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Fatty Acid and Tryptophan Changes on Disturbing Groups of Rats and Caging Them Singly

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KNOTT, P. J., P. H. HUTSON AND G. CURZON. *Fatty acid and tryptophan changes on disturbing groups of rats and caging them singly.* PHARMAC. BIOCHEM. BEHAV. 7(3) 245–252, 1977. – The effects of disturbing groups of 24 hr fasted rats on plasma unesterified fatty acid (UFA) and tryptophan concentrations and brain tryptophan concentrations were investigated. Removing rats from cages rapidly increased plasma UFA and corticosterone and decreased plasma and whole blood tryptophan of cage mates. The disturbance also appeared to influence biochemical values of rats in other cages within the same chamber. Effects specific to individual cages were also suggested. In subsequent experiments 24 fasting rats caged together were rapidly transferred to 24 separate cages and killed at intervals. Plasma UFA rose to a maximum by 12 min and then fell toward initial values. Plasma total tryptophan concurrently fell then rose. Its percentage in the free (ultrafilterable) state, and in some experiments the absolute values of free tryptophan rose then fell. When the latter rise was marked then brain tryptophan and the 5-HT metabolite 5-hydroxyindoleacetic acid rose. Tyrosine changes were negligible. Thus altered brain tryptophan level and 5-HT metabolism may be associated with plasma tryptophan changes caused by brief environmental disturbance.

Fatty acid Tryptophan Fasting Stress 5-hydroxytryptamine

THE alteration of plasma tryptophan disposition induced by stress is of particular interest (a) because it can occur by a mechanism unique to this amino acid [19] and (b) because if changes of brain tryptophan concentration follow then resultant changes of 5-hydroxytryptamine (5-HT) synthesis [19] could have behavioural consequences. It is possible therefore that behavioural responses to changes of external or internal milieu could be mediated or modulated by these biochemical changes.

The above changes, unique to tryptophan can be provoked by secretion of hormones which affect lipolysis [29] so that plasma unesterified fatty acid (UFA) concentration rises. This leads to an increase in the free fraction of plasma tryptophan since UFA binds to albumin and thus weakens the binding of tryptophan to it [12,24]. Other amino acids are unaffected as tryptophan is the only plasma amino acid appreciably bound to albumin [25]. When plasma free tryptophan concentration rises it appears to be rendered more available to the brain. Thus immobilization [19] fasting [19], acute liver failure [13,20] and various drugs [9] all increase the concentrations in plasma of both UFA and free tryptophan and also increase brain tryptophan concentration.

In the above studies, plasma total tryptophan concentrations showed relatively small changes. However under other conditions increased plasma UFA concentration is associated with a considerable and rapid fall of plasma total tryptophan e.g., when rats housed in groups and fasted for 24 hr are disturbed by the removal of cage-mates [10]. In these experiments although the percentage of plasma tryptophan in the free state rose the absolute concentration of free tryptophan showed little change. Plasma total tryptophan also fell in human subjects when plasma UFA concentration rose following adrenaline injection [15], although in these circumstances the fall of total tryptophan was not sufficiently great to prevent a rise in the absolute concentration of plasma free tryptophan.

The absence of an increase of the absolute concentration of plasma free tryptophan following cage disturbance may explain why this was not found to increase brain tryptophan concentration [10]. Another possibility is that the 6 min period over which biochemical changes were determined after disturbance may have been too short for plasma tryptophan changes to have influenced its concentration in the brain.

The present paper describes further studies on the effect of cage disturbance on UFA and on tryptophan disposition. The investigations revealed some sources of variation and led to an experimental design which permitted the study of the above changes over a longer period after the initial cage disturbance.

METHOD

Male Sprague-Dawley rats (Anglia Laboratory Animals, Alconbury, Huntingdon, England) were housed on arrival in

the laboratory in cages in an acoustically lagged chamber with a light 6 hr 00: dark 18 hr 00 cycle. The chamber measured 110 × 45 × 80 cm high and was kept at 25 ± 2° except in experiment 3ii (see RESULTS). In Experiment 1 and 2 (see RESULTS) the rats were housed in standard plastic cages, 22 × 36 × 17 cm high. For Experiments 3 and 4, 24 rats were housed in a large two-story cage made from a plastic box measuring 60 × 45 × 30 cm high. The upper story was a grid covering 3/4 of the cage area and supported 15 cm above the floor by steel brackets bolted to the inside of the cage. Food was available from a 1.6 l capacity metal basket on the upper story and water from eight valve outlets (NKP Plastics, Dartford Kent, England) connected to a reservoir and passing through a grid covering the cage. Ventilation holes each measuring 6 × 1 cm were protected from gnawing by the above brackets which had similar holes cut in them.

The rats were allowed ALGH Rodent Diet (Grain Harvesters Ltd., Canterbury, England) and tap water ad lib. Except where stated otherwise the animals weighed 120–140 g on arrival and 180–220 g after 6–9 days. Food but not water was then withdrawn and the rats killed by decapitation exactly 24 hr later between 14 hr 00 min and 15 hr 00 min.

