Effect of Polyuridylic and Polyadenylic Acids on Protein Synthesis in Isolated Rat Liver Mitochondria

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A study was made of the effects of polyuridylic and polyadenylic acids on the incorporation of labeled L-phenylalanine, L-lysine, and labeled amino acid mixture into protein by isolated rat liver mitochondria to elucidate the role of extramitochondrial messenger ribonucleic acids in the mitochondrial protein synthesis. Furthermore the uptake of labeled polyuridylic acid by mitochondria was followed. The effects of polyuridylic acid on the respiration and oxidative phosphorylation in the mitochondrial preparations were also controlled.

The addition of polyuridylic acid (up to 200 μg/ml) to reaction mixtures containing either no other amino acids but the tritiated L-phenylalanine, one amino acid (L-glutamic acid) or a balanced amino acid mixture had neither a stimulating nor an inhibiting effect on the incorporation of L-phenylalanine into mitochondrial protein, which was very slow in amino acid poor media and about four times higher in a balanced amino acid medium. Neither had the addition of similar amounts of polyadenylic acid any effect on the mitochondrial incorporation of L-lysine. Both polyuridylic and polyadenylic acids were unable to stimulate the incorporation of the labeled amino acid mixture. But the liver cell sap and its pH 5 fraction had a slight stimulating effect on the incorporation of L-phenylalanine by the isolated mitochondria. The labeled polyuridylic acid added to the different reaction mixtures caused only a minimal labeling of mitochondria which was not accessible to ribonuclease.

The metabolism of isolated mitochondria followed by the oxygen consumption, the coupling of oxidative phosphorylation and the swelling degree during the incubation was not affected by polyuridylic acid (up to 200 μg/ml).

The present results indicate that synthetic ribonucleic acids having messenger activity in ribosomes cannot guide the protein synthesis in isolated mitochondria, having on the other hand no harmful effects on mitochondrial metabolism.

Isolated mitochondria have been shown to incorporate amino acids into protein, but only the structural and some enzyme proteins become labeled.[1-3] Soluble proteins like cytochrome *c* have been reported to be synthesized in the

cytoplasm and to be transferred later into mitochondria.[3-4] Thus some of the mitochondrial proteins may have their genetic codes in the mitochondrial deoxyribonucleic acid (DNA), some in the nuclear DNA. On the basis of theoretical calculations, the mitochondrial DNA content is too low to carry the codes of all the mitochondrial proteins.[5] Humm and Humm [6] reported that a fraction of the mitochondrial ribonucleic acid (RNA) hybridized with the nuclear DNA and suggested that the mitochondrial protein synthesis might be controlled by messenger RNA's synthesized in the nucleus. An earlier report provides supporting data, since the stimulating effect of the cell sap on the mitochondrial protein synthesis was found to be ribonuclease sensitive.[7]

The aim of the present study was to elucidate the role of extramitochondrial messenger RNA's (polyuridylic and polyadenylic acids) on protein synthesis in isolated rat liver mitochondria.

MATERIAL AND METHODS

Eighty approximately three months old male Wistar rats (180−220 g) fed *ad libitum* were used. They were stunned by a blow on the head and bled by cutting the abdominal aorta. The liver was made free and cooled in ice cold 0.25 M sucrose solution. (All the subsequent steps were carried out at 4°C.) The liver was quickly weighed on a torsion balance, and it was homogenized in ten times its weight of 0.25 M sucrose with five rapid down and up motions in a Potter-Elvehjem homogenizer driven by a drill motor (500 rpm) and equipped with a loosely fitting Teflon pestle. The vessel was kept in crushed ice. The unbroken cells and nuclei were spinned down at 700 g, 10 min, and the mitochondria were sedimented at 5000 g, 10 min, from the supernatant fraction. Mitochondria were washed twice by resedimenting after which they were resuspended so that the protein content was 20−30 mg/ml determined by the biuret reaction.[8] Preparations for electron microscopy were also made.

