Myocardial Metabolism
V. Effect of Puromycin on Protein Synthesis and Oxidation of Glucose, Acetate, and Aspartate in Perfused Rat Hearts[1] (34199)

C. CARSON AND GEORGE V. VAHOUNY

Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C. 20005

During studies on membrane and cellular mechanisms of lipid transport, it was of importance to determine whether protein synthesis was intimately involved in these processes. Puromycin, an inhibitor of amino acid incorporation into protein (1), has been used to determine the role of protein synthesis in lipid transport processes (2). However, this and other inhibitors were shown to produce metabolic changes which either precede or are unrelated to inhibition of protein synthesis. Several laboratories reported that increases in glycogen metabolism due to puromycin administration were unrelated to the effects on protein synthesis (3–5). Appelman and Kemp (6), furthermore, showed that puromycin inhibits cyclic AMP phosphodiesterase in rat diaphragm, resulting in increased cyclic nucleotide levels which in turn activate phosphorylase β-kinase and increase glycogenolysis. It was shown that inhibition of liver protein synthesis by ethionine is secondary to a decrease in the concentration of ATP (7, 8), due in part to inhibition of oxidative phosphorylation (8). Puromycin also causes swelling of neuronal mitochondria due presumably to the formation of peptidyl-puromycin (9). Since many agents which uncouple oxidative phosphorylation also show this effect on mitochondrial swelling (10), it was important to determine the effect of this protein synthesis inhibitor on heart metabolism before studies on myocardial lipid transport processes could be initiated.

Methods and Materials. Glucose-U-^{14}C, sodium acetate-1-^{14}C and aspartic acid-1-^{14}C were obtained from Amersham (Nuclear Chicago Corp.). Enzymes, cofactors and substrates for measurement of tissue aspartic acid were obtained from Calbiochem or P-L Biochemicals. Puromycin was purchased from Nutritional Biochemicals Corp.

Perfusion technique and experimental design. The perfusion medium consisted of modified Krebs bicarbonate buffer (1.27 m*M* Ca^{2+}), pH 7.4. containing either 9 m*M* glucose-U-^{14}C, 10 mM sodium acetate-1-^{14}C or tracer levels of aspartic acid-1-^{14}C. In certain groups, 0.46 m*M* puromycin was included in the media at the beginning of perfusion.

The method of heart perfusion in the open recirculation apparatus has been reported (11). Male albino rats (200–250 g) of the Carworth Wistar strain were fed *ad libitum* prior to use. After sacrifice, the heart was removed, placed in 37° buffer, attached to the perfusing cannula, and perfused with 5 ml of buffer which had previously been equilibrated with O_2–CO_2 (95:5, v/v). Perfusion was carried out for 60 min during continuous gassing with O_2–CO_2. The effluent gasses were passed through 6 *N* KOH to trap evolved $^{14}CO_2$.

Samples were removed from the perfusate initially and at 15-min intervals for determination of substrate uptake and $^{14}CO_2$ production. Heart rate and coronary flow were monitored throughout the perfusion (11), and heart rates greater than 150 beats/min, and flows of 5 ml/min or greater, were considered satisfactory.

Extraction of protein and analysis of leucine-1-^{14}C incorporation. Following perfusions with leucine-1-^{14}C, 5 ml of Krebs buffer

[1] This study was supported in part by USPHS Grants HE-09489 and HE-5311.

were rapidly forced through the coronary vessels to remove the labeled substrate, and the heart was rapidly weighed, minced, and homogenized in 9 vol of 5% trichloroacetic acid (TCA). The protein was sedimented by centrifugation at 1000 *g* for 10 min, and the precipitate was resuspended in 10 ml of 5% trichloracetic acid. This was maintained at 90° for 15 min, recentrifuged and the precipitate was washed twice with hot 5% TCA. The DNA-free precipitate (12) was resuspended in 5 ml of ethanol–ether (3:1, v/v), maintained at 50° for 10 min to remove lipid, and recentrifuged. The precipitate, now free of DNA and lipid, was dissolved in distilled water containing 1 drop of 0.1 *N* NaOH. Aliquots of this solution were used for protein determination according to Lowry *et al.* (13) and for isotope analysis by liquid scintillation counting (14).

