Biochemical Pharmacology, 1964, Vol. 13, pp. 353–360. Pergamon Press Ltd., Printed in Great Britain.

# INHIBITION BY PUROMYCIN AND ITS REVERSAL BY PEPTIDES*

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(*Received* 13 *June* 1963; *accepted* 19 *September* 1963)

**Abstract**—Purines and their derivatives did not reverse inhibition by puromycin of the growth of *Tetrahymena pyriformis* W. Certain crude materials were capable of reversing the inhibition, and their nature indicated that peptides might be active. An enzymatic digest of casein proved to be effective, as were certain dipeptides. Presence of an aromatic amino acid residue in the peptide is not required for activity. Peptides having glycine as the C-terminal residue are inactive; with glycine as the N-terminal, amino acid activity increases as the polarity of the peptide decreases. Peptides having a racemic mixture as the N-terminal group are inactive. It is suggested that puromycin interferes with the uptake of free amino acids but not that of peptides, and that intracellular peptides are hydrolysed by an aminopeptidase relatively specific for glycyl peptides. The free intracellular amino acids released by the peptidase are then able to exchange for free extracellular amino acids, a process presumably insensitive to puromycin.

PUROMYCIN has been found to be very potent as an amebacide[1] or trypanocide, although in the latter case the aminonucleoside derived from puromycin appears to be somewhat more effective.[2, 3] The aminonucleoside is equally as effective as puromycin as an amebacide.[4] The effects of puromycin against trypanosomes are said to be reversed by the simultaneous administration of adenine or adenine analogs. Guanosine, guanylic acid, adenosine, or adenylic acid is ineffective.[2] Reversal of the amebacidal effect could be obtained only with adenylic acid or partially with adenine.[4]

Bortle and Oleson[5] have reported that the growth of *Tetrahymena pyriformis* is inhibited by puromycin but neither by the aminonucleoside (except at very high concentrations) nor by methoxyphenylalanine. The inhibition by puromycin was said to be reversed by guanylic acid and less well by guanine or guanosine. In 1956 puromycin was tested against Tetrahymena in our laboratory as one of a series of compounds under study by the Cancer Chemotherapy National Service Center and found to be extremely inhibitory to growth.[6, 7] As a routine part of the investigation of these compounds the normal ingredients of the medium (including guanylic acid) were tested for their ability to reverse the effects of the most inhibitory compounds. None had any effects against puromycin. These results were at variance with those of Bortle and Oleson,[5] and it was therefore decided to continue the study of puromycin to determine whether or not we could reverse the inhibitory effect, using crude materials. The results indicated that peptides, among other things, were active.

* Supported by Grants AM 01005 and CA 02924 from the National Institutes of Health, U.S Public Health Service, and Grant T-130D from the American Cancer Society.

## MATERIALS AND METHODS

*T. pyriformis* W. was grown in medium A[8] containing Tween 80 at 10 mg/ml. To this medium were added the various substances* to be tested for antagonism of the inhibition due to puromycin. Growth was measured after 4 days at 25° with a Lumetron colorimeter with a red filter. All experiments were done in triplicate.

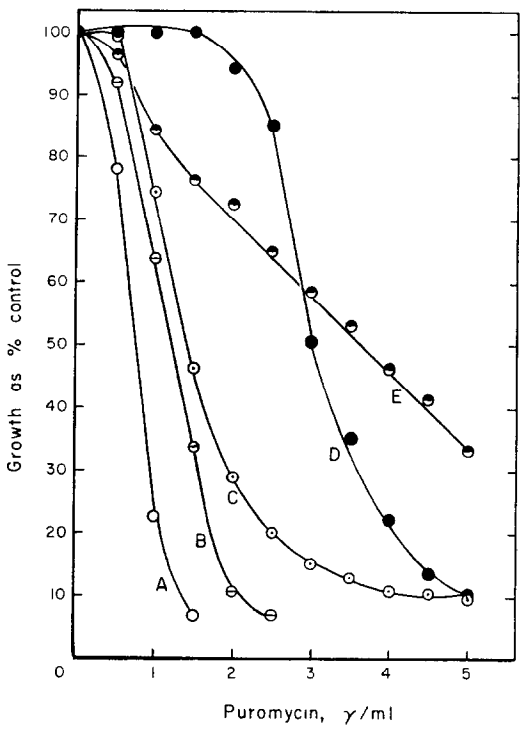


FIG. 1. Dose response to puromycin alone (A) or in the presence of yeast extract, 1 mg/ml (B); distillers solubles, 1 mg/ml (C); proteose-peptone, 1 mg/ml (E); or liver fraction L, 1 mg/ml (D).

