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EFFLUX FROM ISOLATED RAT RETINA OF AMINO ACIDS FORMED FROM DIFFERENT SUBSTRATES

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Abstract—The efflux of GABA, glutamine, glutamate and aspartate was studied in isolated superfused rat retinae which had been incubated with radioactive glucose, acetate or GABA. Apart from the glutamate derived from GABA, amino acid efflux was always biphasic in nature and possibly represented the outflow from two separate tissue compartments. The corresponding rate constants for the two phases were calculated from the efflux curves. These showed that the outputs of all of the amino acids occurred at different rates with each of the three precursors, suggesting that different amino acid pools were being labelled by the different substrates. With exogenous GABA as the precursor, but not with glucose or acetate, aspartate and glutamine were particularly firmly bound by the retinae. With GABA also, amino acid release took place equally well in the presence of AOAA (0·1 mM), implying that outflow was not affected by the continuing metabolism of GABA within the tissue. Addition of cold GABA (0·4 mM) to the superfusion medium enhanced the release of radioactive GABA and glutamine derived from each of the substrates, probably by homoexchange and heteroexchange respectively. Glutamate efflux was always suppressed under these conditions, while aspartate release was not modified. Once again, there were considerable differences in the magnitudes of these responses depending on the radioactive substrate used to label the tissue, further suggesting the separate identity of the pools of amino acids involved.

It has been shown recently that the metabolism of various substrates in retina, as in brain tissue, is compartmented [17]. This means that separate pools of the same metabolites, such as amino acids, exist in the retina. The phenomenon may have considerable functional significance since a number of amino acids have been postulated to be transmitter substances within the retina [1, 5, 8, 16, 20]. Particular attention has been paid to 4-aminobutyric acid (GABA) [9–14, 19, 21, 22]. One of the problems posed by GABA is how to distinguish between that tissue pool of it which is involved simply in the energy metabolism of the cell and a separate, 'transmitter' pool [2].

Logically, one might expect that if different tracer substrates are selectively metabolised within separate metabolic compartments, then the efflux of any one metabolite from these compartments should occur at characteristically different rates, which should be independent of the substrate used to label it in the first place. One might also expect that the barriers to release will vary from pool to pool, depending on the type of cell or subcellular unit involved, and that this could form a further basis for distinguishing between such pools. Conversely, one could argue that similarities in the rates of efflux of a given metabolite, for example GABA, which had been derived initially from different precursors, may indicate that these substrates have penetrated and been metabolised within a common tissue pool. This approach has been used in the present study in an attempt to determine the fate of exogenous GABA by comparing the patterns of efflux of several amino acids synthesised from GABA by rat retina with those derived from incubation with glucose and acetate, as it has been shown that the latter substrates normally label different amino acid pools in this tissue [17].

MATERIALS AND METHODS

Wistar albino rats (Tuck and Charles River) of either sex and weighing 150–250 g were killed by cervical dislocation and the eyes rapidly enucleated. The retinae were quickly dissected out and two retinae placed together in 1 ml Krebs bicarbonate medium (for details of composition see Starr and Voaden [19]) containing 2·78 mM glucose and gassed with 5% carbon dioxide in oxygen. The retinae were given a preincubation at 37° lasting 15 min, then 10 μCi of D-[U-^{14}C]glucose (168 mCi/m-mole) or 20 μCi of [1-^{14}C]sodium acetate (58 mCi/m-mole) or 5 μCi of [1-^{14}C]GABA (3·14 mCi/m-mole) were added and the incubation continued for a further 60 min. After this time the retinae were washed in 20 ml fresh Krebs solution for 2 min and then placed in a plastic gauze holder suspended inside a small perspex perfusion chamber, capacity 1 ml. The chamber was kept at 37° by a heating jacket. A reservoir of Krebs solution was gassed continuously with the CO_2/O_2 mixture and the fluid pumped through the perfusion chamber via a warming coil at the rate of 1 ml/min by means of a roller pump. Samples of the perfusate were collected every 10 min by a fraction collector for analysis. These were acidified by the addition of 0·1 ml 5 N perchloric acid and the radioactive amino acids present in these samples and in the retinae at the end of the experiment were extracted and separated by a combination of column and paper chromatography as described in detail elsewhere [17]. The radioactivity