The incorporation of L-phenylalanine was followed in an amino acid poor reaction mixture (A): potassium phosphate buffer (pH 7.4) 20 mM, KCl 50 mM, MgCl$_2$ 8 mM, adenosine triphosphate (sodium salt, Sigma Chemical Company, St.Louis, Mo, U.S.A.) 2 mM, sucrose 75 mM, glucose 30 mM, hexokinase (Type II from yeast, Sigma) 0.6 mg, uniformly tritiated L-phenylalanine (specific activity 750 Ci/mole, The Radiochemical Centre, Amersham, England) 0.011 mM, substrate (succinate or L-glutamate brought with potassium hydroxide to pH 7.4) 14 mM and an amount of mitochondria corresponding to 2 mg of protein. Unlabeled polyuridylic acid (ammonium salt, S=6.16, Miles Chemical Company, Eckhard, Ind., U.S.A.) was added in amounts of 25 to 200 μg/ml. The final volume was 1 ml.

In addition an amino acid rich reaction mixture (B) [9] was used. One mg of a balanced amino acid mixture [10] containing L-leucine in 0.5 molar proportion and lacking either L-phenylalanine or L-lysine was added into each reaction mixture followed by either tritiated L-phenylalanine as described above or an equal amount of tritiated L-lysine (as monohydrochloride, specific activity 256 Ci/mole, The Radiochemical Centre) and polyuridylic acid as described above or polyadenylic acid (potassium salt, S=13.1, Miles Chemical Co.). In some experiments (C) the amino acid mixture was replaced by labeled amino acids, 14 nCi per reaction mixture (CFB. 104-amino-acids-C^{14} mixture, The Radiochemical Centre), and succinate (14 mM) was added to promote respiration.

The reactions, which were made in duplicate or triplicate, were carried out in open centrifuge tubes at 37°C in a shaker, which made 150 oscillations (27 mm) per min. The reactions were stopped after different time intervals by adding 2 ml of a 0.5 N perchloric acid solution containing unlabeled DL-phenylalanine or L-lysine or balanced amino acid mixture, respectively, (200 mg/l). The precipitates were spun down at room temperature, and washed twice by resuspending in the perchloric acid-amino acid solution after which they were dissolved in 1 ml of 1 N NaOH by incubating for 1 h at 37°C. Reprecipitation was performed by adding 2 ml of 6 N HCl and keeping the tubes 5 min

in boiling water. The precipitates were spun down and washed by resuspending subsequently with acetone, ethanol-ether (3:1, v/v) and ether, 2 ml of each. For counting, the precipitates were dissolved by adding 0.2 ml of a 1 M solution of Hyamine hydroxide in methanol (Nuclear Enterprises Ltd., Edinburgh, Scotland) and warming to 60°C for 1—2 h with occasional agitation after which they were transferred into counting vessels by adding 0.5 ml of methanol and 5 ml of scintillation medium (4 g of PPO (2,5-diphenyloxazolone) and 0.1 g of POPOP (1,4-bis[2-(4-methyl-5-phenylazolyl)]benzene, obtained from Beckman Instruments Inc., Fullerton, Calif., U.S.A., in one liter of toluene). The specimens were counted 24 h later in a scintillation spectrometer (Beckman Instruments Inc., Model 1650). The counting efficiency for tritium was 55 % and for C-14 95 %. The quenching was corrected by S-standardization by adding various amount of methanol into counting vessels containing a certain amount of the labeled compound to be counted in the scintillation medium.

To study the transfer of polyuridylic acid into mitochondria uniformly tritiated polyuridylic acid (potassium salt, molecular weight 9×10^4 daltons, specific activity 12.7 Ci/mole, nucleoside and nucleotide free, Schwarz BioResearch Inc., Orangeburg, New York) was added to the reaction mixtures (A and B), 25—50 nCi per vial, omitting the labeled L-phenylalanine. The incubation was stopped by cooling the reaction mixtures in crushed ice for 2 min. The mitochondria were resedimented at 8000 g, 10 min (4°C). They were resuspended in 1 ml of 0.25 M sucrose containing ribonuclease (20 μg/ml; from bovine pancreas, Type II—A, Sigma) and incubated 10 min at room temperature after which they were again resedimented and washed twice by resuspending in 0.25 M sucrose. In some experiments a similar treatment was performed prior to the incubation with labeled polyuridylic acid, too. After addition of 0.5 mg of carrier RNA the mitochondria were treated with acetone, ethanol-ether, and ether before dissolving for counting as described above.