Blood was collected into heparinised tubes, immediately centrifuged and the plasma stored at −20°. Brains were removed and similarly stored. Plasma corticosterone, total tryptophan, free tryptophan and tyrosine and brain tryptophan, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and tyrosine were determined as before and blood tryptophan determined by the method previously applied to packed red cells [10]. Plasma pH rose to 7.8–7.9 during storage so that tryptophan binding was increased [26]. Therefore, free tryptophan values obtained were lower than in vivo to a comparable extent in all experiments. Plasma unesterified fatty acid (UFA) were determined by the method of Laurell and Tibbling [21] except in one experiment in which a fluorimetric method was used [11].

The following terms were used in describing results.

Intra-chamber effects. Refers to changes due to removal of rats from the same enclosure but different cages.

Intra-group effects. Refers to changes due to removal of cage-mates.

Cage-specific effects. Refers to changes occurring in only one of the cages of rats studied in an experiment.

Rats were treated according to the procedures described below.

RESULTS

Effects of Removal from Group Housing

Experiment 1. Rats housed 2/cage. The object of this experiment was to determine whether plasma UFA and tryptophan changes following removal of cage-mates of rats housed in pairs were similar to those previously found when rats were housed in groups of 3 and 4. Fourteen rats were caged in pairs and 7 days later the animals in the first 4 cages were killed sequentially at 2 min intervals. (Both rats in each of these cages were killed before the animals in subsequent cages.) A rat from each of the other 3 cages was then killed at 2 min intervals. The remaining rats were killed 8 min after their cage-mates.

Intra-chamber effects. Results in Fig. 1a show that plasma UFA was lowest in the first killed rats and rose significantly with times of killing up to 28 min later while

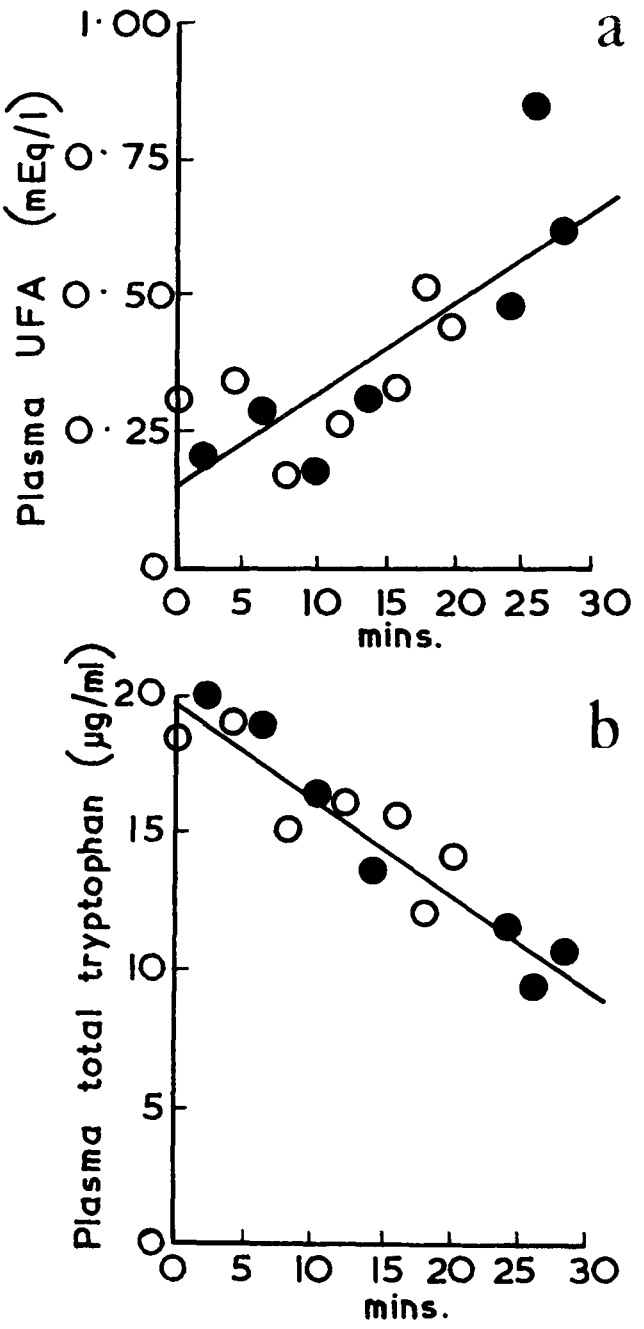

FIG. 1. Effect of disturbance of fasted rats caged in pairs on (a) plasma unesterified fatty acid (UFA) concentration (b) plasma total tryptophan concentration. (Experiment 1). The x axis shows time (after removal of the first rat from the chamber) at which the animals were killed. First and second animals removed from each cage: ○, ● $n = 14$. Plasma UFA v. time: $r = 0.61$, $p<0.02$. Plasma total tryptophan v. time: $r = -.87$, $p<0.001$. Plasma total tryptophan v. UFA (not shown): $r = -.62$, $p<0.02$. The rats in the first 4 cages were killed at 2 min intervals and those in the remaining 3 cages at 8 min intervals.

plasma total tryptophan fell concurrently and significantly (Fig. 1b), so that there was a significant negative correlation between plasma total tryptophan and UFA concentrations.

