Criddle, R. S., Bock, R. M., and Green, D. E., and Tisdale, H. D. (1961), *Biochem. Biophys. Research Communs. 5*, 75.
Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.
Erikson, S., and Sjöquist, J. (1960), *Biochim. et Biophys. Acta 45*, 290.
Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), in Methods of Biochemical Analysis, Vol. 2, Glick, D., editor, New York, Interscience Publishers, p. 360.
Goldberger, R., Pumphrey, A., and Smith, A. (1962), *Biochem. et Biophys. Acta 58*, 307.
Goldberger, R., Smith, A. L., Tisdale, H. D., and Bomstein, R. (1961), *J. Biol. Chem. 236*, 2788.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Green, D. E. (1956–1957), Harvey Lectures, Academic Press, Inc., New York, 1958, p. 177.
Green, D. E. (1961), Plenary Lecture, 5th International Congress of Biochemistry, August, 1961, Moscow, Preprint 176.
Green, D. E., Jarnefelt, J., and Tisdale, H. D. (1959), *Biochim. et Biophys. Acta 31*, 34.
Green, D. E., and Lester, R. L. (1959), *Fed. Proc. 18*, 987.
Green, D. E., Tisdale, H. D., Criddle, R. S., and Bock, R. M. (1961a), *Biochem. Biophys. Research Communs. 5*, 81.
Green, D. E., Tisdale, H. D., Criddle, R. S., Chen, P. Y., and Bock, R. M. (1961b), *Biochem. Biophys. Research Communs. 5*, 109.
Griffiths, D. E., and Wharton, D. C. (1961), *J. Biol. Chem. 236*, 1850.
Hagihara, B. (1956), *Nature 178*, 629.
Holiday, E. R., and Ogston, A. G. (1938), *Biochem. J. 32*, 1166.
Kauzmann, W. (1959), *Advances in Protein Chem. 14*, 1.
Keilin, D., and Hartree, E. F. (1938), *Proc. Roy. Soc. B. 125*, 171.
Linderstrøm-Lang, K., and Lanz, H. (1938), *Compt. rend. trav. lab. Carlsberg, Ser. chim. 21*, 315.
Linnane, A. W., and Ziegler, D. M. (1958), *Biochim. et Biophys. Acta 29*, 630.
Polson, A. G. (1937), Ph.D. thesis, University of Stellenbosch.
Schachman, H. K. (1960), *Brookhaven Symposia in Biology* No. 13, 49.
Singer, T. P., Kearney, E. B., and Massey, V. (1957), *Advances in Enzymology 18*, 61.
Spackman, D., Stein, W., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Svedberg, T., and Pederson, K. (1940), The Ultracentrifuge, New York, Oxford University Press.
Takemori, S., Sekuzu, I., and Okunuki, K. (1960), *Nature 188*, 593.
Theorell, H., and Äkesen, A. (1941), *J. Am. Chem. Soc. 63*, 1804.
Waugh, D. F. (1954), *Advances in Protein Chem. 9*, 325.
Widmer, C., and Crane, F. L. (1958), *Biochim. et Biophys. Acta 27*, 203.
Ziegler, D. M., and Doeg, K. A. (1959), *Biochem. Biophys. Research Communs. 1*, 344.
Ziegler, D. M., Green, D. E., and Doeg, K. A. (1959), *J. Biol. Chem. 234*, 1916.

Preparation and Biological Activity of Dihydroaminopterin*

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Dihydroaminopterin was synthesized by the reduction of purified aminopterin with sodium dithonite and isolated by fractional precipitation with acid. This compound is 34 to 92 times more potent than aminopterin as a growth inhibitor of *Streptococcus faecalis* and *Pediococcus cerevisiae* respectively. It has, however, the same activity as aminopterin against cultured sarcoma 180 cells and as an inhibitor of folic acid reductase from chicken liver or *Streptococcus faecalis*. Paper chromatographic analysis of a preparation of tetrahydroaminopterin revealed that the inhibitory activity of this compound is mainly due to the presence of some substance in the preparation of the former compound other than tetrahydroaminopterin itself. It is suggested that this substance is dihydroaminopterin.

