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Effect of stress and sampling site on metabolite concentration in rat plasma

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The effect of mild stress on various plasma metabolites in the rat has been studied. Mild stress resulted in significant decreases in liver size and glycogen content, as well as in an increase of blood glucose. In addition, plasma lactate, insulin, glycerol and urea, as well as a number of amino acids were altered by stress. These data indicate that minimal stress can have major effects upon the composition of blood, and suggest the need for strict precautions on the handling of animals during blood sampling. The site of blood extraction — tail tip *vs.* neck — was also found to have a significant effect on plasma lactate, glucose and urea concentrations. In stressed animals the differences between tail- and neck blood composition were increased.

Introduction

The problem of blood and tissue sampling as a potential source of artifact in different experimental setups has recently received attention in the literature (FAUPEL *et al.*, 1972; SEITZ *et al.*, 1973; DEPOCAS & BEHRENS, 1977); particularly in studies on the effect of anaesthesics (FODOR & GRAFNETTER, 1960; MERIN *et al.*, 1971; FURNER *et al.*, 1972; AYNSLEY-GREEN *et al.*, 1973; KRAL, 1974; KRAL & FELLENIUS, 1976; MÄNNISTÖ *et al.*, 1976).

In the present study we have examined the effects of mild stress and sampling site on a variety of blood parameters in the rat. The nature and intensity of the mild stress studied is similar to that found in many experimental situations. We have found a significant effect of such stress on a wide variety of blood parameters; in addition, differences were found in blood taken from different sites.

Materials and Methods

Fed female virgin Wistar rats (weight range in Table I) were housed in collective cages in a light cycle and temperature controlled room (12 h light/12 h darkness; 23 ± 2 °C). Three groups of rats were used. The first two groups (1 and 2) were treated gently in order to avoid any unnecessary source of stress; the first group of rats were bled from the cut tail tip with extreme care to minimize stress (bleeding time never exceeded 20 sec). The rats of the second group were killed by decapitation using a guillotine. The third group of animals were kept in a cage on a labora-

tory bench for 90 min; then a blood sample was obtained cutting the tail tip (tail sample) and exactly 15 min after sampling they were beheaded with guillotine and blood was sampled from the neck wound (neck sample) as with group one. Thus, the neck sample of the third group was taken from rats that suffered successive stresses. All animals were sacrificed 2 h after the beginning of the light cycle.

Blood obtained from the tail was collected in dry heparinized china plates. Blood from the neck was collected into dry heparinized beakers. Pieces of both liver and hind leg striated muscle were dissected — within 15 sec after decapitation — and frozen in liquid nitrogen until glycogen determination.

Plasma samples were deproteinized with perchloric acid for the enzymatic determination of lactate (HOHORST, 1965) and glycerol (EGGSTEIN & KÜHLMAN, 1974). An aliquot of whole blood was deproteinized with the Somogyi procedure (SOMOGYI, 1945) and used for the estimation of glucose (HUGGET & NIXON, 1957). Insulin was estimated in plasma with a rat insulin radioimmunoassay kit (HALES & RANDLE, 1963) (kindly provided by Novo Industri, Copenhagen). Glycogen in liver and muscle was determined after alkaline digestion and ethanol precipitation (GOOD *et al.*, 1933), followed by glucose determination (HUGGET & NIXON, 1957). Urea was measured with the indophenol procedure (FAWCETT & SCOTT, 1960). Aliquots of plasma were deproteinized with cold acetone (AROLA *et al.*, 1977) and used for individual amino-acid determination by means of a radiochemical method (AROLA *et al.*, 1976). Labelled Dns-Cl was purchased from the Radiochemical Center, Amersham, England; non-radioactive Dns-Cl and all enzymes and standards were obtained from Sigma Chemical Co., St. Louis, MO, USA.

All glassware was cleaned with the process outlined by LOWRY & PASSONEAU (1972). Samples were counted in a toluene-Triton X-100 with PPO/POPOP-based liquid scintillation cocktail (TURNER, 1971). Counts were corrected for quenching using the external standard channels ratio method. Statistical differences between groups were determined using Student's *t* test.

