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Measurement of the Protein-Synthetic Activity *in vivo* of Various Tissues in Rats by using [^{3}H]Puromycin

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The validity of a new technique was examined for estimating the protein-synthetic activity of various tissues *in vivo*. The basic assumption underlying the method is that the number of peptide chains growing on each active ribosome would increase as the protein-synthetic activity of each tissue increases. The principle of the procedure, which was devised originally by Wool & Kurihara [(1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2401–2407] to determine *in vitro* the number of functional ribosomes in skeletal muscle, is as follows. Puromycin is known to bind easily to the *C*-terminal end of the growing peptide on ribosomes and thus stop further chain elongation. Hence, if the number of puromycin molecules attached to the nascent peptide is determined by using radioactive puromycin as a tracer, one can estimate the number of growing peptides, i.e. the activity of tissue protein synthesis. By using this technique, it is shown that both starvation and the feeding of a protein-free diet caused marked decreases in the relative rate of formation of peptidyl-puromycin, i.e. activity of protein synthesis in liver, skeletal muscle, heart, spleen, testis, lung, kidney and intestine.

Estimating the protein-synthetic rate in various organs *in vivo* may be considered an artificial concept, since it is the sum of the rates for various proteins with fast and slow turnover rates. However, it is of great value, since one can evaluate by using these techniques the relative role of each organ in regulation of protein turnover on a whole-body basis in response to changes in various nutritional or hormonal conditions. Furthermore, it is well known that the efficiency of protein synthesis in a cell-free system is far less than that in the whole animal. Measurements *in vivo* are therefore required to substantiate the conclusion that the effects measured *in vitro* are a true reflection of events in the cell. However, there are many problems encountered in using radioactive amino acid as a tracer for estimating the protein-synthetic rate *in vivo*. The most important are the problems of precursor pools and that of recycling of labelled amino acid. Many kinds of techniques have been devised to eliminate or minimize these problems, e.g. use of less-reutilizable amino acids or their precursors, such as [*guanidino*-^{14}C]arginine (Swick, 1958), [^{14}C]aspartic acid (Young *et al.*, 1971) and $Na_2{}^{14}CO_3$ (Millward, 1970), and injection of massive amounts of radioactive amino acid (Henshaw *et al.*, 1971; Scornik, 1974). Some other workers developed techniques to administer radioactive amino acid at constant rate for a relatively long period, i.e. constant intravenous (Waterlow & Stephen, 1968) or intragastric (Picou & Taylor-Roberts, 1969) infusion, or giving it with diets (Harney *et al.*, 1976).

The purpose of the present study is to examine the validity of a new method for estimating *in vivo* the protein-synthetic activity in various organs by using radioactive puromycin.

Experimental and Results

Male rats of Wistar strain were purchased from Shizuoka Experimental Animals and Agricultural Co-operative (Hamamatsu, Shizuoka, Japan) at 35–40g body wt., and were fed on a commercial stock diet (CE-2; Japan CLEA Co., Urawa, Saitama, Japan) for 1 or 2 days before use. The radioactive puromycin {[8(*n*)-^{3}H]puromycin dihydrochloride, specific radioactivity 5.7Ci/mmol, from The Radiochemical Centre, Amersham, Bucks., U.K.)} was combined with unlabelled puromycin dihydrochloride (Boehringer Mannheim G.m.b.H., Mannheim, West Germany) to give the final specific radioactivity quoted in each Figure and Table, dissolved in saline (0.9% NaCl) and injected intraperitoneally. Animals were killed by cervical dislocation and their entire ventral wall was ripped open. The carcass with its externalized organ was immediately immersed in ice-cold saline. Hind-leg muscle, liver, heart, kidney, spleen, testis, intestine and lung were removed quickly and rinsed with ice-cold saline. After adhering saline was removed with a filter paper, the organs were crushed between two pieces of solid CO_2 and frozen. A portion of each tissue was homogenized with 9vol. of cold 5%

(w/v) trichloroacetic acid. Separation of peptidyl-puromycin from unchanged free [^{3}H]puromycin was carried out by passing the trichloroacetic acid homogenate through a membrane filter (from Sartorius-Membrane Filter G.m.b.H., Goettingen, West Germany; pore size 40μm). We showed previously that about 92–93% of total peptidyl-puromycin formed could be trapped on the filter (K. Nakano & H. Hara, unpublished work). After being washed with 50ml of 5% (w/v) trichloroacetic acid, each filter was put into a counting vial. The protein was dissolved with 0.7ml of 88% (v/v) formic acid, and 10ml of scintillation fluid [7g of 2,5-diphenyloxazole (PPO) and 0.4g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene (POPOP) per litre of toluene/methylCellosolve (1:1, v/v)] was added. The radioactivity was measured in a liquid-scintillation counter (type LSC-653; Aloka Co., Mitaka, Tokyo, Japan), the efficiency of counting being approx. 12%. Another portion of tissue was homogenized with 9vol. of water and used for analysis of RNA. Tissue RNA was determined by the orcinol method (Nakano & Hara, 1979) after treatment as described by Fleck & Munro (1962).