The role of different tetrahydrofolate co-factors in several biosynthetic reactions has directed increasing attention to the synthesis of reduced analogues of folic acid (pteroylglutamic acid) as potential metabolic antagonists. Both synthetic and biological work, however, are complicated by the instability of these compounds. Weygand *et al.* (1951) reported that catalytic hydrogenation of aminopterin resulted in partial loss of inhibitory activity for *Pediococcus cerevisiae* and *Streptococcus faecalis*. Only the crude reaction mixtures were tested in these experiments and the product of the reaction was not isolated. Since that time more has been learned about the properties of tetrahydro and dihydrofolic acid and techniques have been developed for the isolation and stabilization

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of these compounds (Jaenicke, 1955; Kisliuk, 1957; Blakley, 1957, 1958; Hatefi *et al.*, 1960; Mathews and Huennekens, 1960; Silverman and Noronha, 1961). According to Kisliuk (1960), preparations of tetrahydroaminopterin and tetrahydroamethopterin appeared to be much stronger inhibitors of *S. faecalis* and *P. cerevisiae* than the corresponding unreduced compounds.

This paper describes the synthesis of dihydroaminopterin and comparison of its properties with those of tetrahydroaminopterin. The observations reported by Kisliuk with respect to the biological activity of tetrahydroaminopterin were not confirmed, while dihydroaminopterin was found to be a more potent inhibitor than aminopterin in certain test systems. Preliminary reports on part of this work have been presented (Nichol *et al.*, 1961; Zakrzewski, 1961).

Methods

Synthesis of Tetrahydroaminopterin.—Commercial aminopterin was purified by chromatography on cellulose *N,N*-diethylaminoethyl ether. The procedure for purification of aminopterin on *N,N*-diethylaminoethyl ether cellulose (Oliverio and Loo, 1960) was modified by Werkheiser (unpublished data) in the following way: 40 g of *N,N*-diethylaminoethyl ether cellulose was suspended in 0.1 M NaOH and the slurry was poured into a column 4.5 cm in diameter. The cellulose was washed with water, then with 0.1 M phosphate buffer of *p*H 5.5, and again with water. Two hundred mg of aminopterin was dissolved in 20 ml of 1.0 M solution of K_2HPO_4 and this solution was allowed to percolate into the column bed. The gradient elution was conducted under pressure with 4 liters of 0.1 M phosphate of *p*H 5.5 in the mixing flask and 4 liters of 0.8 M phosphate of *p*H 5.5 in the reservior. The progress of the elution was followed by measuring the optical density of the fractions of the eluate at 370 mμ. The fractions containing aminopterin were combined and acidified to *p*H 2.8. After standing overnight in the refrigerator the precipitate was collected by centrifugation, washed with water, and dried with acetone and ether. The yield was about 50%. Tetrahydroaminopterin was prepared by hydrogenation of the purified aminopterin (O'Dell *et al.*, 1947) as described by Kisliuk (1957). The product of the reaction was stored as a suspension in absolute ether.

Tetrahydroaminopterin was analyzed by descending chromatography on Whatman No. 1 paper strips with 0.1 M phosphate buffer, *p*H 6.5, containing 1% mercaptoethanol as the solvent. Owing to the partial decomposition of tetrahydroaminopterin during the chromatography, several fluorescing and ultraviolet-absorbing spots appeared on the paper. The reduced analogue, however, could be located as a purple spot after the paper strips were sprayed with reagents for the detection of diazotizable amines (Blakley, 1957; Zakrzewski and Nichol, 1956). By this technique tetrahydroaminopterin was located as a faintly ultraviolet-absorbing spot at R_F 0.64. In addition, small amounts of a diazotizable amine, presumably free *p*-aminobenzoylglutamic acid, migrated closely behind the solvent front. Only 8% of the total diazotizable amine recovered from the paper strips was due to free *p*-aminobenzoylglutamic acid.