Determination of substrate uptake and oxidation. Glucose was determined using the anthrone method of Carroll *et al.* (15), after extraction of 0.5 ml of perfusion medium in 9 vol of 5% TCA and removal of protein.

Perfusate acetate-1-^{14}C was determined by isotope analysis after acidification of the sample to eliminate $^{14}CO_2$. The method was verified by thin-layer silicic acid chromatography of the perfusate in a solvent system of ethanol:ammonium hydroxide (100:1, v/v) and quantitative recovery of isotope in the silicic acid area corresponding to authentic acetate. Perfusate aspartic acid-1-^{14}C and leucine-1-^{14}C were determined similar to the method for acetate.

Samples of the perfusate (1 ml) and the NaOH trap (1 ml) were acidified with 2 ml of 6 *N* H_2SO_4, and $^{14}CO_2$ was trapped in hyamine hydroxide as described by Cuppy and Crevasse (16). This was counted in a Nuclear Chicago liquid scintillation spectrometer (model 720), using 10 ml of scintillant mixture containing 4 g of 2,-5-diphenyloxazole and 500 mg of *p*-bis-2′-(5′-phenyloxazolyl)benzene/liter of toluene.

Extraction and determination of tissue aspartate. Following removal of residual labeled perfusate in the coronary arteries as described earlier, the heart was weighed and minced in 4.5 ml of 7% ice-cold perchloric acid. The mixture was homogenized and then sonicated (MSE ultrasonic disintegrator) for 30 sec in an ice bath. The supernatant fluid from centrifugation was neutralized to pH 7.0 with 5 *N* KOH and allowed to stand for 10 min in an ice bath. The precipitate of potassium perchlorate was removed by centrifugation and discarded. The L-aspartic acid was determined on this final supernatant by using coupled reactions involving conversion of aspartate to oxalacetate in the presence of excess α-ketoglutarate and glutamic-oxalacetic transaminase, and the subsequent conversion of oxalacetate to malate in the presence of malic dehydrogenase and NADH (17). Oxidation of NADH was determined spectrophotometrically at 366 mμ. The procedure was standarized by recovery of authentic aspartate from heart homogenates.

Calculations. All counts were corrected to dpm by external standardization and the channels ratio method (17). Glucose and acetate uptakes are expressed as micromoles of substrate taken up/g of heart/30 min. Oxidation is expressed as micromoles of glucose or acetate equivalents, or as percentage. All values represent means ± SEM.

Results and Discussion. In the initial study, the effect of circulating puromycin on protein synthesis in the isolated perfused rat heart was determined. Hearts were perfused in the continuous recirculation apparatus with Krebs bicarbonate buffer, pH 7.4, containing 9 m*M* glucose and, in the experimental group, 0.46 m*M* puromycin. After 30-min perfusion, leucine-1-^{14}C (8×10^5 dpm) was introduced directly into the perfusate entering the heart, and recirculation was continued for an additional 30 min. As shown in Table I, total protein in both groups was identical, and these values compare favorably with those obtained in earlier studies (18). Puromycin also had no effect on the percentage uptake of labeled leucine during the second 30 min. of perfusion. However, incorporation of labeled leucine was markedly depressed from 55% of the amino acid uptake in controls, to 2% in the puromycin-perfused hearts. Despite this marked inhibition of protein synthesis, there was no apparent effect of puromycin on either heart rate

TABLE I. Inhibition of Protein Synthesis in Isolated Perfused Rat Heart.

Perfusion conditions[a]	Total protein (%)	Leucine uptake (%) (30 min)	Uptake (%) into protein	Sp act (dpm /mg of protein)
Leucine-1-^{14}C (final 30 min)	12.8 ± 1.3[b]	20.8 ± 4.0	55.4 ± 7.9	741 ± 12
Puromycin (60 min) + leucine-1-^{14}C (final 30 min)	12.7 ± 0.3	20.8 ± 2.0	1.7 ± 0.2	25 ± 4
Significance	NS	NS	$p < 0.01$	$p < 0.01$

[a] Perfusion media consisted of modified Krebs bicarbonate buffer (1.27 m*M* Ca^{2+}), pH 7.4 containing 9 m*M* glucose. In the experimental group perfusion, the medium also contained 0.46 mm puromycin HCl. Perfusions were carried out for 60 min at 37° and 50 mm Hg pressure. After 30 min, leucine-1-^{14}C (8×10^6 dpm) was added to the circulating perfusates and perfusion was continued for 30 min. Removal of heart and extraction and analysis of protein are described in the text.