Intra-group effects. Plasma UFA and total tryptophan concentrations of second killed rats from pairs of animals were not significantly different from values for their cage-mates killed either 2 or 8 min before. However these results were obtained using only 3 or 4 cages at each of these times. In another experiment of similar design but with 8 cages of paired rats each weighing 90–120 g plasma UFA concentrations were found to be significantly higher, total tryptophan concentrations significantly lower and free tryptophan significantly higher in rats killed 2 min after their cage-mates (Table 1).

TABLE 1

EFFECT OF ORDER OF KILLING ON PLASMA UNSTERIFIED FATTY ACID (UFA) AND TRYPTOPHAN OF RATS CAGED IN PAIRS

	Plasma UFA meq/l	Plasma Tryptophan μ g/ml	
		Total	Free
Killed initially (8)	0.62 ± 0.10	16.3 ± 1.7	2.55 ± 0.57
Killed 2 min later (8)	0.79 ± 0.17	13.2 ± 2.0	3.31 ± 0.40
P	<0.05	<0.01	<0.02

Results are expressed as means ± one SD. Numbers of determinations shown in parentheses. Results compared by Student's *t*-test. Rats weighing 90–120 g were deprived of food but not water 24 hr before killing.

These results indicated that the interpretation of experiments on paired rats was likely to be difficult as both intra-chamber and intra-group disturbances appeared to influence metabolism. As intra-chamber effects were not noted in earlier work on 3 or 4 rats/cage killed at 2 min intervals [10] the next experiment was done using 4 rats/cage.

Experiment 2. Rats housed 4/cage. The object of this experiment was to study the effects of removal of cage-mates over a longer period than previously. Twenty-four rats were caged in groups of 4. Seven days later the rats in each cage were killed at 5 min intervals so that the time between killing the first and last rat in any cage was 15 min. Rats from different cages were killed at overlapping times so that all 24 animals were decapitated within 1 hr. In this experiment plasma UFA concentrations were determined fluorimetrically [11].

Intra-chamber effects. As in Experiment 1 but in contrast with the previous study [10] in which the rats were killed at shorter intervals, intra-chamber influences are indicated. Thus plasma UFA but not corticosterone values of the first rats removed from each cage rose in parallel with order of removal from the chamber (Fig. 2).

Intra-group effects. In every cage plasma UFA of subsequently removed rats tended to be higher than that of corresponding initially removed cage-mates. There was a qualitatively similar relationship between UFA and order of removal in 5/6 cages – indications of a peak in rats removed secondly followed by a fall in those removed thirdly and a rise in the finally removed rats. The animals in cage 3 showed a different pattern (described under group-specific effects below). A trend was also indicated in the

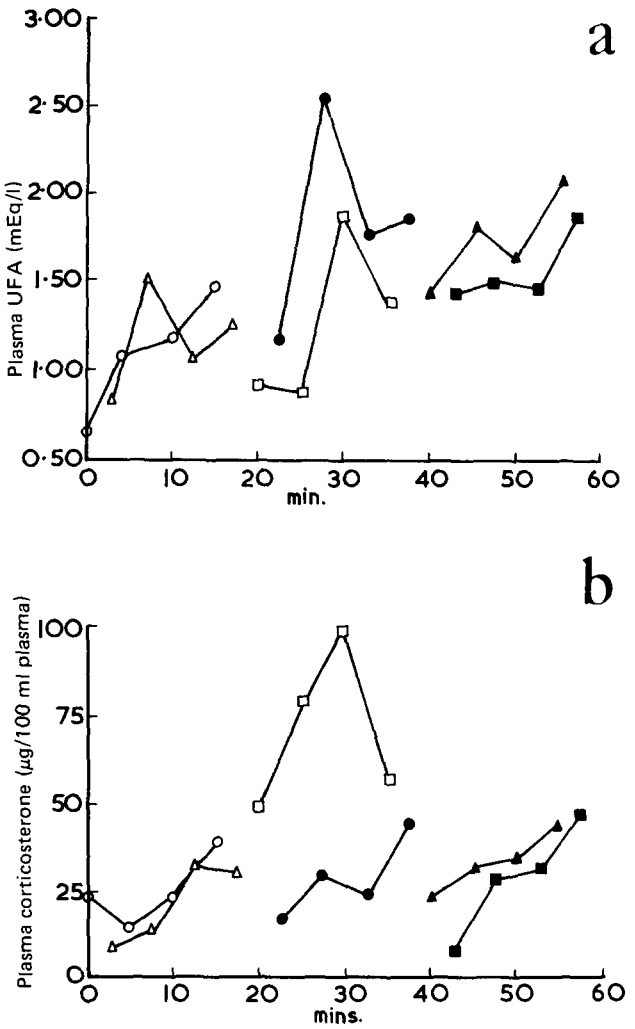

FIG. 2. Effect of disturbance of fasted rats caged in groups of 4 on (a) plasma UFA concentration (b) plasma corticosterone concentration. (Experiment 2). Axes as in Fig. 1. Rats from cages 1–6 are indicated by ○, △, □, ●, ▲, ■, respectively.

differences between values for rats removed initially and secondly. This difference gradually rose, becoming greatest in cage 4 and then falling progressively over the next two cages. As suggested by previous findings [10], plasma corticosterone increased with order of removal from the cage.