Calculations

The number of puromycin molecules bound to nascent peptide, i.e. the number of nascent peptides, P, in μmol per mg of tissue RNA, was calculated from the equation:

$$P = \frac{Q}{S_i \times R}$$

where Q (d.p.m./g of tissue) is the radioactivity of peptidyl-puromycin formed per g of tissue, S_i is the specific radioactivity of puromycin in the original puromycin solution for injection (d.p.m./μmol of puromycin) and R is the concentration of RNA in the tissue (mg/g of tissue).

The concentration of free puromycin in tissue, F (μmol/g of tissue), is the radioactivity in the trichloroacetic acid-soluble fraction (d.p.m./g of tissue) divided by the specific radioactivity of the original puromycin solution for injection (d.p.m./μmol of puromycin).

The protein-synthetic activity of each tissue, i.e. the rate of formation of peptidyl-puromycin as a function of concentration of free puromycin, is therefore:

$$T_1 \text{ (relative rate, i.e. units/mg of RNA)} = \frac{P}{F}$$

$$T_2 \text{ (units/g of tissue)} = \frac{Q}{F}$$

Formation of peptidyl-puromycin

The formation of peptidyl-puromycin in various tissues as a function of time after injection of [^{3}H]-puromycin is shown in Fig. 1. It increases linearly after about 10min lag phase up to 30min and reached a plateau thereafter. Consequently rats were killed 30min after injection in all subsequent experiments.

The second experiment was carried out to estimate the formation of peptidyl-puromycin as a function of dose of puromycin. The method described in the present study assumes that doses of puromycin used in the study do not themselves alter the rate of formation of peptidyl-puromycin. The results illustrated in Fig. 2 show that the amount of puromycin bound to nascent peptide increased linearly as the dose of puromycin increased up to 7.35 or 14.7μmol/100g body wt. It is unlikely therefore that puromycin may exert any harmful effects on formation of peptidyl-puromycin as long as the dose is restricted up to 7.35μmol/100g body wt. It is also noteworthy that the degree of increase in formation of peptidyl-puromycin varied markedly among

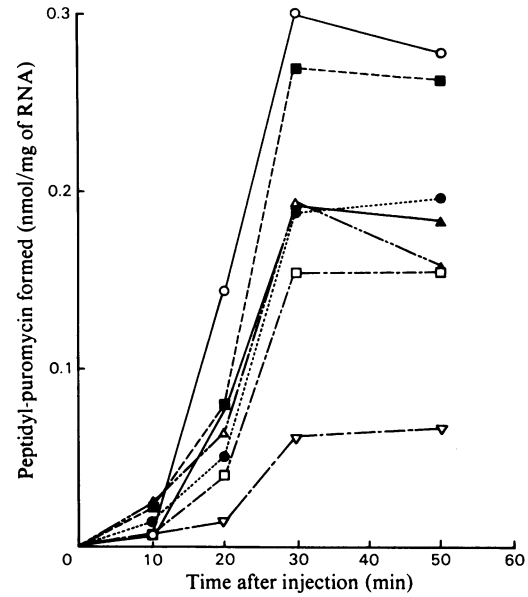

Fig. 1. *Number of puromycin molecules bound to nascent peptide as a function of time after injection of* [^{3}*H*]*puromycin in liver* (○), *kidney* (△), *intestine* (▲), *lung* (□), *testis* (▽), *heart* (■) *and skeletal muscle* (●)
Rats were injected with 0.918μmol of [^{3}H]puromycin (32.7μCi/μmol)/100g body wt. intraperitoneally. The number of puromycin molecules bound to nascent peptide was determined as described in the Experimental and Results section for three animals at each time point.