[b] Values represent means from 6 hearts ± SEM.

(150 beats/min) or coronary flow (5 ml/min). Although no studies have been conducted on the size and turnover of the leucine pool, these data indicate that it is possible to markedly inhibit synthesis of protein without affecting energy metabolism, myocardial contractility or coronary flow during the experimental period used (60 min). Similar conclusions have been drawn from studies on inhibition of intestinal mucosal protein synthesis required for lipid transport (2, 19) but not essential for other functional requirements (2).

Using the same experimental design as above, the myocardial extraction and oxidation of 9 mM glucose-U-^{14}C was studied in the absence and presence of 0.46 m*M* puromycin. The data for glucose uptake and oxidation to $^{14}CO_2$ during 60-min perfusion (Table II) are comparable to those reported earlier (18), and indicate that about 37% of the available circulating glucose was extracted during 60-min perfusion (68 μmoles/g of tissue/hr). Of this, about 12% was recovered as $^{14}CO_2$. The presence of puromycin in the perfusate resulted in almost twice the uptake of glucose (62%) and an almost threefold increase in the level of oxidation to $^{14}CO_2$. As shown in Fig. 1, the effect of puromycin on glucose extraction and oxidation was evident throughout the experimental period.

In order to determine whether puromycin directly influences glucose uptake and/or the rate of glycolysis, or whether these effects were secondary to an increase in tricarboxylic acid cycle activity, a similar experiment was performed using 10 m*M* acetate as substrate. Thus, any effect of puromycin on phosphorylation of glucose or glycolysis was circumvented. As reported elsewhere (20), myocardial uptake of acetate and subsequent oxidation to $^{14}CO_2$ proceed more rapidly than for glucose. Thus, about 46% of the available acetate, or 102 μmoles were extracted/hr and of this, 41% was recovered as $^{14}CO_2$. Addition of puromycin to the circulating medium resulted in an increase in ace-

TABLE II. Effect of Puromycin on Myocardial Extraction and Oxidation of Glucose-U-^{14}C and Acetate-1-^{14}C.

Perfusion conditions[a]	Substrate uptake (μmoles/g/hr)	Substrate oxidation (μmoles/g/hr)	% of Uptake
Glucose-U-^{14}C, 9 m*M*	67.5 ± 3.9	8.4 ± 0.5	12.4 ± 0.7
Glucose + 0.46 m*M* puromycin	113.5 ± 8.2[b]	23.9 ± 1.6[b]	21.1 ± 0.8[b]
Acetate-1-^{14}C, 10 m*M*	101.7 ± 2.6	41.9 ± 2.5	40.7 ± 0.1
Acetate + 0.46 m*M* puromycin	127.5 ± 3.1[b]	58.3 ± 1.5[b]	45.8 ± 0.9[b]

[a] Conditions are described in Table I and the text.

[b] $p < 0.01$ from appropriate controls.