The method used here for amino-acid quantification did not discriminate between the pairs : methionine-ornithine and leucine-isoleucine. Glutamate and glutamine as well as aspartate and asparagine are presented together because of partial deamination (AROLA *et al.*, 1976; PALOU *et al.*, 1977*b*). The value for "cysteine" refers to the total cysteine, cystine and cysteate concentrations in plasma. The allocation of amino acids into groups is the same as outlined previously (PALOU *et al.*, 1977*a*) (Table III).

TABLE 1. *Weights and glycogen content of stressed and control rats.*

Parameter	Controls	Stressed
Body weight (g)	166.8 ± 4.3	165.2 ± 5.9
Liver weight (g)	8.9 ± 0.3	6.7 ± 0.4 ▽▽
Liver weight (as percent of body weight)	5.01 ± 0.11	4.05 ± 0.06 ▽▽▽
Glycogen content in the liver (%)	4.02 ± 0.44	2.66 ± 0.28 ▽▽
Glycogen content in hind leg striated muscle (%)	0.38 ± 0.11	0.34 ± 0.03
Total glycogen content in the liver (mg)	419 ± 9	271 ± 25 ▽▽▽

All data are the mean ± SEM of five different animals.
Significance of the differences *vs.* controls :
▽ ▽ = $P < 0.01$; ▽▽▽ = $P < 0.001$.

TABLE II. *Blood glucose and plasma glycerol, lactate and insulin of controls and stressed rats.*

Parameter	Controls		Stressed Group 3	
	Group 1 tail sample	Group 2 neck sample	tail sample	neck sample
Blood glucose (mM)	5.63 ± 0.02	↑ 6.43 ± 0.14	6.04 ± 0.15	↑ 7.79 ± 0.46
Plasma glycerol (μM)	147.2 ± 14.5	156.7 ± 16.3	138.0 ± 8.5	114.8 ± 14.4
Plasma lactate (mM)	5.22 ± 0.35	↓↓↓ 3.26 ± 0.10	2.99 ± 0.14 ▽▽	↓ 2.19 ± 0.04 ▽
Plasma insulin (mU/litre)	20.6 ± 3.6	18.3 ± 3.8	5.0 ± 2.1 ▽▽▽	7.1 ± 0.6 ▽

All data are the mean ± SEM of five different animals.

Significance of the differences *vs.* controls :
decreases : ▽ = $P < 0.05$; ▽▽ = $P < 0.01$; ▽▽▽ = $P < 0.001$;
increases : △ = $P < 0.05$; △△ = $P < 0.01$; △△△ = $P < 0.001$.

Significance of the differences between tail and neck samples :
tail-neck decreases : ↓ = $P < 0.05$; ↓↓ = $P < 0.01$; ↓↓↓ = $P < 0.001$;
tail-neck increases : ↑ = $P < 0.05$; ↑↑ = $P < 0.01$; ↑↑↑ = $P < 0.001$.

TABLE III. *Plasma amino-acid* (μM) *and urea* (mM) *concentrations in control and stressed rats.*

