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Measurement of Protein Synthesis by the Phenylalanine Flooding Dose Technique: Effect of Phenylalanine and Anaesthesia on Plasma Electrolyte, Enzyme and Metabolite Levels

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Abstract—Ten minutes after an intravenous flooding dose of phenylalanine to rats, plasma sodium and calcium concentrations were slightly reduced (by 2–7%) but no effects on potassium or phosphate were observed. Creatine kinase activities were significantly increased by phenylalanine injection (by 39%), but alkaline phosphatase, alanine aminotransferase, lactate dehydrogenase and aspartate aminotransferase activities were unaltered. Plasma concentrations of total proteins, albumin, cholesterol, triglycerides, urea, creatinine and glucose were also unaffected. In the presence of anaesthesia, phenylalanine injection had almost identical effects, although the increase in creatine kinase activities did not reach statistical significance. Anaesthesia for 10 min reduced plasma potassium concentrations (by 27%), and calcium (by 5%), though phosphate and sodium were unaltered. The activities of lactate dehydrogenase, creatine kinase and aspartate aminotransferase were reduced by between 36–52%, but alkaline phosphatase and alanine aminotransferase activities were unaltered by anaesthesia. Plasma concentrations of total proteins and albumin were also reduced (both by 9%), but glucose concentrations were increased (by 33%). Anaesthesia had no other significant effects on cholesterol, triglycerides, urea or creatinine concentrations. The qualitative effects of anaesthesia in the presence of raised free phenylalanine concentrations were similar. It was concluded that, except for creatine kinase, determinations of plasma constituents in phenylalanine-injected rats could be made without overt interpretational errors. However, caution is required in interpreting data on plasma constituents from anaesthetized rats.

Investigations into the regulation of tissue protein mass by pharmacological agents or treatments are often carried out in parallel with the measurement of protein synthesis and there is a variety of techniques for measuring the kinetics of this process (Waterlow et al 1978). The most widely used technique for measuring the fractional rate of protein synthesis in-vivo is by injection of phenylalanine, in which a "flooding dose" of phenylalanine accompanies the label (Garlick et al 1980). Over 200 published studies have been carried out using this method. These include the effects of steroids (Garlick et al 1987), glucagon (Preedy & Garlick 1985), insulin (Garlick et al 1983), amino acids (Preedy & Garlick 1986) and ethanol (Preedy & Peters 1988; Preedy et al 1988). In many of these experiments there were also concomitant measurements of plasma biochemicals, such as intermediary metabolites, ions or enzymes.

In recent studies on the effects of ethanol, we observed very high plasma activities of creatine kinase in both control and treated animals. This may have been due to muscle damage as a result of ethanol, saline (control) or phenylalanine injection (Preedy & Peters 1988; Preedy et al 1988). In subsequent studies, high creatine kinase activities were also observed in rats which were injected with phenylalanine alone (unpublished observations). This suggested that the raised phenylalanine concentrations may be responsible for increasing creatine kinase activities. Alternatively, muscular activity, produced at the time of death, may also have caused release of muscular creatine kinase. If this were true, then induction of anaesthesia, with an agent that also induces muscular relaxation, should reduce this effect. However, apart from our previous investigations into the effects of phenylalanine injection on insulin, glucagon and corticosterone (Preedy & Garlick 1986) there have been no studies on the effects of the phenylalanine injection itself on plasma metabolites or enzymes. We therefore investigated whether the injection of phenylalanine caused concomitant changes in plasma biochemistry. These experiments were also carried out in the absence or presence of methoxyflurane anaesthesia.

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Materials and Methods

Materials and animals

L-Phenylalanine and other biochemicals were obtained from the Sigma Chemical Company (Poole, Dorset, UK) and BDH (Poole, Dorset, UK) or were of highest quality available. Metofane (methoxyflurane, 99·99% (w/w) was from C-Vet Ltd, (Bury St Edmunds, Suffolk, UK). Male Sprague Dawley rats were obtained from an in-house breeding colony, and had free access to a standard laboratory chow and water up to the period of experimentation.