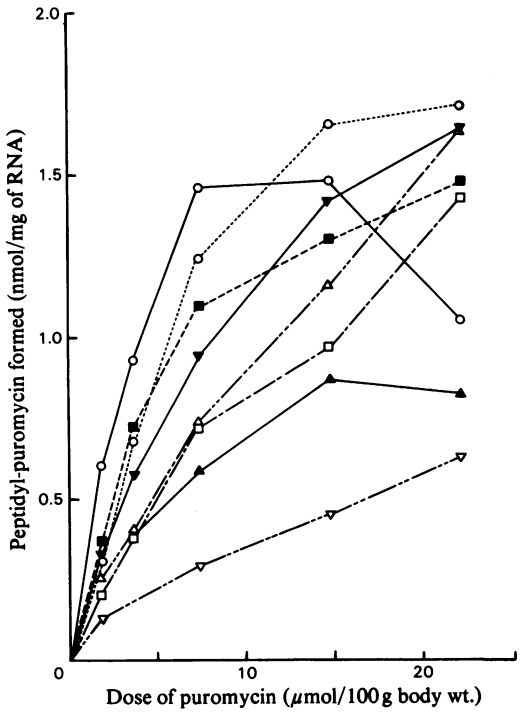

Fig. 2. *Number of puromycin molecules bound to nascent peptide as a function of dose of* [^3H]*puromycin in liver* (○), *kidney* (△), *intestine* (▲), *lung* (□), *testis* (▽), *spleen* (▼), *heart* (■) *and skeletal muscle* (●)

Rats were injected with 1.84–22.0μmol of [^{3}H]-puromycin (2.72μCi/μmol)/100g body wt. intraperitoneally and killed after 30min. The number of puromycin molecules bound to nascent peptide was determined as described in the Experimental and Results section for three animals at each dose.

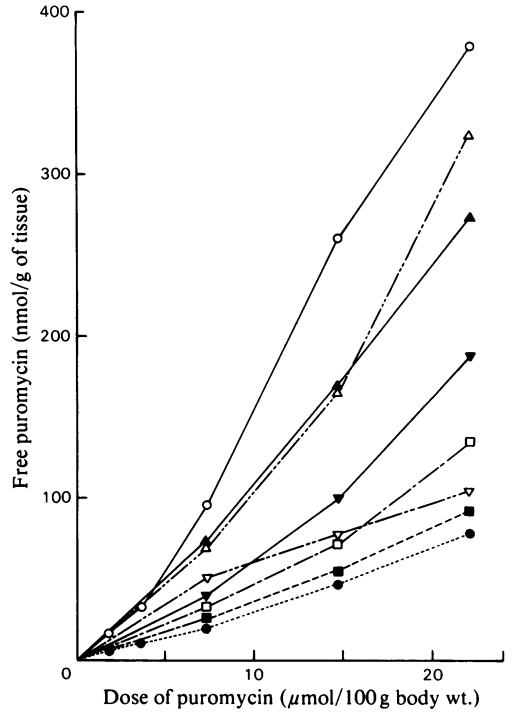

Fig. 3. *Concentration of free puromycin in tissue as a function of dose of* [^3H]*puromycin in liver* (○), *kidney* (△), *intestine* (▲), *lung* (□), *testis* (▽), *spleen* (▼), *heart* (■) *and skeletal muscle* (●)

The experimental conditions were the same as those described in Fig. 2. The concentration of free puromycin in each tissue was determined as shown in the Experimental and Results section.

various tissues examined. Fig. 3 depicts the change in the concentration of free puromycin in tissue as a function of the dose of puromycin injected: it increased linearly with dose up to 22.0μmol/100g body wt. The results shown in Fig. 3 also suggest that the degree of increase in concentration of free puromycin differs greatly among various tissues. It is probable therefore that the results shown in Fig. 2 may not be a true reflection of the actual rate of formation of peptidyl-puromycin in each tissue. To overcome this problem, the results were expressed thereafter on the basis of concentration of free puromycin in each tissue, as illustrated in Fig. 4. One can then compare the rate of formation of peptidyl-puromycin for each tissue and perhaps the activity of protein synthesis. Fig. 4 shows that the relative rate of formation of peptidyl-puromycin, i.e. that of nascent peptide, is greater in muscular tissue such as heart and skeletal muscle than in the visceral tissues, e.g. liver and intestine, which have been considered to have a larger capacity to synthesize protein than the former (Garlick *et al.*, 1975). One possible explanation for this could be that radioactive puromycin might be absorbed more efficiently into the visceral organs than into others, including the muscular tissues, since it was injected intraperitoneally, i.e. into the visceral cavity, throughout the experiments. This may cause an apparent higher concentration of free puromycin in the visceral tissues than in others. Thus the actual uncorrected value of puromycin per unit of RNA as shown in Fig. 2 may be more accurate than the corrected values presented in Fig. 4. It is necessary to compare these results with those obtained in studies with radioactive puromycin being injected through any other routes, e.g. intravenously.