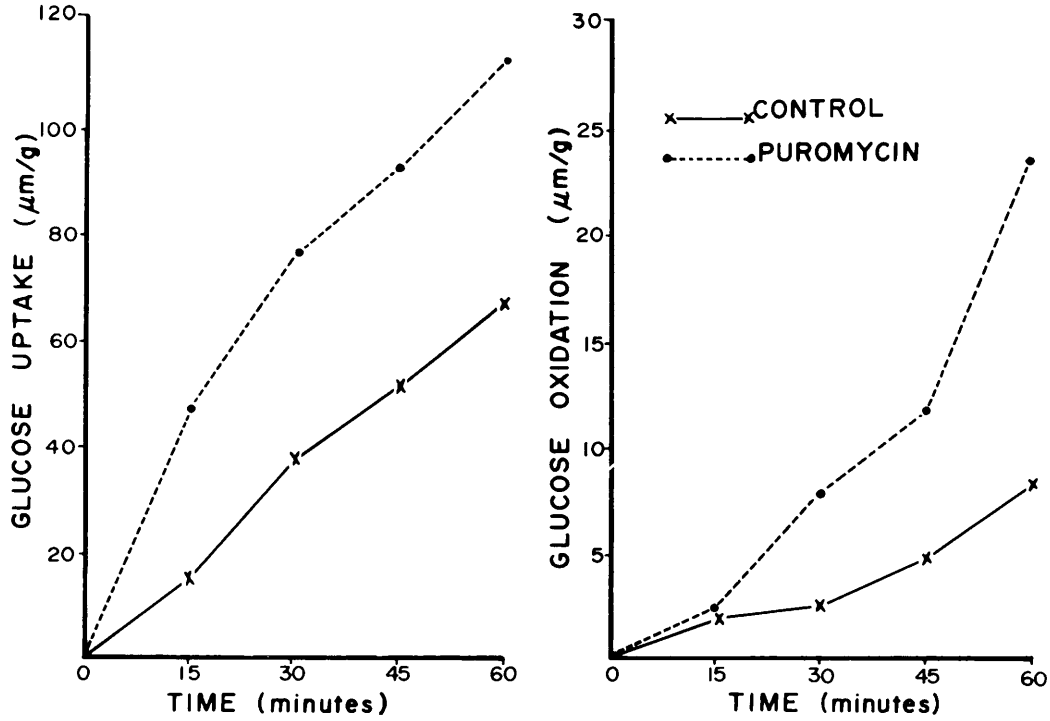

FIG. 1. Effect of 0.46 m*M* puromycin on myocardial extraction and oxidation of 9 m*M* glucose-U-^{14}C. Experimental details are given in Tables 1 and 2 and the text.

tate uptake of about 25% and an increase in acetate oxidation of about 14%. For all data, the effect of puromycin was highly significant, but was not as marked with acetate as with glucose due to the already high levels of uptake and oxidation. It is, however, still possible that puromycin may have a direct effect on glucose uptake and/or on glycolysis which can only be shown by studies directed at these points.

The present study indicated a direct effect of puromycin on Krebs cycle activity and further evidence for this conclusion was obtained by experiments using aspartate-1-^{14}C. Since this amino acid is an immediate precursor of oxalacetate, a Krebs cycle intermediate, increased oxidation of aspartate-1-^{14}C should occur if: (a) transamination of aspartate to oxalacetate was not limiting; and (b) the labeled amino acid was not diluted by increased endogenous aspartate due to puromycin inhibition of protein synthesis. The results, summarized in Table III, indicate that no significant differences in tissue aspartate levels occurred between the control and puromycin-perfused groups. Puromycin resulted in a highly significant increase in aspartate-1-^{14}C uptake by perfused hearts and an even greater increase in oxidation of the extracted amino acid (from 47 to 64%).

Bowman (21) has shown that labeled aspartate is incorporated into malate and citrate by perfused rat hearts, despite an earlier report that the penetration of aspartate is poor (22). However, the addition of 1.5 m*M* L-aspartate to perfusion media containing 4 m*M* acetate had no effect on the acetylation state of CoA, despite the fact that aspartate is a precursor of oxalacetate (23). The similar levels of tissue aspartate in control and puromycin perfused hearts in the present study make it appear highly unlikely that increased oxalacetate levels are responsible for the increased Krebs cycle activity. However, these studies provide no data on turnover rates of this intermediate. Based on the reports of others (7–9), showing the effect of puromycin on mitochondrial swelling and of

TABLE III. Effect of Puromycin on Myocardial Extraction and Oxidation of Aspartic-1-^{14}C Acid.

Perfusion conditions[a]	Tissue aspartic acid (μmoles)	Aspartate-1-^{14}C uptake (%)	% of Uptake to $^{14}CO_2$
Nonperfused	5.31 ± 0.02		
Aspartic-1-^{14}C acid (5 μCi)	5.25 ± 0.23	33.9 ± 0.03	46.9 ± 1.0
Aspartic acid + 0.46 m*M* puromycin	4.99 ± 0.09	40.6 ± 0.6	63.9 ± 1.9
Significance	NS	$p < 0.01$	$p < 0.01$

[a] Conditions are described in Table I and the text. Following perfusion (60 min), hearts were flushed with Krebs bicarbonate buffer, pH 7.4, weighed and homogenized in 4.5 ml of ice-cold 7% perchloric acid. Protein was removed by centrifugation and following neutralization of the supernatant to pH 7.0, the precipitate of potassium perchlorate was removed by centrifugation and discarded. Aspartate was determined on the supernatant by spectrophotometric determination of NADH oxidation during conversion of aspartate to malate (17).

ethionine on uncoupling oxidative phosphorylation, it appears more likely that increases in catabolism of glucose, acetate, and aspartate due to puromycin are a reflection of increased energy requirements of the tissue due to interference with ATP production in mitochondria.