The effect of polyuridylic acid on mitochondrial oxidative phosphorylation was studied both with manometric and polarographic techniques. When performing manometric measurements, the total volume of reaction mixture (A) added to 15 ml Warburg manometer flasks (Model H-9 Precision Scientific Co., Chicago, Ill., U.S.A.) was 2 ml. L-Phenylalanine was omitted. The substrate (either succinate or β-hydroxybutyrate) was added from the side arm after a 10 min equilibration period. The center well contained 0.2 ml of 20 % KOH. The uptake of oxygen was followed for 30 min at 37°C, during which time it proceeded linearly. The reaction was stopped by adding 1 ml of 5% trichloroacetic acid. The remaining phosphate was determined by a modified Fiske and SubbaRow method.[11] In the polarographic experiments the reaction mixture (A) (1 ml) was kept in an open vessel revolving 50 times per min, and the Clark-type oxygen electrode was placed at the centrum to a depth of 2—3 mm. The electrode was attached to a Beckman Oxygen Analyzer, Model 160 coupled to an external recorder.

The swelling of mitochondria was followed during incubation in the different reaction media described above containing polyuridylic acid and in 0.25 M sucrose, too. During incubation at 37°C 0.1 ml samples were taken and diluted with 0.9 ml of 0.25 M sucrose. The absorbance was measured in a Beckman DU spectrophotometer at wave length of 550 nm.

The number of bacteria in the reaction mixtures before and after the incubation was determined by spreading 50 μl of the reaction mixture in three dilutions on culture agar plates (Uricult, Lääketehdas Orion Oy, Helsinki, Finland) and counting the colonies after an incubation of 24 h at 38°C.

RESULTS

The electronmicroscopic examination revealed that the mitochondrial fractions used were free of nuclei and essentially free of endoplasmic reticulum.

When the mitochondria were incubated in a succinate containing medium (A) with the labeled L-phenylalanine, the rate of incorporation was very slow (Fig. 1 A). A similar curve was obtained, if the succinate was replaced by L-glutamate (Fig. 1 B). In the presence of the balanced amino acid mixture
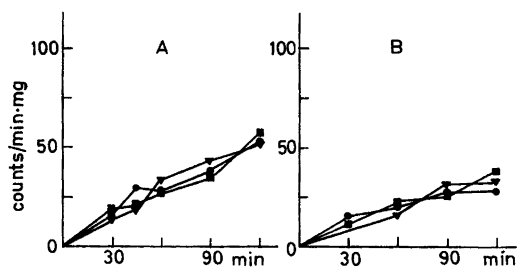
Fig. 1. The incorporation of tritiated L-phenylalanine into protein by isolated rat liver mitochondria in a medium (*A*) containing as oxidizable substrate, succinate: A, and L-glutamate: B with the addition of polyuridylic acid 200 µg/ml: black squares, 100 µg/ml: triangles or without any polyuridylic acid: black spots. Similar results were obtained with lower amounts of polyuridylic acid (25 and 50 µg/ml).

Fig. 2. The incorporation of tritiated L-phenylalanine into protein by isolated rat liver mitochondria in a medium (*B*) containing 1 mg of a balanced amino acid mixture.[10] The effect of polyuridylic acid on the incorporation rate: 0, 50, 100, and 200 µg/ml, black spots, circles, triangles, and black squares, respectively.
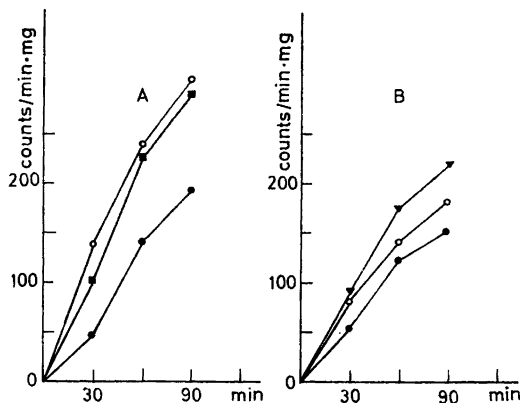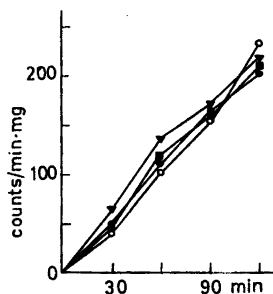
Fig. 3. The incorporation of tritiated L-phenylalanine into protein by isolated mitochondria in a medium (*B*) containing in addition to the balanced amino acid mixture (black spots), A: rat liver supernatant fraction (60 min, 105 000 *g*), 3.3 mg of protein per tube (circles) and further 200 µg/ml of polyuridylic acid (black squares), B: rat liver pH 5 fraction obtained from the supernatant fraction used above by adding potassium acetate buffer (pH 4.5) to pH 5 and collecting by spinning the precipitate, 0.53 mg of protein/ml (circles) and 1.3 mg of protein/ml (triangles).
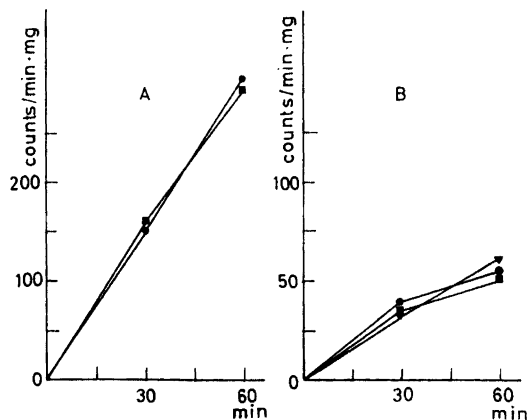
Fig. 4. A: The incorporation of L-lysine into protein by isolated mitochondria in a medium (*B*) containing the balanced amino acid mixture (black spots) and in addition polyadenylic acid (200 μg/ml) (black squares). B: The effect of polyuridylic and polyadenylic acids (200 μg/ml), black squares and triangles, respectively, on the incorporation of the labeled amino acid mixture (*C*) into protein by isolated mitochondria; black spots: without any added RNA.

