydrochloric acid; (*b*) the 2-methyl homogogue (II) should not have the phenyl group completely planar with the phosphorous group, and hence some interference with the resonance should be manifested by lower ε max values; (*c*) the 2,5-dimethyl homologue (III) should have very little resonance, if any, other than the usual Kekule resonance of the benzene ring. No effect should be noted when hydrochloric acid is added to this compound. All these predictions are borne out (Table 1).[3]

TABLE 1

	I*	I in HCl†	II*	II in HCl†	III*	III in HCl†
λ(mμ)	250	251	249	247	252	No
ε max	6,500	2,830	4,050	3,020	865	Change

* I and II as 2 mg % and III as 10 mg % in 95% ethanol.
† For second measurement, one drop of concentrated hydrochloric acid was added.

An alternate explanation (B) for the high ε max values compared to aniline (*1*) may lie in the extreme ease of oxidation of these compounds by the oxygen of the air. These phosphorous compounds may tend to exist partly as free radicals. The lone electron can resonate with the benzene ring. This hypothesis should predict that on standing the ε max should rapidly decrease. Observations show this, too.

Additional work is being done to see if the phosphorous group will direct a new group to the *ortho* and *para* positions on the benzene ring in support of hypothesis A, and if these compounds are associated in support of hypothesis B.

The values reported here were taken from compounds kept in sealed tubes, but opened occasionally for withdrawal of samples. It is possible that measurements taken under completely anhydrous and oxygen-free conditions would show that the ε max values for these compounds would be much higher.

Details of preparation will be published elsewhere.

Reference

1. KUMLER, W. D., and STRAIT, L. A. *J. Am. Chem. Soc.*, **65**, 2349 (1943).

[1] A Frederick Gardner Cottrell grant from the Research Corporation is gratefully acknowledged.

[2] Taken from the M. S. thesis of Robert J. Horvat.

[3] The authors wish to thank Michael K. Hrenoff for the spectra data.

The Effect of Aureomycin and Vitamins on the Growth Rate of Chicks

Jacob Biely and B. March

Poultry Nutrition Laboratory,
The University of British Columbia, Vancouver

Following the discovery by Stokstad and Jukes (*1*) that the feeding of aureomycin had a stimulating effect on the growth of the chick, several reports have appeared on the effects of feeding antibiotics to chicks and to various other animals. It was found that with animals other than ruminants the inclusion of an antibiotic in the feed almost invariably resulted in a marked increase in the rate of growth. There are, however, reports of instances where the addition of antibiotics to a ration had no effect on the growth rate. Speer *et al.* (*2*), for example, reported an experiment in which the addition of aureomycin to a ration fed to young pigs had no effect on the growth rate. The