Treatment of animals

The rats formed a homogeneous group with a range of 191–222 g body weight. On the day of the experiment the rats were divided into 4 groups of equal mean body weight, as follows. *Group 1: untreated controls.* Rats were decapitated and mixed (Venous and arterial) blood collected into cold (0–4°C) lithium heparin tubes (kept in ice) via heparinized funnels.

Group 2: phenylalanine injected. Rats were briefly wrapped in a paper towel and injected with a flooding dose of phenylalanine (150 μmol/100 g), via a lateral tail vein (Garlick et al 1980). Rats were then immediately unwrapped and placed in an animal cage until they were decapitated 10 min later. Collection of blood was as above.

Group 3: anaesthesia. Rats were placed in an anaesthetic jar containing methoxyflurane soaked in cotton. Animals were subjected to anaesthesia for 10 min, then decapitated and blood collected as above.

Group 4: phenylalanine plus anaesthesia. Rats were injected with phenylalanine (150 μmol/100 g) and immediately subjected to methoxyflurane anaesthesia. After 10 min, rats were decapitated and mixed (venous and arterial) blood collected.

In both groups treated with methoxyflurane, there was a noticeable reduction in limb twitching immediately after the animals were killed. However, this was not quantified.

All plasma was separated by centrifugation at 0–4°C (2000 *g*, 10 min) and plasma stored at −70°C until analysis.

The effects of phenylalanine on analytical measurements of sodium, calcium and creatine kinase were also investigated. Plasma (0·18 mL) from five untreated rats was mixed with 0·02 mL distilled water (control) or 0·02 mL of either 15 or 150 mM phenylalanine. These final plasma concentrations, i.e. 1·5 and 15 mM, respectively, cover the range of values to be expected in rats injected with phenylalanine. The normal range is 50–100 μM (Preedy 1981).

Analytical methods

Plasma sodium and potassium were assayed using a routine indirect ion-selective electrode. Other assays were as follows: calcium (Connerty & Briggs 1966); phosphate (Daly & Ertingshausen 1972); urea (Talke & Schubert 1965); creatinine (Lustgarten & Wenk 1972); glucose (Cooper 1973); triglycerides (Fossati & Prencipe 1982); alanine aminotransferase [EC 2.6.1.2] (Bergmeyer & Horder 1980); alkaline phosphatase [EC 3.1.3.1] (Bowers & McComb 1975); aspartate aminotransferase [EC 2.6.1.1] (Bergmeyer & Horder 1977); creatine kinase [EC 2.7.3.2] (Rosalki 1967); albumin (Webster 1977); total plasma proteins (Gornall et al 1949); haemoglobin by absorption spectrometry on a clinical Coulter SPLUS STKR, (Coulter Electronics Ltd, Luton, Beds) by reaction with cyanide and subsequent absorption measurement at 525 nm; cholesterol (Katterman et al 1984); lactate dehydrogenase [EC 1.1.1.2.7] (Keiding et al 1974).

Statistical analysis

For the in-vivo studies differences between means were initially assessed by an analysis of variance followed by Student's *t*-test, using the pooled estimate of variance. For in-vitro studies, Student's *t*-test for paired samples was used for assessing whether differences between control and phenylalanine-treated plasma samples were significant. Significances were indicated for $P < 0.05$.

Results

Effects on plasma sodium, potassium, calcium and phosphate

The results (Table 1) show that phenylalanine injection caused small (2–7%), but statistically significant, reductions in plasma sodium and calcium. In anaesthetized rats, phenylalanine injection had a similar effect (3 and 5% reductions in sodium and calcium, respectively). Phenylalanine injection had no effect on plasma phosphate concentrations.