## RESULTS

Since increasing the concentrations of amino acids, nucleic acid derivatives, or vitamins had no effect on growth in the presence of puromycin, the effect of crude materials on the inhibition was tested.† Their effect can be seen in Fig. 1. Both proteose

* The following peptides were obtained from Nutritional Biochemicals Corp.: DL-alanyl-DL-alanine, DL-alanyl-DL-asparagine, DL-alanylglycine, DL-alanylglycylglycine, DL-alanyl-DL-phenylalanine glycylglycine, glycylglycylglycine, glycyl-L-leucine, glycyl-L-phenylalanine, glycyl-DL-phenylalanine glycyl-L-tryptophan, glycyl-L-tyrosine, glycyl-DL-valine, L-histidyl-L-histidine, DL-leucyl-DL-leucine DL-leucyl-DL-phenylalanine, L-leucyl-L-tyrosine, and the enzymatic digest of vitamin-free casein. The Mann Laboratories supplied the following peptides: glycyl-DL-leucine, glycyl-D-leucine, glycyl DL-norleucine, glycyl-L-proline, DL-leucylglycine, D-leucylglycine, L-leucyl-L-leucine, L-leucyl-L phenylalanine, L-phenylalanylglycine, and L-phenylalanyl-L-phenylalanine. Glycyl-DL-alanine glycyl-DL-serine, and L-leucylglycine were products of H.M. Chemicals Ltd., California Foundation for Biochemical Research and of Hoffmann-La Roche Co. respectively. Proteose-peptone and yeast extract were obtained from Difco, liver fraction L from the Wilson Laboratories, and distillers' solubles from the Hiram Walker Co. N-acetyl-, N-propionyl-, and N-butyrylleucine were a gift of Dr. Alfred Gellhorn.

† It was later found that omission of Tween 80 from the medium decreased the inhibitory effect of puromycin; however, for the sake of uniformity Tween was present in all media reported here.

peptone and liver fraction L were active, but their modes of action must differ, to judge by the shapes of the response curves. Since it was felt that the feature common to these two materials was that they contain peptides, an enzymatic digest of casein was then tried. As may be seen (Fig. 2) this material is also active and resembles proteose-peptone in its effect. The reversal appears to be of the competitive type.

It was assumed from the structure of puromycin that reversal by casein digest might

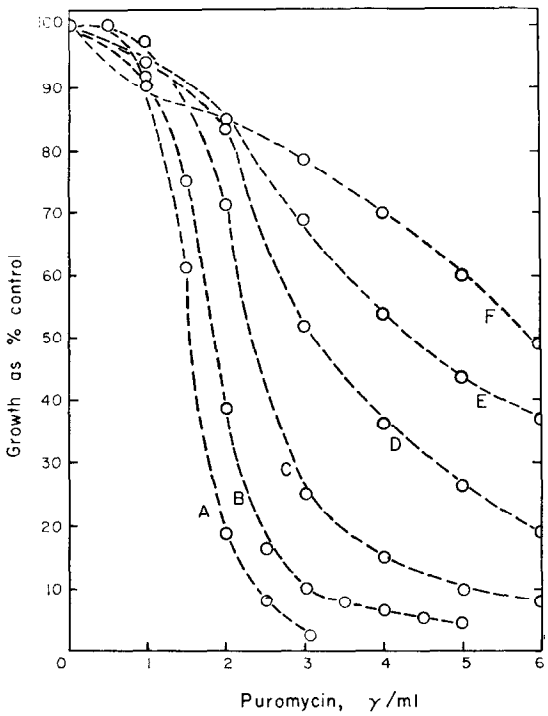


FIG. 2. Dose response to puromycin alone (A) or in the presence of an enzymatic digest of vitamin-free casein, 0·125 mg/ml (B); 0·25 mg/ml (C); 0·5 mg/ml (D); 1 mg/ml (E); and 2 mg/ml (F).

be due to the presence of peptides of phenylalanine and tyrosine in the mixture. Accordingly, simple dipeptides of these two amino acids containing either glycine or leucine as the other amino acid residue were tested. As controls, such peptides as leu-gly* or gly-leu were also included. The surprising lack of specificity of the responses led to the testing of a number of other peptides (see Tables).