Synthesis of Dihydroaminopterin. — Dihydroaminopterin was prepared by the reduction of purified aminopterin with sodium dithionite under conditions described by Futterman (1957). Two compounds were formed which were separated by paper chromatography. In contrast, the reduction of folic acid by this procedure produces a single product as identified by paper chromatography which is presumably 7,8-dihydrofolic acid (Osborn and Huennekens, 1958). One of the compounds derived from aminopterin appears on the paper chromatograms as a bright blue fluorescing spot at R_F 0.12 when developed with 0.1 M phosphate buffer of *p*H 7.0 containing 1% mercaptoethanol, and in this respect resembles dihydrofolic acid. The second compound was located by its dark blue fluorescence at R_F 0.33, and is very unstable even in the presence of mercaptoethanol. It loses its fluorescence rapidly and at the same time it becomes a UV-absorbing yellow compound. The rate of migration of the former compound on paper chromatograms and its fluorescence suggest that it is 7,8-dihydroaminopterin.

The following procedure was used for the preparation of pure dihydroaminopterin. Twenty mg of purified aminopterin and 500 mg of ascorbic acid were dissolved in a mixture of 2.16 ml water and 3.84 ml 1.0 M KOH, giving a solution with *p*H 10.5–11.0. To this solution 200 mg sodium

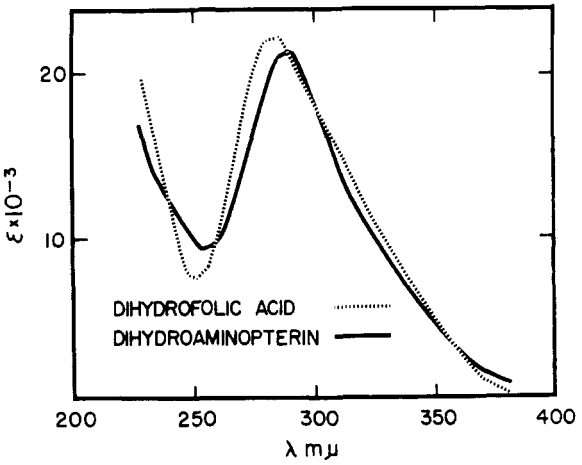

FIG. 1.—Ultraviolet spectra of dihydroaminopterin and dihydrofolic acid in 0.001 M NaOH containing 0.002% of mercaptoethanol by volume. The molar extinction coefficients are based on the assumption that the products were 100% pure and their molecular weights were calculated as free acids, monohydrates.

dithionite was added and the mixture was allowed to stand at room temperature for 15 minutes. During this time, a fine precipitate formed. After standing in ice for 1 hour, the solution was clarified by centrifugation in a refrigerated centrifuge and the precipitate was discarded. The supernatant solution was adjusted to *p*H 3 with glacial acetic acid. A white, partially crystalline precipitate formed while the solution was stored in a refrigerator overnight. This material was collected by centrifugation and resuspended twice in ice-cold 1% aqueous solution of mercaptoethanol, twice in acetone, once in ether, and finally dried at room temperature under vacuum.

The dry powder could be kept at −5° with little or no change in color for several weeks. The product migrated on the paper as a single blue fluorescing spot (R_F 0.12 in 0.1 M phosphate, *p*H 7.0, containing 1% mercaptoethanol). This compound could be oxidized by iodine in alkaline but not in acidic solution, and two atoms of iodine were used per mole[1] of the compound. The ultraviolet spectrum of the isolated compound is compared with that of dihydrofolic acid in Figure 1.