Parameter	Controls		Stressed Group 3	
	Group 1 tail sample	Group 2 neck sample	tail sample	neck sample
Composite "gluconeogenic"	2 651 ± 116	2 794 ± 101	1 985 ± 95 ▽▽▽	↑↑ 2 390 ± 77
Alanine	671 ± 32	641 ± 18	406 ± 23 ▽▽▽	525 ± 42
Glutamate + glutamine	978 ± 50	949 ± 24	804 ± 31 ▽▽	↑ 903 ± 22
Aspartate + asparagine	152 ± 23	162 ± 13	156 ± 13	181 ± 18
Glycine	233 ± 15	257 ± 14	208 ± 18	201 ± 19
Serine	277 ± 27	330 ± 17	204 ± 19 ▽▽	↑ 278 ± 25
Threonine	341 ± 30	395 ± 17	169 ± 27	229 ± 15
Composite imino acids	513 ± 22	459 ± 15	349 ± 27 ▽▽▽	370 ± 37
Proline	467 ± 17	424 ± 17	292 ± 17 ▽▽▽	304 ± 32
Hydroxyproline	46 ± 7	43 ± 4	64 ± 6	↓ 44 ± 3
Composite branched chain	711 ± 30	674 ± 30	534 ± 28 ▽▽▽	482 ± 18
Leucine + isoleucine	476 ± 20	463 ± 20	384 ± 21 ▽▽	388 ± 25
Valine	236 ± 26	222 ± 16	176 ± 15 ▽	169 ± 12
Composite basic	840 ± 94	923 ± 39	704 ± 36	677 ± 21
Arginine	303 ± 34	370 ± 33	355 ± 28	331 ± 26
Histidine	104 ± 14	116 ± 9	95 ± 9	129 ± 18
Lysine	433 ± 62	430 ± 18	295 ± 22 ▽▽	267 ± 8
Composite aromatic	374 ± 14	393 ± 14	422 ± 32	362 ± 12
Tryptophan	201 ± 9	193 ± 11	183 ± 7	183 ± 11
Tyrosine	90 ± 6	108 ± 7	113 ± 9	90 ± 9
Phenylalanine	79 ± 7	79 ± 2	127 ± 17	91 ± 4
Composite sulphur	475 ± 63	580 ± 36	685 ± 51 △△	650 ± 57
"Cysteine"	45 ± 4	38 ± 5	83 ± 11 △△	↓ 50 ± 4
Methionine + ornithine	75 ± 5	90 ± 7	183 ± 30 △△△	135 ± 10
Taurine	355 ± 62	433 ± 21	472 ± 30	518 ± 54
Composite total	5 564 ± 159	5 802 ± 118	5 213 ± 96 ▽▽	5 140 ± 135
Urea	8.97 ± 0.88	↑ 11.38 ± 0.31	8.21 ± 0.41	↑↑↑ 16.02 ± 0.69

All data are the mean ± SEM of five different animals.
The meaning of significance is the same as in Table II.

Results

The changes in liver weight found in group 3 as compared with group 2 controls are seen in Table I. Part of this decrease can be attributed to a decrease in glycogen content, which is reduced by about one third with stress. Muscle glycogen did not change.

The concentrations of blood glucose, glycerol, lactate and radioimmunoassayable insulin in the plasma of both controls and stresssed rats are shown in Table II. Glucose concentrations are significantly lower (14 %) in the blood of group 1 than in that of group 2. The difference is magnified in group 3 animals in which there is a 29 % difference between the tail and neck sample. In addition, there is a significant increase in blood glucose and a significant decrease in plasma glycerol concentrations in the neck sample (15 min after the extraction of the tail blood sample) of the stressed *vs.* controls (Table II).

There is a significant drop in lactate concentrations (Table II) in stressed rats as compared with the controls.

There is a substantial drop in plasma insulin levels in the stressed *vs.* control rats.

The composite and individual amino-acid and urea concentrations in plasma are presented in Table III. There are no differences between groups 1 and 2 except for urea, which is higher in the group 1 plasma. Again, in the third group animals, neck plasma urea concentration is higher than that of the tail. In the stressed state, the neck but not the tail plasma had a higher urea concentration as compared to the controls.

The pattern of change in amino-acid concentrations in the stressed rats *vs.* the corresponding values of the group 1 and 2 controls is different for the tail and for the neck samples. Alanine, proline, leucine plus isoleucine, valine, lysine and the composite gluconeogenic, imino acids, branched chain and total amino acids show significant decreases in group 3 animals (Table III). In general, the gluconeogenic amino-acids levels are higher in tail rather than neck plasma. This is best illustrated by the pattern of glutamate plus glutamine and serine.

The levels of aspartate plus asparagine, glycine, threonine, arginine, histidine, tyrosine, tryptophan, taurine and the composite aromatic amino acids were not affected by stress and sampling site. There is a significant tail *vs.* neck difference in the levels of hydroxyproline in group 3 animals. The significant differences in the tail *vs.* neck samples found in "cysteine" and methionine plus ornithine concentrations can be attributed to the type and duration of stress. The same can be said about phenylalanine and composite sulphur amino-acid values, as here also the only significant difference is found in the tail samples.