(medium *B*) the incorporation rate was at least four times higher (Fig. 2). The mitochondrial phenylalanine incorporation was stimulated slightly after the addition of cell sap from homogenized rat liver or its pH 5 fraction (Fig. 3). The incorporation proceeded in all cases almost linearly with time for at least one hour.
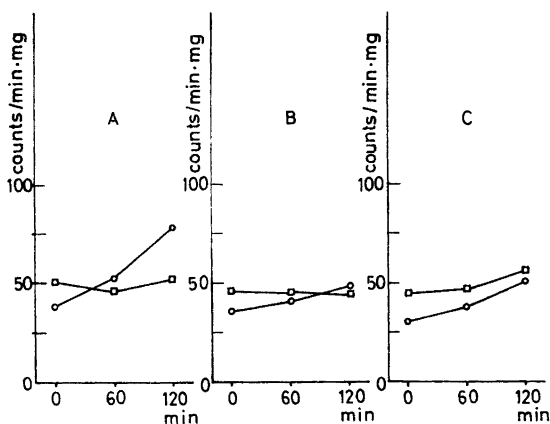
Fig. 5. The ribonuclease resistant uptake of tritiated polyuridylic acid by isolated mitochondria, A: incubated in 0.25 M sucrose, B: respiring on succinate containing medium (*A*), and C: in the balanced amino acid medium (*B*) without any labeled amino acid. The mitochondria were either pretreated with ribonuclease during isolation (squares) or used without any such pretreatment (circles).
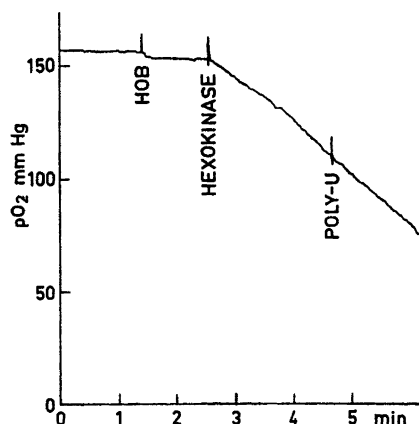
Table 1. The effect of polyuridylic acid on the coupling of oxidative phosphorylation in isolated mitochondria respiring on β-hydroxybutyrate in medium (*A*). The experiment was carried out by using manometric techniques. The figures are P/O values.

| | Polyuridylic acid (µg/ml) | | | | |
	0	25	50	100	200
P/O	2.75	2.62	2.87	2.61	2.71

The addition of polyuridylic acid in concentrations of 25—200 µg/ml neither stimulated nor lowered the incorporation rate of tritiated phenylalanine in reaction mixtures containing succinate (Fig. 1 A), L-glutamate (Fig. 1 B), balanced amino acid mixture (Fig. 2 B), or rat liver cell sap (Fig. 3 A). The addition of polyadenylic acid to the reaction mixtures containing the balanced amino acid mixture and labeled L-lysine did not increase the L-lysine incorporation (Fig. 4 A); the addition of either polyuridylic or polyadenylic acids to reaction mixtures containing the labeled amino acid mixture did not result in increased uptake of the label (Fig. 4 B).