Microbial Inhibition.—The inhibitory activities of the reduced analogues of aminopterin were determined for two organisms, *Streptococcus faecalis* (8043) and *Pediococcus cerevisiae* (8081); either solid medium (Burchenal *et al.*, 1952) or the usual tube assay technique (Flynn *et al.*, 1951) was used. In the latter case the solutions of the analogues were added aseptically to the autoclaved media. All of the test compounds were dissolved in 0.1 M solution of Na_2HPO_4 containing 0.1% of 2-mercaptoethanol by volume. Also, all of the dilutions were made with 0.1% aqueous solution of 2-mercaptoethanol.

Inhibition of Mammalian Cell Growth in Culture—Sarcoma 180 cells were grown in Eagle's medium (Eagle, 1959). Mercaptoethanol was found to be toxic to these cells at concentrations above 2.0 μg per ml (0.0002%). Therefore, when the inhibitory effect of dihydroaminopterin was tested, a freshly prepared 0.1 mM solution of the inhibitor in 0.1 M Na_2HPO_4 containing 0.05% mercaptoethanol was made each day. Further dilutions were made in water and added to the medium so that the highest final concentration of mercaptoethanol in the medium was one-fourth of the tolerated concentration.

S-180 cells (200,000 cells in 2 cc medium) were placed in T-15 culture flasks. After 24 hours were allowed for the attachment of the cells to the glass, the experimental medium was applied and thereafter changed three times during the one week of growth. Growth was measured by the increase in total protein (Oyama and Eagle, 1956) over that of the inoculum.

Inhibition of Folic Acid Reductase.—The preparation of the partially purified folic acid reductase from chicken liver was described previously (Zakrzewski, 1960). An enzyme with similar characteristics occurs also in *Streptococcus faecalis* 8043 (Zakrzewski, 1960). Since the amethopterin-resistant subline of this organism (*S. faecalis*/A), like amethopterin-resistant lines of Sarcoma 180 (Hakala *et al.*, 1961), contains considerably larger amounts of folic acid reductase than the parent cells,[2] the enzyme preparation used in these experiments was extracted from *S. faecalis*/A cells which grew well in the presence of 10 μg/ml of amethopterin. Frozen cells (3 to 5 g) were mashed in a Hughes press (Rodgers and Hughes, 1960). The cell paste was suspended in 4 volumes of cold 0.9% saline and centrifuged. The supernatant solution was dialyzed overnight in a refrigerator against 100 volumes of saline containing 0.01 M phosphate buffer of *p*H 6.8. Nucleic acids were precipitated with protamine (0.15 ml of 2% protamine sulfate per ml of dialyzed extract). The supernatant solution obtained after centrifugation was used as the enzyme preparation. In each case, reductase activity was determined after incubation with folic acid and TPNH by measurement of the diazotizable amine derived from tetrahydrofolate by the colorimetric procedure described previously (Zakrzewski, 1960). Solutions of dihydro- and tetrahydroaminopterin were stabilized by the addition of mercaptoethanol, 0.1% by volume. The concentration of mercaptoethanol in the reaction mixture did not exceed 0.02%. Mercaptoethanol was also added to the vessels incubated in the presence of aminopterin.

Results

Paper chromatograms of tetrahydroaminopterin and dihydroaminopterin were placed on agar medium seeded with *Pediococcus cerevisiae* 8081. Tracings of the zones of inhibition are shown in Figure 2. When a paper strip with tetrahydroaminopterin freshly developed with 0.1 M phosphate of *p*H 5.5 in the presence of mercaptoethanol was placed on the plate, the main zone of inhibition appeared at R_F 0.12 and not at R_F 0.60 as would be expected for tetrahydroaminopterin. However, a trailing area of material between the main zone and the position where tetrahydroaminopterin should be located indicated that

[1] Samples of 5 to 10 mg of dihydroaminopterin were suspended in 1.0 ml standardized iodine solution, about 0.1 N. Three drops of 2 M NaOH and 0.25 ml water were added. The mixture was allowed to stand for 15 minutes at room temperature. Thereafter, 5 drops 2 M HCl were added and the solution was titrated with 0.1 N solution of sodium thiosulfate.