Anaesthesia significantly reduced plasma potassium and calcium by 27 and 5%, respectively. When rats were injected with phenylalanine, additional anaesthesia caused an apparent decrease (21%) in plasma potassium level and a decrease (5%) in plasma calcium.

Effects on plasma enzyme activities

Phenylalanine injection itself caused a significant (39%) increase in creatine kinase activity (Table 2). However, it had no significant effect on any of the other enzyme activities, (alkaline phosphatase, alanine aminotransferase, lactate dehydrogenase, aspartate aminotransferase).

Anaesthesia reduced the activities of lactate dehydrogenase, creatine kinase and aspartate aminotransferase, by 36, 52 and 41%, respectively. When the effects of anaesthesia on phenylalanine-injected rats were examined, significant

Table 1. Effect of a flooding dose of phenylalanine, with or without anaesthesia, on plasma ion concentrations (mM). Male rats were either decapitated (controls) or injected with a flooding dose of phenylalanine, then after 10 min decapitated, and blood collected. In the anaesthetized group, rats were subjected to metofane anaesthesia for 10 min, at the end of which rats were decapitated. In the phenylalanine plus anaesthesia group, the amino acid was injected, the anaesthesia immediately administered, and the rats killed after 10 min. All data are presented as mean ± s.e.m. of six rats in each group.

Ion	Control	Phenylalanine	Anaesthesia	Phenylalanine +anaesthesia
Sodium	139 ± 1	137 ± 1*	141 ± 1	137 ± 1**$^{\Delta\Delta}$
Potassium	8·95 ± 0·54	8·73 ± 0·56	6·53 ± 0·32**	6·92 ± 0·40**†
Phosphate	2·79 ± 0·10	2·62 ± 0·07	2·78 ± 0·08	2·86 ± 0·08
Calcium	2·93 ± 0·05	2·72 ± 0·02***	2·77 ± 0·02**	2·62 ± 0·02***$^{\Delta}$†

Differences between untreated controls and the other 3 groups: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Differences between phenylalanine injected and phenylalanine plus anaesthesia: † $P < 0.05$. Differences between anaesthesia injected and phenylalanine plus anaesthesia: $^{\Delta}$ $P < 0.01$, $^{\Delta\Delta}$ $P < 0.001$.

Table 2. Effect of a flooding dose of phenylalanine, with or without anaesthesia, on plasma enzyme activities (int. units L^{-1}).

Enzyme	Control	Phenylalanine	Anaesthesia	Phenylalanine +anaesthesia
Alkaline phosphatase	524±31	469±17	500±21	468±37
Alanine aminotransferase	57±4	62±6	49±4	48±3†
Lactate dehydrogenase	1290±160	1400±150	820±65*	960±100†
Creatine kinase	12600±2000	17500±2200*	6100±780**	7400±880*††
Aspartate aminotransferase	229±10	260±19	135±8***	153±8***††

Experimental details are in the legend to Table 1 and in Methods. All data are displayed as mean ± s.e.m. of 6 observations in each group. Differences between untreated controls and the other 3 groups: * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Differences between phenylalanine injected and phenylalanine plus anaesthesia: † $P<0.05$, †† $P<0.001$. There were no differences between anaesthesia and phenylalanine plus anaesthesia.

Table 3. Effect of a flooding dose of phenylalanine, with or without anaesthesia, on plasma proteins and other plasma biochemicals.

	Control	Phenylalanine	Anaesthesia	Phenylalanine +anaesthesia
Total proteins (g L^{-1})	54·1±1·1	56·8±0·6	49·2±1·2*	48·2±1·2**††
Albumin (g L^{-1})	25·5±0·5	26·2±0·4	23·3±0·5*	22·5±0·5**††
Cholesterol (mM)	2·27±0·06	2·17±0·07	2·05±0·08	1·92±0·09*†
Triglycerides (mM)	1·74±0·16	1·40±0·13	1·29±0·23	1·30±0·17
Urea (mM)	5·00±0·21	4·92±0·26	5·03±0·26	4·80±0·13
Creatinine (μM)	38·8±1·7	44·7±2·9	43·2±1·4	45·0±3·6
Glucose (mM)	8·4±0·2	9·1±0·2	11·2±0·4**	12·1±0·5**††