Group-specific effects. Rats in cage 3 had markedly different patterns of both UFA and corticosterone concentrations from those noted above in the other cages. Thus the rise of plasma UFA concentrations, although large, was retarded until the thirdly removed rat and corticosterone concentrations of the whole group were high.

The above results indicated intra-chamber, intra-group and group specific influences on the two stress-dependent variables plasma UFA and corticosterone. Therefore in subsequent experiments intra-chamber and group specific differences were eliminated by taking all the rats from one large cage within as short a time as possible and housing then singly for different periods before killing.

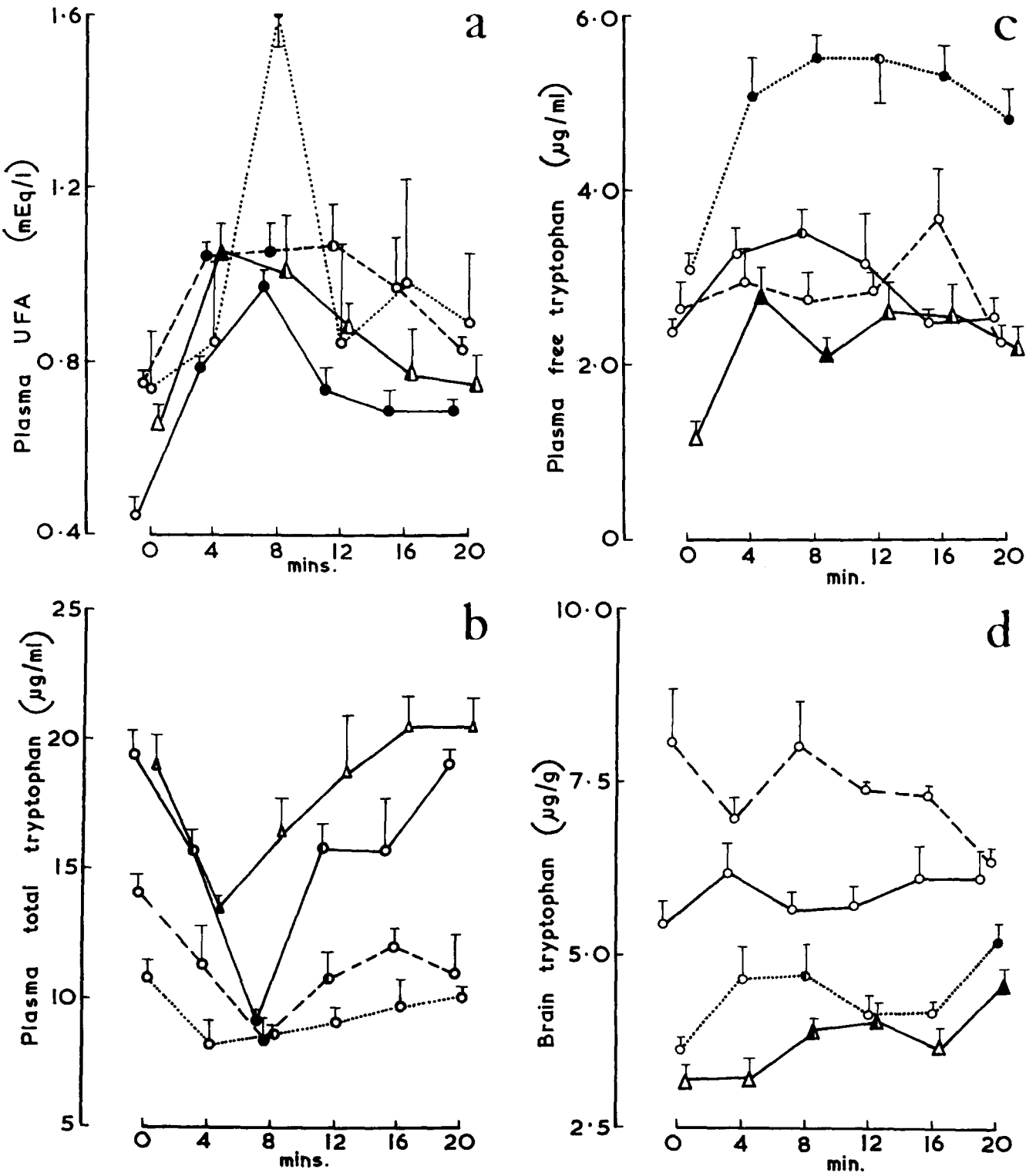

FIG. 3. Effects of removal of fasted rats from group of 24 and rehousing singly, on concentrations of (a) plasma UFA, (b) plasma total tryptophan, (c) plasma free tryptophan and (d) brain tryptophan. (Experiments 3 and 4). In Experiments 3i (——○——), ii (— — —○— — —) and iii (. . . .○. . . .) rats were rehoused singly and killed in groups of 4 at the times indicated. Experiment 4 (——△——) was performed similarly except that after the group of rats were killed at 12 min the remaining animals were rehoused singly for a second time. Symbols are slightly displaced laterally to enhance clarity. Means ± one SE are shown. Results on each group compared with those obtained on the initially killed group by Students *t*-test are indicated as follows: NS, ○, △; $p<0.05$ ◐, ◭; $p<0.001$, ●, ▲.

Effect of Removal from Group Housing (24/Cage) and Rehousing Singly

Experiment 3. Rehousing Once. Twenty-four rats were housed in the large 2 story cage (see METHOD). Nine days later 4 rats were removed and rapidly killed. The remaining rats were concurrently removed and singly housed in twenty 28 × 20 × 18 cm high cages so that 90 sec after opening the large cage all 20 animals had been recaged. They were killed in groups of 4 at 4, 8, 12, 16 and 20 min after the initial cage disturbance. Each group of 4 rats was killed within 20 sec.