Summary. The recirculation with 0.46 m*M* puromycin in Krebs bicarbonate buffer containing 9 m*M* glucose had no effect on the myocardial uptake of leucine-1-^{14}C, but depressed leucine incorporation into protein from 55 to 2%. The rate of myocardial contraction and coronary flow were unaffected. Myocardial removal of glucose from the medium was increased from 70 μmoles/hr to 114 μmoles/hr, and the oxidation of glucose to $^{14}CO_2$ was increased from 8 to 24 μmoles/hr. To eliminate glycolysis as a site of puromycin action, similar studies were carried out with perfusions of 10 mM acetate. As in the case of glucose perfusion, the circulation of puromycin stimulated acetate uptake from 100 to 128 μmoles and increased acetate oxidation from 42 to 58 μmoles. Since the data suggested an effect of puromycin on mitochondrial Krebs cycle activity, labeled aspartate was perfused in the presence of acetate and puromycin. There was a 20% increase in uptake and a 30% increase in the oxidation of aspartate due to puromycin perfusion. However, there was no change in the levels of endogenous aspartate. The results suggest that puromycin affects mitochondrial oxidative pathways, either directly or via an effect on mitochondrial permeability and oxidative phosphorylation.

1. Yarmolinsky, M. and De la Haba, G., Proc. Natl. Acad. Sci. U. S. **45**, 1721 (1959).

2. Sebesin, S. M. and Isselbacher, K. J., Science **147**, 1149 (1965).

3. Hofert, J. and Boutwell, R. K., Arch. Biochem. Biophys. **103**, 338 (1963).

4. Garren, L. D., Davis, W. W., Crocco, R. M., and Ney, R. L., Science **152**, 1386 (1966).

5. Sovik, O., Acta Physiol. Scand. **66**, 307 (1966).

6. Appleman, M. M. and Kemp, R. G., Biochem. Biophys. Res. Commun. **24**, 564 (1966).

7. Villa-Trevino, S., Shull, K. H., and Farber, E., J. Biol. Chem. **238**, 1757 (1963).

8. Singal, S. A., Knowles, V., Marks, W., Taylor, W., and Agee, J., Federation Proc. **24**, 362 (1965).

9. Gambette, P. and Gonatas, N. K., Science **161**, 900 (1968).

10. Weinbach, E. C., Garbus, J., and Sheffield, H. G., Exptl. Cell Res. **46**, 129 (1967).

11. Vahouny, G. V., Katzen, R., and Entenman, C., Proc. Soc. Exptl. Biol. Med. **121**, 923 (1966).

12. Siekevitz, P., J. Biol. Chem. **195**, 549 (1952).

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

14. Bray, G. A., Anal. Biochem. **1**, 279 (1960).

15. Carroll, N. V., Longley, R. W., and Roe, J. H., J. Biol. Chem. **220**, 583 (1956).

16. Cuppy, D. L. and Crevasse, L. E., Anal. Biochem. **5**, 462 (1963).

17. Pfleiderer, G., *in* "Methods of Enzymatic Anasis" (H. U. Bergmeyer, ed.), p. 381. Verlag Chemie, Berlin; and Academic Press, New York (1963).

18. Vahouny, G. V., Liljenquist, J., Wilson, R., Liao, A., and Rodis, S. L., Arch. Biochem. Biophys. **125**, 809 (1968).

19. Vahouny, G. V., Ito, M., and Treadwell, C. R., Federation Proc. **28**, 687 (1969).

20. Rodis, S. L., D'Amato, P. H., Koch, E., and Vahouny, G. V., Arch. Biochem. Biophys., in press.

21. Bowman, R. H., J. Biol. Chem. **241**, 3041 (1966).

22. Hicks, R. M. and Kerly, M., J. Physiol. **150**, 621 (1960).

23. Pearson, D. J. and Tubbs, P. K., Biochem. J. **105**, 953 (1967).

Received April 9, 1969. P.S.E.B.M., 1969, Vol. 132.