Sample	Iodine Used
	(moles × 10^6)
1.21	2.77
1.40	2.77

[2] Nichol, C.A., unpublished data.

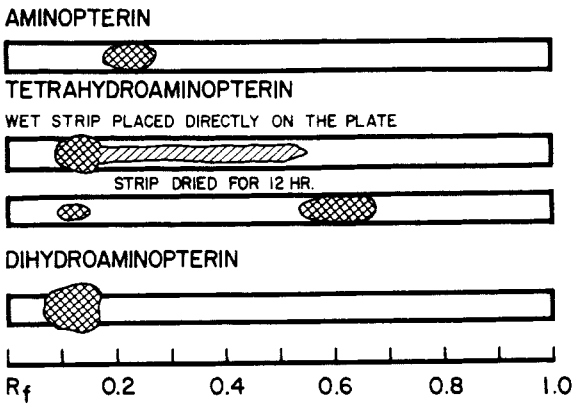

FIG. 2.—Tracings of bioautographs of aminopterin, tetrahydroaminopterin, and dihydroaminopterin. Test organism: *Pediococcus cerevisiae* 8081.

an inhibitory compound was formed during chromatography. Only after the developed paper strip was allowed to dry for 12 hours at room temperature and then placed on an agar plate did the main zone of inhibition appear at R_F 0.61. The secondary zone was still present at R_F 0.12. Under these conditions aminopterin moved to R_F 0.22. Dihydroaminopterin appeared as a single zone located at R_F 0.12. Similar results were obtained when *Streptococcus faecalis* 8043 was used as the test organism. These experiments indicated that the inhibitory activity of tetrahydroaminopterin preparation in these biological test systems is due to the presence of some substance other than tetrahydroaminopterin itself or aminopterin.

The effect of tetrahydroaminopterin on the growth of *S. faecalis* and *P. cerevisiae* was determined by the pad-plate assay based on the diameter of zones of inhibition. The results obtained by this technique were very inconsistent and varied greatly from experiment to experiment. In general, the inhibitory potency of tetrahydroaminopterin as compared to that of aminopterin was much lower in *S. faecalis* assays and somewhat higher in *P. cerevisiae* assays. In contrast, the results obtained with dihydroaminopterin were quite reproducible in both solid and liquid medium assays. As shown in Table

TABLE I
COMPARISON OF INHIBITORY POTENCY OF AMINOPTERIN AND DIHYDROAMINOPTERIN AGAINST *Streptococcus faecalis*, *Pediococcus cerevisiae*, AND CULTURED SARCOMA 180

Organism	Aminopterin	Dihydro-aminopterin
	(mμg/ml for 50% inhibition)	
S. faecalis[a]	2.72	0.08
P. cerevisiae[b]	182.0	1.99
S-180[a]	2.9	2.5

[a] Grown in the presence of folic acid. [b] Grown in the presence of folinic acid (5-formyl-tetrahydrofolic acid).

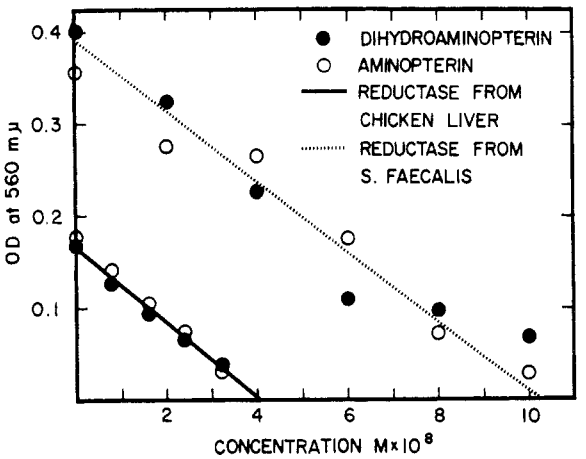

FIG. 3.—Comparison of aminopterin and dihydroaminopterin by titration with folic acid reductase preparations from chicken liver and *Streptococcus faecalis*. The conditions for incubation of the chicken liver enzyme and the analytical procedures were the same as described previously (Hakala *et al.*, 1961). The reductase preparation from *S. faecalis*/A (144 μg of protein) was added to a medium containing 1×10^{-4} M folate and 3.8×10^{-4} M TPNH in 0.1M citrate buffer of *p*H 6 along with aminopterin or dihydroaminopterin, as indicated, in a total volume of 0.5 ml. The incubation was continued for 30 minutes at 37° under helium and was terminated by the addition of 0.1 ml of 5 N HCl.