Experimental details are in the legend to Table 1 and in Methods. All data are mean ± s.e.m. of 6 observations in each group. Differences between untreated controls and the other 3 groups: * $P<0.01$, ** $P<0.001$. Differences between phenylalanine and phenylalanine plus anaesthesia † $P<0.05$, †† $P<0.001$. There were no differences between anaesthesia and phenylalanine plus anaesthesia.

Table 4. Effect of phenylalanine in-vitro on plasma sodium, calcium and creatine kinase determinations.

	Exogenous phenylalanine concentrations (mM)		
	0 (Control)	1.5	15
Sodium (mM)	128±1	129±1	127±1
Calcium (mM)	2·44±0·06	2·42±0·05	2·38±0·08
Creatine kinase (int. units L^{-1})	8130±1540	7940±1470	8350±1570

Plasma from control rats was mixed with distilled water or isovolumeric phenylalanine solutions to a final concentration of 1.5 or 15 mM, as described in the Methods section. All data are presented as means ± s.e.m. of five determinations in each group. Differences between means were assessed by Student's *t*-test for paired samples. There were no significant differences due to any treatment ($P>0.05$).

decreases in the activities of lactate dehydrogenase, creatine kinase and aspartate aminotransferase, were similarly observed (31, 58 and 41%, respectively). In addition, the activities of alanine aminotransferase were also reduced by 23%.

There were no statistically significant differences between anaesthetized rats and those treated with anaesthesia plus phenylalanine.

Effects on plasma proteins, lipids, urea, creatinine and glucose

Phenylalanine injections had no significant effect on total plasma proteins, albumin, cholesterol, triglycerides, urea, creatinine or glucose (Table 3). Furthermore, phenylalanine treatment had no effect in the presence of anaesthesia. Anaesthesia decreased total plasma proteins and albumin (both by 9%) and glucose was significantly increased (33%).

When the effects of anaesthesia were assessed in phenylalanine injected rats, there was also a reduction in plasma protein and albumin (both by approximately 15%), and an increase in glucose (33%). In addition, plasma cholesterol was reduced (12%).

In-vitro effect of phenylalanine on plasma calcium, sodium and creatine kinase

The effects of addition of phenylalanine to plasma from untreated animals are shown in Table 4. Phenylalanine had no effect on the levels of sodium, calcium or creatine kinase.

Discussion

Use of large flooding dose of phenylalanine

Many studies investigating the pharmacological actions of agents on protein synthesis have used the flooding dose technique with concomitant measurement of plasma metabolites and enzyme activities (Preedy & Peters 1988; Preedy et al 1988). The advantages of using phenylalanine, rather than other amino acids or amino acid mixtures, are that its assay does not require complex analytical procedures, it is not considered to be a major regulatory amino acid, it is relatively soluble and its endogenous levels are relatively low. However, it has been assumed that the injection of phenylalanine itself does not alter the plasma variables being

measured. This study was therefore confined to the effects of injecting phenylalanine, rather than the injection process (i.e. brief immobilization and injection of a volume of solute) itself.

Our results show that phenylalanine injection slightly decreases plasma sodium and calcium, even in the presence of anaesthesia. The only other effect we observed was a marked increase in creatine kinase activities. In the presence of anaesthesia, however, the phenylalanine-induced increase in creatine kinase activities was not statistically significant. In all groups the activities of creatine kinase were high compared with the study of Spargo (1984). Although we have measured creatine kinase activities in previous publications (Preedy et al 1988), we argued that its apparent high value may be due to the injection of phenylalanine itself. The results of the present study tend to exclude this possibility, as high values were obtained in the control group in which rats were not injected.