The inhibition of the growth of Tetrahymena by puromycin tends to vary from one experiment to another. For example, the average values (with standard error of the mean) for a series of ten experiments were: 0·76 $\pm$ 0·14 $\mu$g/ml for 25 per cent inhibition, 1·09 $\pm$ 0·16 $\mu$g/ml for 50 per cent, and 1·43 $\pm$ 0·21 $\mu$g/ml for 75 per cent. In a second series of ten experiments the values were: 0·77 $\pm$ 0·29, 1·10 $\pm$ 0·35, and 1·42 $\pm$ 0·40

* Abbreviations used are alanine, ala; asparagine, asp$NH_2$; glycine, gly; histidine, his; leucine, leu; norleucine, norleu; phenylalanine, phe; proline, pro; serine, ser; tryptophan, try; tyrosine, tyr; valine, val.

respectively. For this reason the data in the tables are presented as per cent change in amount of puromycin required for a given degree of inhibition in the presence of a peptide as compared to a control run at the same time.

TABLE 1. EFFECT OF PEPTIDES CONTAINING ONLY NATURAL ISOMERS ON INHIBITION BY PUROMYCIN

| Per cent inhibition | 25 | | 50 | | 75 | |
|---|---|---|---|---|---|---|
| Peptide (μmole/ml) | 0·25 | 0·50 | 0·25 | 0·50 | 0·25 | 0·50 |
| | per cent change in amount required for inhibition compared to control* | | | | | |
| Gly-L-phe | 75 | 45 | 122 | 153 | 178 | 295 |
| L-ala-L-phe | 28 | 54 | 32 | 76 | 69 | 136 |
| L-leu-L-phe | 60 | 112 | 54 | 105 | 58 | 131 |
| L-phe-L-phe | 30 | 30 | 43 | 48 | 95 | 159 |
| L-phe-gly | 13 | 18 | 15 | 30 | 10 | 26 |
| Gly-L-tyr | 36 | 43 | 58 | 86 | 72 | 104 |
| L-leu-L-tyr | 25 | 46 | 49 | 88 | 51 | 79 |
| Gly-L-leu | 48 | 72 | 52 | 79 | 85 | 149 |
| L-leu-gly | 8 | 20 | 44 | 16 | 0 | 33 |
| L-leu-L-leu | 17 | 30 | 26 | 46 | 36 | 80 |
| Gly-L-pro | | 18 | | 9 | | 3 |
| Gly-L-try | | 89 | | 74 | | 104 |
| L-his-L-his | | 20 | | 10 | | 6 |
| Gly-gly | | 4 | | 15 | | 26 |
| L-ala-L-ala | | 5 | | 3 | | 10 |
| Gly-gly-gly | | 80 | | 82 | | 91 |

* These values were derived by dividing the amount of puromycin required for the given percentage of inhibition in the presence of the peptide by the amount of puromycin required for the same degree of inhibition in its absence (control). These figures were then converted to per cent by substracting 1·00 and multiplying by 100.

It may be seen from Table 1 that, while peptides containing an aromatic amino acid residue are active in reversal of inhibition, this is not absolutely essential, since gly-L-leu, gly-DL-leu and gly-DL-val (Table 3) have considerable activity. What appears to be of more importance is the nature and position of the amino acid residues. When glycine occupies the C-terminal position, activity is much reduced when compared to that of peptides in which glycine is N-terminal (e.g. gly-L-phe and L-phe-gly or gly-L-leu and L-leu-gly). On the other hand, peptides having N-terminal glycine are more active than those in which other amino acids occupy this position (e.g. gly-L-phe and L-leu-L-phe or gly-L-leu and L-leu-L-leu).

Additional evidence of the importance of the nature of the N-terminal residue may be seen in Table 2. The presence of a racemic mixture in this position completely eliminates the activity of the dipeptides. This is not the case with the tripeptide DL-ala-gly-gly.

Data are presented for only one concentration of these peptides, since the results were almost identical at other concentrations. Although the peptides containing a racemic acyl residue appear to increase the inhibitory effect of puromycin, it is doubtful that this inhibition is great enough to be significant. At the concentrations tested, these peptides alone have no effect on the growth of the ciliate.

In contrast, the nature of the C-terminal residue (Table 3) is of far less importance for activity. Even gly-D-leu has a trace of activity and gly-DL-norleu is surprisingly

active. It is the polarity of the C-terminal residue that appears to be most effective in determining activity. Increasing chain length and therefore increasing nonpolarity confers greater activity on the peptide.