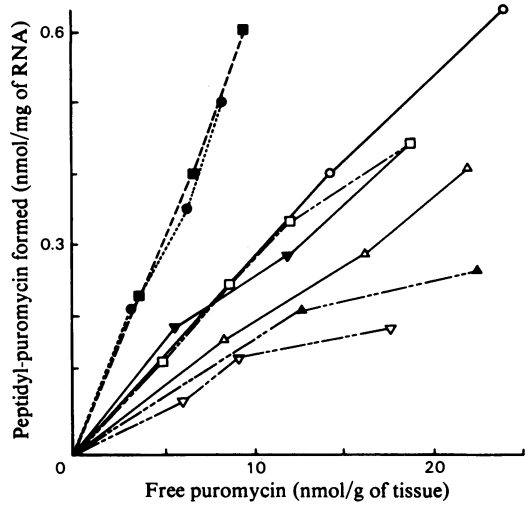

Fig. 4. *Number of puromycin molecules bound to nascent peptide as a function of concentration of free puromycin in liver* (○), *kidney* (△), *intestine* (▲), *lung* (□), *testis* (▽), *spleen* (▼), *heart* (■) *and skeletal muscle* (●)
The values for the numbers of puromycin molecules bound to nascent peptide were taken from Fig. 2. The values for the concentration of free puromycin in each tissue were taken from Fig. 3.

Effect of starvation and feeding of a protein-free diet

On the basis of the preliminary experiments described above, the effects of starvation and feeding of a protein-free diet were then examined on the relative rate of formation of peptidyl-puromycin in various tissues in rats. It is well known that the rate of protein synthesis decreases in many tissues under these dietary conditions (Garlick *et al.*, 1975; Nakano, 1978). All rats were previously fed on a 25%-casein/starch diet (Nakano, 1978) for 4–5 days. The rats of the control group were given the same diet for another 1 or 3 days, whereas the starved group was kept for 24h without food before being killed. The protein-depleted group was fed on a protein-free diet (Nakano, 1978) for 1 or 3 days. The results obtained show that both starvation for 1 day and feeding of a protein-free diet for 1 or 3 days caused a significant decrease in the rate of formation of peptidyl-puromycin in almost all tissues examined irrespective of the way of expression of the results, i.e. per mg of RNA or per g of tissue (Table 1).

Discussion

The technique for estimating the number of nascent peptides by using [^{3}H]puromycin was first devised by Wool & Kurihara (1967) and adapted to a cell-free system using ribosomal preparations isolated from skeletal muscle of rats.

One of the basic assumptions underlying the method described here is that the number of nascent peptide chains growing on each active ribosome would change in parallel with the changes in protein-synthetic rate in each tissue. Wool & Kurihara (1967) have shown by using this technique that the decrease in protein-synthetic activity of skeletal muscle of diabetic rats is attributed to the decrease in the number of active ribosome particles in these animals. It was shown by Whelly & Barker (1974) that the oestradiol-induced increase in rate of protein synthesis by uterine ribosomes *in vitro* is due both to increase in number of peptide chains growing on the active ribosomes and to the increase in activity of each active ribosome, i.e. rate of peptide-chain elongation. On the other hand, Kostyo & Rillema (1971) have found, by using the same method, that the stimulatory action *in vitro* of somatotropin on protein synthesis in isolated rat diaphragm is due not to the increase in number of active ribosomes, but to the enhanced ability of each active ribosome to promote peptide-chain formation. We showed previously (K. Nakano & H. Hara, unpublished work) by using the same method that there is a close correspondence between the ability of a ribosomal preparation obtained from skeletal muscle to incorporate radioactive amino acid and the number of nascent peptides present in these ribosomal preparations under various hormonal and dietary conditions such as diabetes, starvation and re-feeding the starved rats with a protein meal.

The advantage of the method proposed here for estimating *in vivo* the protein-synthetic activity by using [^{3}H]puromycin would be that one avoids the problems of precursor specific radioactivity, since this compound does not occur naturally in any tissues at all, nor from the evolution of any radioactive gas such as $^{14}CO_2$ during the treatment of animals. Furthermore, attention should be paid in choosing the kind of amino acid in the studies where any amino acid is used as a tracer, since the tissues where each amino acid is mainly metabolized are different for different amino acids. Thus the branched-chain amino acids such as leucine, isoleucine and valine are metabolized in extrahepatic tissues, whereas others are degraded in liver (Miller, 1962). Indeed, the use of branched-chain amino acid was recommended in a study for examining the changes in protein-synthetic activity in liver (Woodside & Mortimore, 1972). On the other hand, tyrosine was used as a tracer in the studies of protein turnover in skeletal muscle, since it is neither synthesized nor degraded in this tissue (Fulks *et al.*, 1975).