Two other explanations for high creatine kinase activities are therefore possible. The first is that upon decapitation reflex contraction of hindlimb and other muscles may have caused acute ischaemic- or hypoxaemic-induced release of creatine kinase. When we attempted to minimize this with anaesthesia, plasma creatine kinase activities were reduced, but values were still comparatively high. The second explanation is that the assay procedure used (largely developed for routine clinical use) is not appropriate for experimental work on the rat. However, the standard methods for measuring creatine kinase activities in man and the rat are identical. Spargo (1984) used analytical methods similar to those used by us and found that plasma creatine kinase activities in rats anaesthetized with sodium phenobarbitone were approximately 110 int. units L^{-1}; in that study blood was sampled via the femoral artery. More recently we have determined that in diethyl-ether-anaesthetized rats, in which blood was sampled via the vena cava in the thoracic cavity, creatine kinase activities were between 8 and 112 int. units L^{-1} (V. R. Preedy et al unpublished). This would strongly suggest that high creatine kinase activities are due solely to the decapitation procedure. An interesting finding of Spargo's study was the observation that cardiac-specific creatine kinase isoenzymes were not detectable in rat plasma.

A possible explanation for the phenylalanine-induced changes in the concentrations of sodium, calcium or creatine kinase activities was the direct effect of the amino acid on the analysis of these compounds. However, despite the fact that very high levels were added in-vitro, no change in creatine kinase activities or calcium and sodium concentrations were observed. The concentrations of phenylalanine that were added in-vitro, (i.e. 15 and 1·5 mM) were far higher than the concentrations observed in-vivo (approx. 50–100 μM, Preedy 1981). If approximately 150 μmol of phenylalanine is injected into a 100 g rat, then assuming a plasma volume of 10%, concentrations of 15 mM are attained. However, in practice, the phenylalanine is rapidly transported into the tissue and extracellular space and in addition a substantial proportion of it is converted to tyrosine (Garlick et al 1980).

It is likely that the changes in sodium, calcium and creatine kinase homeostasis were due to the pathological effects of the phenylalanine injection. The results of these studies do not inform us of whether it is the injection process or the phenylalanine which alters plasma sodium, calcium and creatine kinase; we were primarily concerned with the technique of measuring protein synthesis and therefore separate injection studies with saline were not carried out. Nevertheless, the experiments above provide overwhelming evidence that the injection of phenylalanine has minimal or negligible effects on numerous other plasma biochemicals. This suggests that the measurement of these variables in the same group of rats used for protein turnover studies (i.e. phenylalanine injected) is valid.

Use of anaesthesia

The rationale for using methoxyflurane anaesthesia was based on its co-properties as a muscle relaxant (Green 1979). It was argued that, as decapitation stimulates autonomically-induced muscular contractions, then any changes in blood biochemistry due to the decapitation procedure itself may be reversed by anaesthesia. Methoxyflurane is a general non-selective anaesthetic that causes generalized depression of the CNS, with accompanying depression of reflex activity and relaxation of striated muscles (Green 1979). It is an agent recommended for induction of anaesthesia in small laboratory animals (Green 1979) and is widely used in numerous research establishments. The induction of anaesthesia is fairly rapid. However, our results show that anaesthesia partially blocked the phenylalanine-induced increase in creatine kinase activity, though it also induced a number of other changes. This included reductions in potassium, calcium, total proteins and albumin, though glucose concentrations were increased. The activities of lactate dehydrogenase, aspartate aminotransferase and creatine kinase were decreased. It is difficult to ascribe these changes to any one particular process, largely because of the complex nature in which anaesthetics are metabolized. According to Green (1979), specific hepatotoxic effects of methoxyflurane have not been established. Methoxyflurane is largely excreted unmetabolized, but some is metabolized to carbon dioxide, fluoride ion, dichloroacetic acid and methoxyfluoracetic acid (Lee & Atkinson 1973). It is possible that some of the changes we have described may be due to the generation of these compounds and ensuing metabolic effects. It is also important to mention that other anaesthetics, with greater muscle relaxant properties than methoxyflurane, may induce effects on blood biochemistry, but these responses may well be different to those reported here.