The experiment was performed 3 times under somewhat different conditions (Experiments 3i, ii, iii). Thus the rats in Experiment 3i were subjected to additional disturbance during the week before the experiment began because it was found necessary to modify the cage somewhat during this period to the specifications described under METHOD. Experiment 3ii was done during exceptionally hot weather in which temperature control could not be maintained and the chamber was at 28–34°. Plasma UFA rose and total tryptophan fell on each occasion (Fig. 3) although the magnitude of their changes varied from experiment to experiment. Thus peak values of UFA were 117%, 43% and 120% higher than initial values in Experiments 3i, ii, iii, respectively. However, in all three experiments peak UFA values were reached 8 min after the rats were removed from the large cage and then fell towards the values found for the initially removed animals (Fig. 3a).

Although plasma total tryptophan concentrations of the initially removed rats were considerably different in the three experiments (Fig. 3b) they fell to almost identical minima at 8 min and then rose towards the values found for the initially removed animals.

The rise of UFA concentrations was paralleled by large increases of the percentage of plasma tryptophan in the free form (not shown). However, as before [10] the associated fall of plasma total tryptophan led to the changes of absolute free tryptophan concentration being smaller than those of percentage free tryptophan so that absolute increases were significant in Experiments 3ii and iii only (Fig. 3c). The rise in Experiment 3iii was considerably greater and more prolonged than in Experiment 3ii.

Brain tryptophan changes (Fig. 3d) had some parallelism with those of plasma free tryptophan (Fig. 3c) although they were relatively small. Thus appreciable and significant brain tryptophan increases occurred in Experiment 3iii while small increases and decreases were found in Experiment 3ii and 3i, respectively. These relationships are clearer when shown as percentages changes (Fig. 4). The increase of brain tryptophan in Experiment 3iii was not associated with clear changes of brain 5-HT or 5-HIAA concentrations (Fig. 5)..

Plasma tyrosine concentrations unlike those of tryptophan showed small and inconsistent changes following disturbance (Fig. 6a) while brain tyrosine also did not show clear changes (Fig. 6b) (results not shown).