I, dihydroaminopterin was a much more powerful inhibitor than aminopterin for *S. faecalis* and *P. cerevisiae*. In cultures of sarcoma 180, its inhibitory potency was equal to that of aminopterin.

Dihydroaminopterin has the same potency as aminopterin as an inhibitor of folic acid reductase obtained from chicken liver or *S. faecalis* (Fig. 3). Both compounds inhibited the reductase preparations stoichiometrically (Werkheiser, 1961), and the same amount of each antagonist was required for complete inhibition of a given amount of the enzyme. In contrast, tetrahydroaminopterin had only 40% of the expected activity in comparison with aminopterin on a molar basis.

DISCUSSION

Paper chromatography of the product derived from aminopterin by catalytic hydrogenation demonstrated at first a lack of inhibition on the bioautogram at the location of tetrahydroaminopterin. The appearance of an inhibitory zone on bioautograms at the R_F of tetrahydroaminopterin which occurred when the developed paper strips were dried overnight can be easily explained when the assumption is made that the behavior of reduced derivatives of aminopterin is similar to that of the reduced derivative of folic acid. Both dihydrofolic acid and tetrahydrofolic acid are unstable when exposed to air (O'Dell *et al.*, 1947). Both compounds, however, can be stabilized considerably in the presence of reducing agents such as mercaptoethanol, dimercaptopropanol, or ascor-

bate (Blakley, 1958; Hatefi *et al.*, 1960; Mathews and Huennekens, 1960; Silverman and Noronha, 1961). When an unprotected solution of tetrahydrofolic acid is exposed to air at neutral *p*H, the ultraviolet spectrum of the former compound changes gradually to that of dihydrofolic acid (Osborn and Huennekens, 1958). Dihydrofolic acid, although more stable than tetrahydrofolic acid, is in turn oxidized further to folic acid and degradation products (Blakley, 1957). Thus, it is suggested that the formation of an inhibitory zone at R_F 0.6 is due to the oxidation of tetrahydroaminopterin to dihydroaminopterin. At the same time small quantities of dihydroaminopterin originally present in the preparation of tetrahydroaminopterin gradually decompose to biologically less active or inactive products. Such events are consistent with the observed decrease in the size of the zone at R_F 0.12. Whether tetrahydroaminopterin possesses only weak inhibitory activity or none cannot be concluded with certainty because of the difficulty of excluding the presence of dihydroaminopterin. Tetrahydroaminopterin was much weaker than aminopterin as an inhibitor of folic acid reductase.

Kisliuk (1960) observed that tetrahydroaminopterin preparations were more potent than aminopterin when standard microbial assays (Flynn *et al.*, 1951) were performed with *S. faecalis* and *P. cerevisiae* in liquid medium. In this laboratory, the results of Kisliuk could not be confirmed when a solid medium technique was used (Burchenal *et al.*, 1952). The inconsistent results obtained in microbial assays with tetrahydroaminopterin were most likely due to the varying amounts of dihydroaminopterin formed in the course of decomposition of the former compound.

The reduction of folic acid with dithionite yields a single compound, presumably the 7,8-dihydroisomer (Futterman, 1957; Osborn and Huennekens, 1958). When aminopterin is reduced by dithionite under identical conditions at least two compounds are formed. The properties of one of the products closely resemble those of dihydrofolic acid and accordingly it is most likely to be the 7,8-dihydroisomer.