Due to the nature of the experiment we did not deliver precise quantities of methoxyflurane, but instead used the standard procedure for induction of anaesthesia within our laboratories. It is possible that some of the changes described in this paper may have been due to respiratory changes, such as hypoxia or even carbon dioxide retention. We do not believe this to be so, as both these physiological disturbances are known to exert profound changes in muscle biochemistry such as alterations in protein turnover (Preedy et al 1984, 1985; Preedy & Garlick 1988; Preedy & Sugden 1989). However, we could find no effect of methoxyflurane anaesthesia on skeletal muscle protein synthesis, albeit after acute induction (V. R. Preedy & T. J. Peters unpublished results).

The occurrence of relatively high levels of plasma potassium (observed in all groups) may be indicative of mild haemolysis. Under the experimental conditions described in

the methods section, this was unavoidable. All blood samples were simultaneously processed for plasma separation and subsequent biochemical analysis, therefore similar degrees of haemolysis would be expected in all groups. We attempted to confirm this by measurement of free haemoglobin in the plasma. It is pertinent to state that the assay procedure we used does not distinguish between myoglobin and haemoglobin. Apparent haemoglobin levels were as follows (mean $\pm$s.e.m. of 6 observations, mg L^{-1}): control, $3{\cdot}2\pm0{\cdot}5$; phenylalanine-injected, $3{\cdot}2\pm0{\cdot}5$; anaesthesia, $1{\cdot}8\pm0{\cdot}2$; anaesthesia- and phenylalanine-injected, $2{\cdot}0\pm0{\cdot}4$. This would suggest that anaesthesia reduced haemolysis ($P<0{\cdot}05$ when controls were compared with the two anaesthetized groups). The data tentatively imply that anaesthesia has erythrocytic membrane stabilizing properties, either as a direct effect on erythrocyte structure or indirectly via some biochemical process (for example, 2,3-diphosphoglycerate, which appears to determine spontaneous haemolysis rates, Preedy 1981). Alternatively, it is possible that anaesthesia reduces myoglobin release and this would be in accordance with other features of this study, i.e. methoxyflurane reductions in creatine kinase activities. The reduction in plasma potassium by methoxyflurane may therefore be related to the possible muscle damage caused by contractions. This may have caused perturbations in muscle membrane stability and thereby induced potassium (Preedy 1981) and myoglobin release. The reduction of plasma potassium concentrations by anaesthesia would be in accordance with its muscle relaxant and/or CNS depressant properties.

The most surprising effect of the anaesthesia was the reduction in plasma proteins. In the absence of enhanced catabolism, an apparent change in hydration is suggested. However, plasma calcium and potassium were also reduced, but not phosphate and sodium, implying that alterations in hydration were not a single causal factor. The nature of those changes in plasma protein concentrations are therefore perplexing, but the 10 min period of treatment is insufficient for enhanced plasma protein degradation, or reduced plasma protein synthesis, to manifest itself as alterations in absolute amounts. An important consideration is the fact that the plasma proteins are distributed intra- and extra-vascularly. Physiological perturbations precipitated by anaesthesia may have caused alterations in the plasma protein compartmentation (Ahlinder et al 1970; Plantin et al 1971).

We conclude that plasma taken from rats injected with a large dose of phenylalanine remains amenable for the determination of a large number of plasma constituents, apart from creatine kinase. However, methoxyflurane causes alterations in plasma biochemistry that precludes its use as a routine anaesthetic agent in protein turnover studies. This may be compounded by pharmacological treatments, such as ethanol administration, which are known to alter the production of toxic by-products of methoxyflurane metabolism (Van Dyke 1983; Rice et al 1983).

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