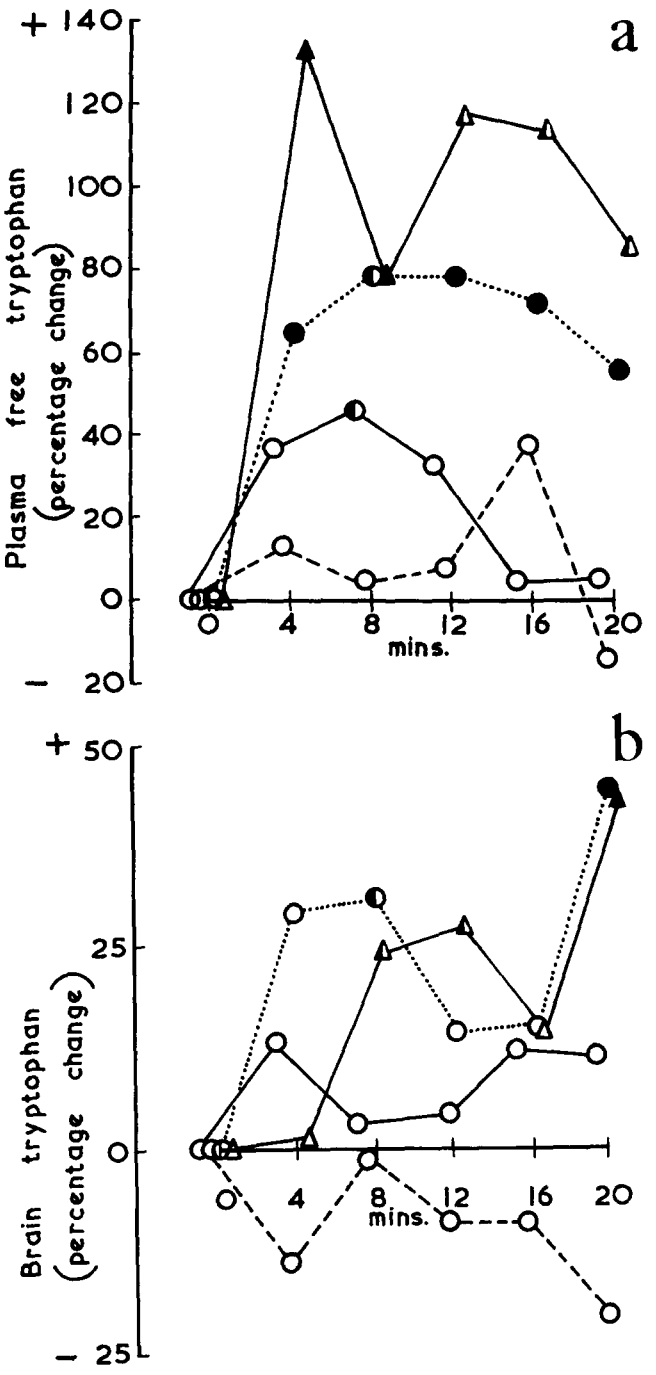

FIG. 4. Effect of removal of fasted rats from a group of 24 and rehousing singly on percentage changes of (a) plasma free tryptophan (b) brain tryptophan (Experiments 3 and 4). Results from Fig. 3 are expressed as percentage changes from values for corresponding initially killed groups. See legend to Fig. 3 for details.

Experiment 4. Recaging Rats Twice

The main object of this experiment was to attempt to investigate whether the biochemical changes shown in Experiment 3 resulted from the initial removal of the rats from the large cage or from their subsequent caging singly. The experimental design was exactly as in Experiment 3 except that immediately after killing the 4 rats which had been caged singly for 8 min the remaining 12 singly caged animals were transferred again to a second series of individual cages. They were then killed in groups of 4 at 12, 16 and 20 min after removal from the large cage.

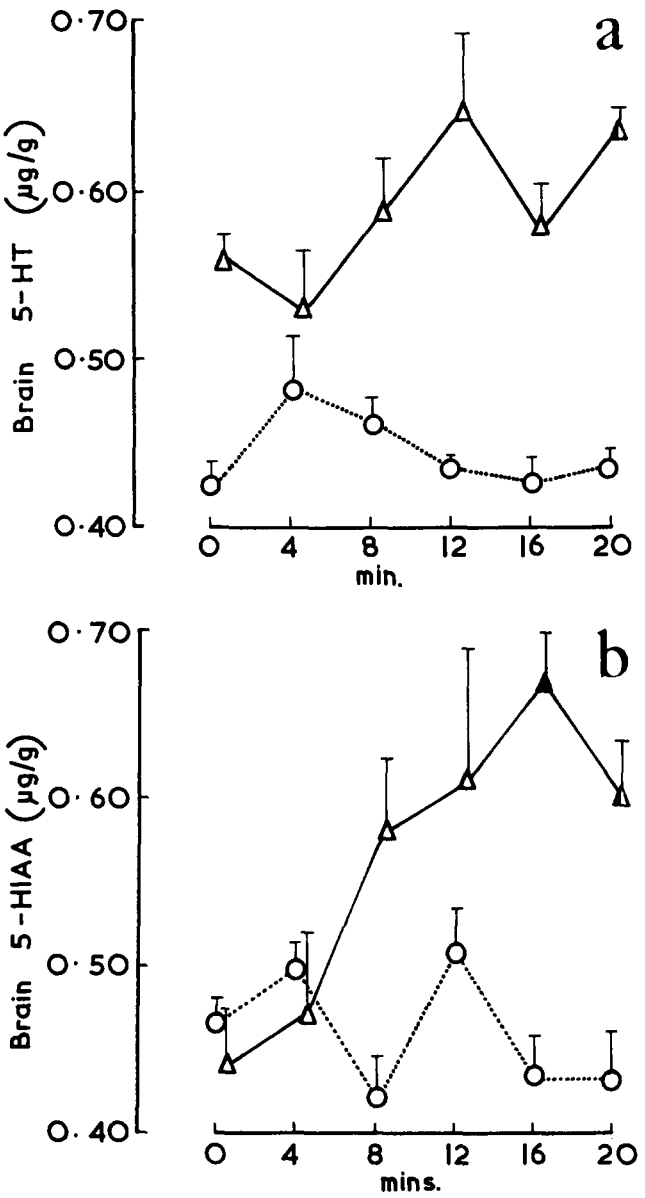

FIG. 5. Effect of removal of fasted rats from group of 24 and rehousing singly on concentrations of (a) brain 5-HT (b) brain 5-HIAA (Experiments 3iii, 4). See legend to Fig. 3 for details.