When mitochondria were incubated in 0.25 M sucrose solution in the presence of uniformly tritiated polyuridylic acid, a slight ribonuclease resistant labeling was found, but if the mitochondria were pretreated before the experiment with ribonuclease, no uptake of label did occur (Fig. 5 A). The uptake of labeled polyuridylic acid was not stimulated, if the reaction mixture (*A*) was used to maintain respiration or the mixture (*B*) to provide amino acids for the active protein synthesis (Fig. 5 B and C).

The failure of the synthetic nucleic acids to stimulate the amino acid incorporation might also be explained by their possible inhibitory effect on the mitochondrial metabolism. The different amounts of polyuridylic acid

Fig. 6. The effect of polyuridylic acid (POLY—U) (100 µg/ml) on the oxygen consumption by the isolated rat liver mitochondria (corresponding to 250 mg of fresh liver) respiring on β-hydroxybutyrate (HOB) in medium (*A*). Similar results were obtained, if β-hydroxybutyrate was replaced by succinate or L-glutamate. Oxygen was determined polarographically by using a Clark-type oxygen electrode.

(25—200 μg/ml) had not, however, any effect on the oxygen consumption or on the coupling of the oxidative phosphorylation either studied by manometric of polarographic techniques (Table 1 and Fig. 6). The mitochondrial swelling was followed during the incubation in the different media: 0.25 M sucrose, succinate containing medium (*A*), and the balanced amino acid medium (*B*). It was not affected by the addition of various amounts of polyuridylic acid (Table 2).

Table 2. The effect of polyuridylic acid on the swelling of mitochondria incubated in different reaction mixtures. The figures are absorbancies ($\times 10^{-3}$ *A*) at 550 nm.

	Medium								
	0.25 M sucrose			Medium (*A*), succinate			Medium (*B*)		
Time				Polyuridylic acid (μg/ml)					
(min)	0	50	100	0	50	100	0	50	100
0	531	538	550	504	492	488	618	645	628
30	404	423	418	466	472	468	585	600	595
60	374	390	385	467	453	482	573	580	565

The bacterial cultures revealed that the number of bacteria giving viable colonies varied from 2000 to 12 000 per reaction mixture, and during a 60 min incubation no increase of the number was observed. By adding 2 mg of serum albumin as a carrier after an incubation of the reagents only, no uptake of labeled amino acids from the reagent mixture (*C*) was observed.

DISCUSSION

The addition of synthetic polyuridylic and polyadenylic acids to media containing varying number of amino acids had no stimulating effect on the incorporation of tritiated L-phenylalanine, L-lysine, or ^{14}C labeled amino acid mixture in isolated mitochondria. This lack of stimulation was not due to depression of mitochondrial metabolism, since the addition of polyuridylic acid up to 200 μg had no effect on the oxygen uptake or the coupling of mitochondrial oxidative phosphorylation. The metabolic state of mitochondria studied by the swelling degree [12] was also unaffected by the addition of various amounts of polyuridylic acid. Both rat liver cell sap and its pH 5 fraction had a stimulating effect, as reported in Ref. 7, but the addition of polyuridylic acid had no further stimulating effect.

Thus, the mitochondrial protein synthesis differs markedly from the ribosomal protein synthesis which is stimulated by polyuridylic and polyadenylic acids.[13] These start the synthesis of polyphenylalanine and polylysine, respectively.

Studies made with tritiated polyuridylic acid showed only a minimal ribonuclease resistant labeling of the mitochondria. The slight incorporation may be due to splitting of labeled polyuridylic acid and incorporation of some low molecular weight fragments. These results do not agree with those obtained by Humm and Humm,[6] if the hybridization of mitochondrial ribonucleic acids is explained by assuming their nuclear origin. According to Brierly and Green [14] molecules larger than 60 000—90 000 daltons do not penetrate the mitochondrial membrane.

The results of penetration studies agree well with the results obtained in the amino acid incorporation experiments. Thus, the failure of the synthetic ribonucleic acids to stimulate the amino acid incorporation was not due to subcompartmentalization of the mitochondria. The experiments with the labeled amino acid mixture were carried out to exclude the possibility that the amino acid codes in mitochondria were different from those of the ribosomal system, but this problem remains to be solved, since the mitochondrial membrane appears too impermeable to the synthetic ribonucleic acids.