Discussion

The animals stressed by standing on a laboratory bench showed substantial changes in many metabolic parameters. As expected blood glucose concentrations were higher and plasma insulin and liver glycogen lower in stressed animals (FURNER *et al.*, 1972). This is very probably due to the action of the catecholamines released during stress upon the glycogenolytic pathway and pancreatic insulin release (PORTE *et al.*, 1966). Haemorrage and handling stress are potent adrenergic stimuli (HAWKINS *et al.*, 1971; SPITZER & SPITZER, 1972; KRAL & FELLENIUS, 1976; DEPOCAS & BEHRENS, 1977) which may explain the differences observed between tail and neck samples of stressed animals. Nevertheless, the tail sample was very small (about 0.2 ml) and not likely to cause significant hypovolemia.

In the stressed animals, the decrease in glycogen levels would seem to suggest that part of the decrease in absolute and relative liver size weight could be due to the loss of glycogen, however, it has been found (KAPLAN & CHAIKOFF, 1936) that changes in liver weight are primarily related to protein and water than to glycogen.

There is a clear inverse correlation between blood glucose and plasma lactate concentrations. This fact, together with the decreased plasma lactate, glycerol and insulin levels in the hyperglycemic stressed animals seems to be related to decreased glucose utilization through the glycolytic pathway and enhanced gluconeogenesis. This is probably a result of increased release of catecholamines as a response to stress. Adrenergic inhibition of muscle amino-acid release (GARBER *et al.*, 1976*a* & *b*) and enhanced gluconeogenesis is consistent with the observed decrease in gluconeogenic amino acids and imino acids, and glycerol in the stressed animals, as all of them are excellent gluconeogenic substrates (EXTON, 1972; FELIG, 1973). Increased utilization of glycerol for gluconeogenesis is further supported by the fact that catecholamines increase glycerol release from adipose tissue which would otherwise be expected to rise plasma glycerol levels (LAURELL & TIBBLING, 1966). Catecholamines are known to inhibit pancreatic insulin release, which would explain the abrupt descent in plasma insulin levels in stressed animals. The decrease in insulin levels will also be expected to increase glycogenolysis, gluconeogenesis and blood glucose concentration.

Amino-acid concentrations in the plasma of control rats are in agreement with other previous data (AROLA *et al.*, 1976; PALOU *et al.*, 1977*a* & 1978). No data are available to compare the results obtained in this short term stress experiment, but plasma hydroxyproline levels did not change in contrast to long term mild stress in which levels decreased (PALOU *et al.*, 1978).

Branched chain amino acids decrease during continuing stress, suggesting that they are utilized perhaps by muscle as alternative energetic substrates (MANCHESTER, 1965). Sulphur amino-acid concentrations in the stressed animals are characterized by a considerable buildup of both "cysteine" and methionine (plus ornithine), with no significant changes in taurine concentration. This seems to be related to increased proteolysis and release of amino acids from the peripheral tissues, together with a relative lack of utilization of sulphur amino acids.

The effect of stress upon aromatic amino acids is in agreement with the postulated increase in circulating amino acids produced by proteolysis. Phenylalanine shows a relative increase, and the lack of changes of tyrosine and tryptophan concentrations could be the consequence of a balance between increased availability and increased disposal of these amino acids due to stress-induced activation of their main catabolizing enzymes (SCHEPARTZ, 1973; STEFAN *et al.*, 1974 & 1975).

There are important differences in glucose, lactate and urea concentrations depending on the site of extraction. These differences may be in part a result of higher stress (*e.g.* lactate) or to different composition in the arterial/venous blood collected, together with possible contamination with lymph and other fluids in the neck sample. As a tentative conclusion, it can be postulated that utmost care must be taken to avoid changes in the environment of the animals, and that a painless and swift method of sacrifice must be used if experiments dealing with changes in plasma or blood components are to be of real value. Furthermore, the data highlight the importance of sampling site as a potential source of variability of blood components.

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