The patterns of plasma UFA and of total and free tryptophan changes (shown in Fig.. 3) are qualitatively similar to those found in Experiment 3 except that they were greatest at 4 min instead of at 8 min after the initial disturbance. Whole blood tryptophan concentration was also determined in this experiment and in common with plasma total tryptophan fell to a minimum at 4 min and then rose (Fig. 6). Brain tryptophan concentration first appeared elevated 4 min later than plasma free tryptophan (Figs. 3c and d) while brain 5-HT although it did not show significant changes did tend to parallel those of brain tryptophan while 5-HIAA increased significantly with a peak value at 16 min after the initial disturbance. Results in general did not suggest any clear influence of the second recaging on the biochemical parameters measured.

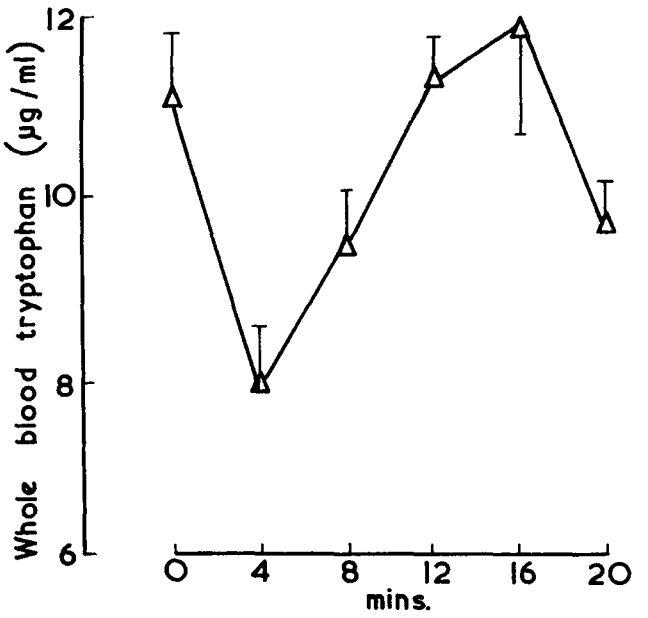

FIG. 6. Effect of removal of fasted rats from group of 24 and rehousing singly on blood tryptophan concentration (Experiment 4). See legend to Fig. 3 for details.

DISCUSSION

Results confirm the previous finding that rats deprived of food for 24 hr and disturbed by removal of cage-mates show rapid increases of plasma UFA concentration and of the percentage of plasma tryptophan in the free state and a rapid decrease of plasma total tryptophan concentrations [10]. The latter change was not simply due to uptake of newly freed tryptophan by red blood cells because the concentration of tryptophan fell in whole blood. This result supports the previous experiment [10] in which red blood cell tryptophan was not appreciably altered by disturbance. As before, tyrosine was unaffected which is consistent with the tryptophan changes being due to a mechanism unique to this amino acid. In the earlier work cage disturbance of fed rats provided only one of the above biochemical changes, i.e., the increase of the percentage of tryptophan in the free state. Recently however in agreement with work by others [3] we have also found significantly increased plasma UFA concentrations on disturbing fed rats (unpublished work).

The present experiments show that the biochemical changes found are influenced not only by removal of cage-mates (intra-group effects) but also by removal of rats from other cages in the same enclosure (intra-chamber effects). Thus concentrations of UFA in the plasma of rats removed first from each cage within a chamber rose with their order of removal from the chamber which contained the cages (Fig. 2a). Superimposed on this intra-chamber trend a characteristic pattern of plasma UFA changes related to the order of removal from each cage is indicated, i.e., an increase of UFA at 5 min after removing the first rat from the cage followed by a fall at 10 min and a final rise at 15 min. A possible explanation of these changes is that the rats responded to the removal of a cage-mate by a considerable rise of plasma UFA 5 min later but that the removal of a second rat had little further effect so that

UFA fell (cages 2, 4, 5, 6) or rose less rapidly (cage 1) by 10 min. However, as removal of the third rat at 10 min isolated the fourth animal for 5 min before killing its environment had been altered in a qualitatively different manner from that of its former cage-mates. This may have led to plasma UFA concentrations being higher in last removed rats. It suggests that plasma UFA may also be elevated in last removed rats from groups of other sizes.

The above pattern was not shown by the rats in cage 3. As these animals also had strikingly higher plasma corticosterone values than the rats in the other 5 cages the results obtained were probably not merely fortuitous but may have reflected a different response of this group of rats related to their behavioural state at the time of disturbance.

The results in Fig. 2a reveal not only intra-chamber and intra-group effects. They also suggest that the magnitude of the latter effect may depend on the former one. Thus the differences between UFA values of rats removed at 5 min and those removed initially increased from cage 1 to cage 4 and then declined from cage 4 to cage 6 with the exception of cage 3 (see above). It may be conjectured that previous disturbances of the chamber at first cumulatively increases awareness of the removal of cage-mates but eventually the rats become accustomed to it. An early increase of responsiveness following disturbance of the chamber is not surprising in experiments done during the light period when rats are normally quiescent, as the disturbance of the chamber due to removal of rats from other cages may have made animals more aware of the removal of their own cage-mates.