While fatty acyl amino acids at high concentration (Table 2) show some activity against low concentrations of puromycin, they have little effect against higher concentrations (75 per cent inhibition). This is in contrast to the behavior of peptides, which are generally most active against the higher doses of the antibiotic.

TABLE 2. EFFECT OF COMPOUNDS HAVING A RACEMIC AMINO ACID OR A FATTY ACID AS THE ACYL RESIDUE OR BOTH RESIDUES ON INHIBITION BY PUROMYCIN

| Percent inhibition | 25 | 50 | 75 |
|---|---|---|---|
| | Per cent change in amount required for inhibition compared to control* | | |
| Peptide (0·5 μmole/ml) | | | |
| DL-ala-gly | −13 | −14 | −3 |
| DL-ala-DL-ala | −13 | −14 | −12 |
| DL-ala-DL-asp$NH_2$ | −13 | −9 | −1 |
| DL-ala-DL-phe | −11 | −5 | −1 |
| DL-leu-gly | −5 | −4 | −7 |
| DL-leu-DL-leu | −6 | −4 | −3 |
| DL-leu-DL-phe | −10 | −15 | −11 |
| (L-leu-L-phe; 0·125 μmole/ml | −50 | +44 | +34) |
| DL-ala-gly-gly | −29 | +79 | +83 |
| D-leu-gly | +11 | +2 | −1 |
| Acylaminoacids (1·0 μmole/ml) | | | |
| N-acetyl-leu | +64 | +30 | +5 |
| N-propionyl-leu | +58 | +26 | +3 |
| N-butyryl-leu | +57 | +26 | +3 |

* See footnote to Table 1.

TABLE 3. EFFECT OF COMPOUNDS HAVING A RACEMIC AMINO ACID OR AN UNNATURAL AMINO ACID AS THE C-TERMINAL RESIDUE ON INHIBITION BY PUROMYCIN

| Per cent inhibition | 25 | | 50 | | 75 | |
|---|---|---|---|---|---|---|
| Peptide, μmoles/ml | 0·25 | 0·50 | 0·25 | 0·50 | 0·25 | 0·50 |
| | Per cent change in amount required for inhibition compared to control* | | | | | |
| Gly-DL-ser | | 36 | | 17 | | 22 |
| Gly-DL-ala | | 49 | | 35 | | 47 |
| Gly-DL-val | | 57 | | 69 | | 120 |
| Gly-DL-leu | 50 | 94 | 56 | 104 | 70 | 126 |
| Gly-D-leu | 17 | 29 | 6 | 16 | 4 | 16 |
| Gly-DL-norleu | 14 | 56 | 8 | 37 | 4 | 34 |
| Gly-DL-phe | 88 | 128 | 78 | 118 | 80 | 137 |

* See footnote to Table 1.

## DISCUSSION

The mode of action of puromycin in amebae and trypanosomes appears to differ from that in the ciliate, since its effect can be reversed by purines in the first two cases but not in the third. However, amebae and trypanosomes are as sensitive, if not more

so, to the aminonucleoside derived from puromycin as they are to the parent compound. On the other hand Tetrahymena is almost completely insensitive to the aminonucleoside (unpublished results and Ref. 5). It might be expected, therefore, that inhibition of Tetrahymena by puromycin would be insensitive to purines. We have no explanation for the failure of our results to agree with those of Bortle and Oleson.[5]

Numerous reports[9-18] confirm the fact that puromycin inhibits protein synthesis both *in vitro* and *in vivo*. Yarmolinsky and de la Haba[9, 10] point out that the site of interference with protein synthesis by puromycin is in the transfer of amino acids from aminoacyl-s-RNA to m-RNA. According to Allen and Zamecnik[11] puromycin, as an analog of the aminoacyl end of s-RNA, inhibits incorporation by the fact that it itself, or the methoxyphenylalanyl residue, is incorporated into the growing polypeptide chains, some of which are then prematurely released from the ribosomes and some retained. This mode of action might also account for the increased deacylation of aminoacyl-s-RNA by ribosomes in the presence of puromycin described by Nathans and Lipmann.[12] It is difficult, however, to fit into this picture the fact that peptides can reverse the inhibition caused by puromycin. If only peptides that contain phenylalanine or tyrosine were active, the inhibition might be explained on the basis that puromycin is a structural analog of these peptides. The activity of such compounds as gly-leu or gly-val precludes the acceptance of this hypothesis.