The *in vitro* results reported here speak against the possibility that the cell nucleus could direct the mitochondrial protein synthesis by sending appropriate messenger ribonucleic acids *via* the cytoplasm to the mitochondrial system of protein synthesis, and the present data do not agree with the conclusions drawn by Humm and Humm.[6] The nuclear genetic information must be supplied in some other way.

The results published by Haldar, Freeman and Work [3] and Kadenbach [4] present another possibility; they indicate that some of the mitochondrial proteins are synthesized outside the mitochondria, and are transported *in statu nascendi* through the mitochondrial membranes. In Kadenbach's experiments *in vitro*, however, only minimal amounts of the protein synthesized by the cytoplasmic ribosomes were found in the mitochondria. A highly ordered anatomy is apparently necessary for efficient transport, and this cannot be obtained *in vitro* except in tissue slices, where a very high labeling of mitochondrial enzymes was observed.

The bacterial cultures revealed that the reaction mixtures contained bacteria although the reagents were stored frozen in small amounts. Sandell and coworkers [15] have reported that sterile mitochondria are not able to incorporate amino acids. The synthesis of specific mitochondrial proteins even by the isolated mitochondria indicates, however, that at least some of the observed incorporations have taken place in mitochondria.[16] Kroon and coworkers [17] did not find any difference in the leucine incorporation by bacteria-free mitochondrial preparations and preparations containing from 5000 to 99 000 bacteria per mg of protein, and 250 000 bacteria were required to give a 10 % increase in amino acid incorporation in a 60 min experiment. Our experiments with different media support this, since the amino acid incorporation into protein was sensitive to medium changes even though all of them were good for bacterial growth.

The fact that some incorporation of L-phenylalanine took place even in the absence of other added amino acids is explained by the endogenous amino acids found in mitochondria.[10] Mitochondria are also able to synthesize some amino acids with the aid of their aminotransferases.

Preliminary reports on the above work were published earlier.[18],[19]

REFERENCES

1. Simpson, M. V., Skinner, D. M. and Lucas, J. M. *J. Biol. Chem.* **236** (1961) PC 81.
2. Kalf, G. F. and Grece, A. M. *Biochem. Biophys. Res. Commun.* **17** (1964) 674.
3. Haldar, D., Freeman, K. and Work, T. S. *Nature* **211** (1966) 9.
4. Kadenbach, B. *Biochim. Biophys. Acta* **134** (1966) 430.
5. Nass, S., Nass, M. M. K. and Hennix, U. *Biochim. Biophys. Acta* **95** (1965) 426.
6. Humm, D. G. and Humm, J. H. *Proc. Natl. Acad. Sci. U.S.* **55** (1966) 114.
7. Greengard, O. and Campbell, P. N. *Biochem. J.* **72** (1959) 305.
8. Gornall, A. G., Bardawill, C. J. and David, M. M. *J. Biol. Chem.* **177** (1948) 751.
9. Kroon, A. M. *Biochim. Biophys. Acta* **76** (1963) 165.
10. Truman, D. E. S. and Korner, A. *Biochem. J.* **83** (1962) 588; **85** (1962) 154.
11. Hänninen, O., Hartiala, K. and Nurmikko, V. *Acta Chem. Scand.* **18** (1964) 937.
12. Azzi, A. and Azzone, G. T. *Biochim. Biophys. Acta* **105** (1965) 253.
13. Reunanen, M., Hänninen, O. and Hartiala, K. *Nature* **213** (1967) 918.
14. Brierley, G. and Green, D. E. *Proc. Natl. Acad. Sci. U.S.* **53** (1965) 73.
15. Sandell, S., Löw, H. and von der Decken, A. *Biochem. J.* **104** (1967) 575.
16. Wheeldon, L. *Biochim. Biophys. Res. Commun.* **24** (1966) 407.
17. Kroon, A. M., Saccone, C. and Botman, M. J. *Biochim. Biophys. Acta* **142** (1967) 552.
18. Hänninen, O. and Alanen, K. *Scand. J. Clin. Lab. Invest.* **19** *Suppl.* **95** (1967) 42.
19. Hänninen, O. *Ann. Acad. Sci. Fennicae A IV* **128** (1968) 40.