The disadvantage of the method proposed here for studying protein turnover in whole animals is

Table 1. *Effect of* 1 *day starvation or* 1 *day or* 3 *days of feeding of a protein-free diet on protein-synthetic activity in various tissues*

The activity of protein synthesis, i.e. the rate of formation of peptidyl-puromycin as a function of concentration of free puromycin in each tissue [(munits/mg of RNA (T_1) or/g of tissue (T_2), ± S.E.M., $n = 4$] was measured in rats fed on 25%-casein diet, in 1-day-starved rats and in rats fed on protein-free diet for 1 day or 3 days. Rats were injected with 1.84 μmol of [^{3}H]puromycin (24.5 μCi/mmol)/100g body wt. and the number of puromycin molecules bound to nascent peptide and the concentration of free puromycin were determined in each tissue of animals killed at 30min as described in the Experimental and Results section. Statistical analyses of the difference between the groups were by Student's t test; $^*P < 0.05$.

Expt. no. ...	I ($n = 2$)			II ($n = 4$)			III ($n = 4$)		
	Fed on 25%-casein diet	Starved 1 day	Increase or decrease (%)	Fed on 25%-casein diet	Fed on protein-free diet 1 day	Increase or decrease (%)	Fed on 25%-casein diet	Fed on protein-free diet 3 days	Increase or decrease (%)
Liver									
T_1	26.4	15.6	−40.9	29.1 ± 2.5	22.4 ± 2.0	−23.0	24.0 ± 2.4	19.6 ± 0.7	−18.3
T_2	307	184	−40.0	331 ± 25	200 ± 19*	−39.6	296 ± 32	170 ± 6*	−42.6
Kidney									
T_1	21.2	12.9	−39.1	21.9 ± 0.4	11.5 ± 0.5*	−47.5	18.7 ± 0.5	10.4 ± 0.1*	−44.3
T_2	136	74.1	−45.6	134 ± 1	68.1 ± 2.6*	−49.3	129 ± 3	61.3 ± 1.6*	−52.7
Intestine									
T_1	16.7	5.78	−65.4	12.9 ± 1.7	9.85 ± 1.2	−23.6	12.8 ± 1.4	8.58 ± 0.49*	−33.0
T_2	163	56.6	−65.6	119 ± 14	75.2 ± 0.9*	−37.0	136 ± 15	77.2 ± 4.3*	−43.4
Lung									
T_1	35.4	18.2	−40.6	32.7 ± 1.0	26.8 ± 1.3*	−18.0	27.6 ± 0.3	26.1 ± 0.2	−5.4
T_2	121	51.8	−57.0	130 ± 8	85.8 ± 6.6*	−33.8	134 ± 3	79.8 ± 5.8*	−40.4
Testis									
T_1	16.6	7.18	−56.7	18.5 ± 1.1	13.7 ± 0.3*	−25.9	12.7 ± 1.2	9.76 ± 0.53*	−23.1
T_2	88.2	40.5	−54.1	83.3 ± 1.8	65.7 ± 1.4*	−21.1	65.0 ± 4.7	49.0 ± 2.5	−24.6
Spleen									
T_1	35.4	18.8	−46.9	31.9 ± 1.5	30.5 ± 2.3	−4.4	25.5 ± 1.8	30.4 ± 1.7	+19.2
T_2	376	153	−59.3	307 ± 19	212 ± 16*	−30.9	269 ± 17	180 ± 3*	−33.1
Heart									
T_1	84.1	35.9	−57.3	68.1 ± 0.9	63.3 ± 1.3*	−7.0	61.3 ± 0.9	56.1 ± 2.2	−8.5
T_2	269	115	−57.2	247 ± 4	197 ± 4*	−20.2	224 ± 7	175 ± 4*	−21.9
Skeletal muscle									
T_1	84.0	16.1	−80.8	63.9 ± 1.6	55.5 ± 1.4*	−13.1	60.7 ± 2.3	46.7 ± 3.9*	−23.1
T_2	214	38.6	−81.8	192 ± 7	139 ± 1*	−29.2	191 ± 7	100 ± 7*	−47.6

that one cannot estimate the absolute or fractional rate of protein synthesis in various tissues, but can evaluate only the relative protein-synthetic activity of each tissue.

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