As a whole, the results in Figs. 1 and 2 illustrate how commonly used experimental designs can lead to quite complex biochemical variations within the same experiment due to intra-chamber, intra-group and group-specific mechanism. For these reasons, stress experiments in which animals to be studied are either singly housed [30] or withdrawn from one large cage may give more clearly interpretable results. When the latter procedure was used in experiments in which the rats were all removed from a large cage within 90 sec and singly housed for various times before killing while the magnitudes of plasma UFA and tryptophan changes were different in different experiments their time courses were comparable. Furthermore, although initial plasma total tryptophan concentrations varied considerably between the experiments the minima to which they fell were almost identical each time Experiment 3 was done suggesting that a compensatory mechanism may be triggered at this tryptophan level. The minimum in Experiment 4 was however not as low.

The results of Experiment 4 suggest that the biochemical changes were precipitated by the initial removal of the rats from the large cage but that their subsequent single housing had no effect as a second period of single housing caused no additional biochemical change. However the first period of single housing might have had a greater effect than the second. Also biochemical response to isolation is suggested by the high UFA values of rats isolated for 5 min in their home cages after removal of cage-mates (Fig. 2).

Quite mild and brief stresses are known to increase plasma concentrations of UFA [3] corticosterone and prolactin [30] in the rat. However the effects of stress on central transmitter metabolism have usually been studied using quite severe or prolonged procedures such as immobilization and electric shock [4,8]. It is therefore of interest that a relatively mild environmental disturbance can lead not only to the changes of plasma UFA and tryptophan concentrations discussed above but also in some but not all experiments to increase concentration in the brain of tryptophan (a 5-HT precursor). This was not found in earlier work [10] in which determinations were only made at times up to 6 min after the initial cage disturbance. In the present experiments brain tryptophan rose only when plasma free tryptophan concentration rose considerably. It did not rise when plasma total tryptophan concentration decreased so markedly that only the percentage of plasma tryptophan in the free state rose (i.e., Experiment 3i). Thus when environmental disturbance leads to liberation of tryptophan from plasma albumin its access to the brain is apparently opposed by its extracerebral transport and catabolism. Responsible mechanisms may include degeneration by hepatic tryptophan pyrrolase or other mechanisms dependent on stress provoked secretion of adrenocortical hormones [8, 16, 17, 18].

Brain tryptophan concentrations may rise not only upon an increase of plasma free tryptophan concentration but also when insulin secretion increases following food intake [14] as this can result in a fall of plasma concentrations of amino acids which compete with tryptophan for transport to the brain. This mechanism can hardly explain the brain tryptophan increases found in the present study as food was not made available. Furthermore, short periods of stress usually lead to decreased plasma insulin [28]. However, the possibility is not excluded that rapid changes of plasma concentrations of competing amino acids due to some unknown stress dependent mechanism could have influenced brain tryptophan concentration. Nevertheless, the indications of a relationship between the changes of plasma free tryptophan and brain tryptophan concentrations revealed when four different cage-disturbance experiments are compared (Fig. 4a,b) strongly suggests that these values are causally related.

While the results obtained show an overall consistency the magnitude of changes varied considerably from experiment to experiment. This may well have reflected environmental differences as not only were the experiments performed at different times of year but also the cage-disturbance necessitated exposure to an uncontrolled milieu. The variability of the changes found is strikingly illustrated by the percentage increases of plasma free tryptophan (Fig. 4a) which ranged from negligible changes in Experiment 3ii (when the animals may have been affected by the high environmental temperature) to the particularly large increases in Experiment 4. The latter experiment was the only one in which a definite increase of brain 5-HT turnover was shown. It is also of interest that brain tryptophan increased after cage disturbance only in the experiments in which the initially killed rats had relatively low brain tryptophan concentrations (Fig. 3d).

However, brain tryptophan and concurrent plasma free tryptophan concentrations were not significantly correlated. This is not surprising in view of the presumably dynamic increase of plasma free tryptophan concentration on cage disturbance but also the associated decrease of plasma total tryptophan (Fig. 3b) may well influence brain tryptophan concentrations as recent evidence shows that tryptophan can be stripped from binding to plasma albumin as it passes through the vascular bed of the brain [32].

Although only very large increases of brain tryptophan affect rodent behavior in the home cage [23,27] activity in an open field of normal rats [5,31] and sensitivity to pain of tryptophan deficient rats [22], are altered by relatively

small changes of tryptophan intake. Therefore it is possible that the magnitude of brain tryptophan and 5-HT changes resulting from environmental disturbance and other stresses may influence the nature and appropriateness of subsequent behavioural responses. Such considerations may be relevant to depressive illness as deficiencies of tryptophan and 5-HT may be important here [1,7]. They could also be involved in abnormalities of appetite control as high brain tryptophan [2,20], and the post-synaptic action of 5-HT [6] are associated with decreased food intake.

ACKNOWLEDGEMENT

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