The structural requirements for activity of a dipeptide in reversing the inhibition by puromycin are: (1) The acyl residue of the peptide should be an amino acid (fatty acyl amino acids are less active). (2) The natural form of the amino acid must be present as the N-terminal (acyl) residue. (3) the N-terminal residue should preferably be glycine. (4) The C-terminal residue should be relatively nonpolar.

These structural requirements suggest that an enzymatic activity is involved and that the enzyme may be an aminopeptidase or a glycine aminopeptidase.

Puromycin itself may be regarded as a peptide in which the amino acyl residue is methoxyphenylalanine and the C-terminal residue the aminonucleoside. This structure is not at all comparable with the structures of the active peptides. It would seem unlikely therefore that puromycin is competing with the dipeptides for the "glycine aminopeptidase" postulated. Indeed it is difficult to envision that such a competition would be deleterious to the growth of the cell in a medium composed of free amino acids.

Another possibility is that puromycin is denied access to the interior of the cell in the presence of peptides which compete with the antibiotic for active sites on the cell surface. The relative lack of stereospecificity of the access mechanisms for amino acids in various types of cell[19] argues against such a mode of action but does not exclude it, since the stereochemistry of the access mechanism for dipeptides has not been described.

Leach and Snell[20] have shown that in the case of *Lactobacillus casei* peptides and free amino acids enter the cell by different routes and that there is no competition between these two types of compound for uptake. If one makes the assumption that puromycin inhibits amino acid uptake generally, perhaps by interfering with the $K^+$ exchange diffusion,[19] but does not affect the uptake of peptides, the reversing activity of peptides could be explained. Peptides that had gained access to the cell interior would be hydrolyzed by the aminopeptidase, thus making available free amino acids which could be used either for protein synthesis or for exchange diffusion with other

amino acids in the medium. The activity of a given dipeptide would then depend upon its suitability as a substrate for the aminopeptidase and the ability of its components to undergo exchange diffusion. It is in this last reaction that the polarity of the amino acids involved would play a part, since diffusion through lipid membranes would occur most readily with nonpolar compounds.

The data presented here agree reasonably well with those of Jacquez[21] on amino acid exchange diffusion in Ehrlich ascites cells. In these cells glycine, alanine, serine, and proline do not exchange with extracellular tryptophan. We have found the glycyl peptides of these amino acids to be of a very low activity against puromycin. Oxender and Christensen[22] report that in ascites cells glycine and amino isobutyric acids do not undergo exchange diffusion to an appreciable extent, whereas the less polar compounds (valine, leucine, and methionine) do so readily. Such a mechanism would explain the lack of activity of gly-gly against puromycin as well as the relative effectiveness of gly-leu.

The activity of the two tripeptides included in this study cannot be explained by the same mechanism as that proposed for the dipeptides. Perhaps a peptidase of different specificity could be invoked, but there are not sufficient data to justify drawing any conclusions.

It is of interest to note that lipid materials have been implicated in amino acid metabolism and protein synthesis in protoplast membranes[23, 24] and hen oviduct minces.[25, 26] Lipid amino acid complexes have been described from a number of tissues,[27–31] and it has been suggested that such complexes are involved in membrane permeability to amino acids.[32] If puromycin interferes with the formation of the amino acid–lipid carrier it must do so at the exterior of the cell unless a different (insensitive) type of carrier transporting amino acids from the inside out, as in exchange diffusion, is postulated. An aminopeptidase has been found associated with the membrane fraction of *Escherichia coli*,[33] and an aminopeptidase isolated from kidney particulates[34] contained 30 per cent lipid of which 70 per cent was phospholipid, suggesting that it was derived from a membranous structure in the cell.

Since the inhibition of growth by puromycin is considerably more sensitive than the inhibition of ribosomal protein synthesis (ca. 10 mμmole/ml as compared to ca. 200 mμmole/ml), there is a possibility that puromycin exerts inhibitory effects at more than one site. This is supported also by the fact that liver fraction L gives a type of reversal curve different from that of peptides or peptide mixtures. The two sites of action cannot be entirely unrelated and differ only in relative sensitivity to the antibiotic and in their responses to different types of reversing compounds. Thus, peptides are relatively ineffective at a site inhibited by low concentrations of puromycin and relatively more effective than liver extract at a site sensitive to higher concentrations of the antibiotic. The nature of the active compound(s) present in liver is under investigation.