Since dihydroaminopterin is no more potent than aminopterin as an inhibitor of folic acid reductase from *S. faecalis*, different rates of penetration of some cellular barrier may account for the marked difference in the toxicity of the two compounds for this organism. More rapid transport of dihydroaminopterin into cells may also be a major factor in its greater potency than aminopterin for *P. cerevisiae*. Preliminary tests indicate that both compounds are of similar potency for a subline of this organism which has much greater capacity than the parent line to take up C^{14}-folic acid and C^{14}-aminopterin.[2]

Preparations of tetrahydroamethopterin were reported to be about 10 to 20 times more toxic than amethopterin in mice (Mead *et al.*, 1961) and chickens (Kisliuk and Fox, 1961). In comparison with aminopterin (which is considerably more toxic for rodents than amethopterin), dihydroaminopterin was about twice as toxic in preliminary trials in mice. Although tetrahydroaminopterin preparations had less activity than aminopterin in inhibiting folic acid reductase, analogues of tetrahydrofolic acid deserve further attention as potential inhibitors of one-carbon transfer reactions.

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References

Blakley, R. L. (1957), *Biochem. J. 65*, 331.
Blakley, R. L. (1958), *Nature 182*, 1719.
Burchenal, J. H., Waring, G. B., Ellison, R. R., and Reilly, H. C. (1952), *Proc. Soc. Exper. Biol. Med. 80*, 516.
Eagle, H. (1959), *Science 130*, 432.
Flynn, L. M., Williams, V. B., O'Dell, B. L., and Hogan, A. G. (1951), *Anal. Chem. 23*, 180.
Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.
Hakala, M. T., Zakrzewski, S. F., and Nichol, C. A. (1961), *J. Biol. Chem. 236*, 952.
Hatefi, Y., Talbert, P. T., Osborn, M. J., and Huennekens, F. M. (1960), *Biochem. Prep. 7*, 89.
Jaenicke, L. (1955), *Biochim. Biophys. Acta 17*, 588.
Kisliuk, R. L. (1957), *J. Biol. Chem. 227*, 805.
Kisliuk, R. L. (1960), *Nature 188*, 584.
Kisliuk, R. L., and Fox, M. R. S. (1961), *Arch. Biochem. Biophys. 93*, 534.
Mathews, C. K., and Huennekens, F. M. (1960), *J. Biol. Chem. 235*, 3304.
Mead, J. A. R., Vendetti, J. M., Schrecker, A. W., Goldin, A., and Kisliuk, R. L. (1961), *Nature 189*, 973.
Nichol, C. A., Hakala, M. T., and Zakrzewski, S. F. (1961), *Pharmacologist 3*, 78.
O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S., and Pfiffner, J. J. (1947), *J. Am. Chem. Soc. 69*, 250.
Oliverio, V. T., and Loo, T. L. (1960), *Proc. Am. Assoc. Cancer Res. 3*, 140.
Osborn, M. J., and Huennekens, F. M. (1958), *J. Biol. Chem. 233*, 969.
Oyama, V. I., and Eagle, H. (1956), *Proc. Soc. Exper Biol. Med. 91*, 305.
Rodgers, A., and Hughes, D. E. (1960), *J. Biochem Microbiol. Tech. Eng. 2*, 70.
Silverman, M., and Noronha, J. M. (1961), *Biochem. Biophys. Res. Communs. 4*, 180.
Werkheiser, W. C. (1961), *J. Biol. Chem. 236*, 888.
Weygand, F., Wacker, A., Mann, H. J., and Rowold, E. (1951), *Z. Naturforsch. 66*, 174.
Zakrzewski, S. F., and Nichol, C. A. (1956), *Fed. Proc. 15*, 390.
Zakrzewski, S. F. (1960), *J. Biol. Chem. 235*, 1776.
Zakrzewski, S. F. (1961), Abstracts of Papers, 140th Meeting of the American Chemical Society, p. 45C.