## REFERENCES

1. D. J. Taylor, J. F. Sherman and H. W. Bond, *J. Amer. chem. Soc.* **76**, 4497 (1954).
2. R. J. Hewitt, A. R. Gumble and W. S. Wallace, *Antibiot. and Chemother.* **4**, 1222 (1954).
3. E. J. Tobie and B. Highman, *Amer. J. trop. Med. Hyg.* **5**, 504 (1956).
4. M. Nakamura and S. Jonsson, *Arch. Biochem.* **66**, 183 (1957).
5. L. Bortle and J. J. Oleson, *Antibiot. Ann.* 770 (1954–55).

6. G. E. FOLEY, R. E. MCCARTHY, V. M. BINNS, E. E. SNELL, B. M. GUIRARD, G. W. KIDDER, V. C. DEWEY and P. S. THAYER, *Ann. N.Y. Acad. Sci.* **76**, 413 (1958).
7. G. E. FOLEY, H. EAGLE, E. E. SNELL, G. W. KIDDER and P. S. THAYER. *Ann. N.Y. Acad. Sci.* **76**, 952 (1958).
8. V. C. DEWEY, R. E. PARKS, JR. and G. W. KIDDER, *Arch. Biochem.* **29**, 281 (1950).
9. M. B. YARMOLINSKY and G. L. DE LA HABA, *Proc. Nat. Acad. Sci. (Wash.)* **45**, 1721 (1959).
10. M. B. YARMOLINSKY and G. L. DE LA HABA, *Antimicrobial Agents Ann.* 320 (1960).
11. D. W. ALLEN and P. C. ZAMECNIK, *Biochim. biophys. Acta* **55**, 865 (1962).
12. D. NATHANS and F. LIPMANN, *Proc. nat. Acad. Sci. (Wash.)* **47**, 497 (1961).
13. L. BOSCH and H. BLOEMENDAL, *Biochim. biophys. Acta* **51**, 613 (1961).
14. J. COHEN, A. R. BRENNEMAN and Y. J. TOPPER, *Biochim. biophys. Acta* **63**, 554 (1962).
15. A. M. NEMETH and G. DE LA HABA, *J. biol. Chem.* **237**, 1190 (1962).
16. J. J. FERGUSON, JR., *Biochim. biophys. Acta* **57**, 616 (1962).
17. M. RABINOVITZ and J. M. FISHER, *J. biol. Chem.* **237**, 477 (1962).
18. A. J. MORRIS and R. S. SCHWEET, *Biochim. biophys. Acta* **47**, 415 (1961).
19. H. N. CHRISTENSEN, *Advanc. Protein Chem.* **15**, 239 (1960).
20. F. R. LEACH and E. E. SNELL, *J. biol. Chem.* **197**, 791 (1952).
21. J. A. JACQUEZ, *Biochim. biophys. Acta* **71**, 15 (1963).
22. D. L. OXENDER and H. N. CHRISTENSEN, *Nature (Lond.)* **197**, 765 (1963).
23. G. D. HUNTER, P. BROOKES, A. R. CRATHORN and J. A. V. BUTLER, *Biochem. J.* **73**, 369 (1959).
24. G. D. HUNTER and R. A. GOODSALL, *Biochem. J.* **78**, 564 (1961).
25. R. W. HENDLER, *Biochim. biophys. Acta* **49**, 297 (1961).
26. R. W. HENDLER, *Biochim. biophys. Acta* **60**, 90 (1962).
27. W. L. GABY, R. N. NAUGHTEN and C. LOGAN, *Arch. Biochem.* **82**, 34 (1959).
28. W. L. GABY and R. SILBERMAN, *Arch. Biochem.* **87**, 188 (1960).
29. W. L. GABY, H. L. WOLIN and I. ZAJAC, *Cancer Res.* **20**, 1508 (1960).
30. J. L. HAINING, T. FUKUI and B. AXELROD, *J. biol. Chem.* **235**, 160 (1960).
31. J. WESTLEY, J. J. WREN and H. K. MITCHELL, *J. biol. Chem.* **229**, 131 (1957).
32. R. W. HENDLER, *Nature (Lond.)* **193**, 821 (1962).
33. A. T. MATHESON, *Canad. J. Biochem.* **41**, 9 (1963).
34. D. S. ROBINSON, S. M. BIRNBAUM and J. P. GREENSTEIN, *J. biol. Chem.* **202**, 1 (1953).