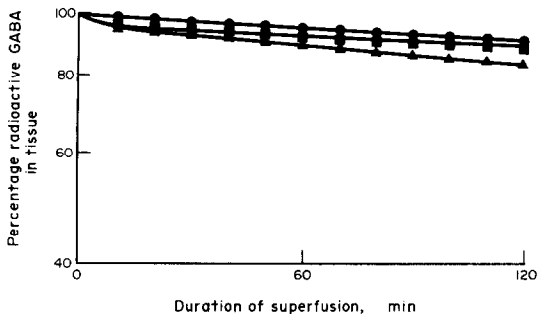

Fig. 1. Time course of the release of GABA from superfused retinae. Pairs of retinae were incubated with 10 μCi D[^{14}C]glucose (60 nmoles), 20 μCi [1-^{14}C]sodium acetate (172 nmoles) or 5 μCi [1-^{14}C]GABA (1·59 μmoles) for 60 min at 37° in 1 ml Krebs solution. They were then washed for 2 min and superfused together and the outputs of the labelled amino acids measured. The figure shows the release of radioactive GABA prepared from labelled glucose (●—●), acetate (▲—▲) and exogenous GABA (■—■). Each curve is the average of at least six experiments.

present in each amino acid spot separated on the paper chromatograms was eluted with 6 ml 2-ethoxyethanol. After allowing to stand for 60 min, 10 ml 0·5% butyl PBD were added to each eluate and the radioactivity counted in a liquid scintillation counter (Nuclear Chicago Model Isocap 300). Counts per min were converted to dis/min after correcting for quenching, background radiation and total recovery. Efflux curves were constructed as before [23].

All radioactive compounds were obtained from The Radiochemical Centre, Amersham; butyl PBD from Fisons Scientific Appliances Ltd., Loughborough; toluene from BDH Chemicals Ltd., Poole; scintillation grade 2-ethoxyethanol from Hopkin and Williams, Chadwell Heath and amino-oxyacetic acid (AOAA) from Aldrich Chemical Company Ltd., Milwaukee, Wisconsin.

RESULTS

GABA efflux. The time-courses for the release of GABA derived from various radioactive precursors are shown in Fig. 1. Each exponential efflux curve was resolved into a fast and a slow linear component and the corresponding rate constants, k_1 and k_2, are listed in Table 1 [4, 23]. It is assumed, as with the other amino acids studied, that these represent the outflow of radioactive material from separate tissue pools [6]. The speed of output of unchanged GABA, previously accumulated from the incubation medium, was very fast over the first 10 min of superfusion, but was then much slower for the remainder of the experiment. The biphasic nature of this and some other curves (see Fig. 2) was more readily apparent when the data were plotted using an expanded log scale. The initial rates of release of GABA synthesised from either glucose or acetate were considerably slower, and in each case it took about 40 min superfusion to exhaust the faster releasing GABA pool and to reveal the slower releasing one. The rate constants obtained for the liberation of GABA from retinae prelabelled with glucose and exogenous GABA were not significantly different from each other ($P > 0·01$), but were both approximately half the rate observed when acetate was the source of GABA carbon.

Glutamine efflux. Glutamine was readily synthesised from all three radioactive precursors and in each case passed out into the superfusion medium in a biphasic fashion (Fig. 2 and Table 1). When GABA was used as the precursor a small fraction of the glutamine (less than 4% of the total) was rapidly lost from the tissue and this was followed by a relatively slow and steady efflux for the rest of the experiment. This slower phase only accounted for a further decrease in the tissue radioactive glutamine content of about 18%, which meant that at the end of the superfusion as much as 78% still remained inside the tissue. With glucose and acetate as the precursors, however, glutamine was released more slowly at the beginning of

Table 1. Release of ^{14}C-amino acids from isolated superfused retinae following incubation with radioactive glucose, acetate or GABA

Amino acid	^{14}C-precursor used to label amino acid	Efflux rate constants ($min^{-1} \times 10^{-3}$) k_1	k_2	k'_2	k'_2/k_2	Radioactivity remaining in amino acids after superfusion with normal medium (%)
GABA	glucose	77·8	0·64	4·49	7·1	92·1
	acetate	44·5	1·07	1·89	1·8	84·1
	GABA	397	0·48	4·58	9·5	90·9
Glutamine	glucose	72·0	15·2	64·5	4·3	10·5
	acetate	89·8	8·48	22·1	2·6	10·3
	GABA	371	2·04	2·40	1·2	78·0
Glutamate	glucose	41·7	1·57	0·22	0·14	72·7
	acetate	55·8	0·66	0·33	0·5	83·4
	GABA	-	2·62	1·23	0·47	71·7
Aspartate	glucose	N.D.	0·93	0·94	1·0	88·5
	acetate	N.D.	1·38	1·39	1·0	83·8
	GABA	N.D.	0·17	0·17	1·0	97·5

Rate constants were calculated from the efflux curves depicted in the figures after resolution of the curves into fast (k_1) and slow (k_2) linear components according to the method described by Brading [4]. In some experiments non-radioactive GABA (0·4 mM) was added to the medium 120 min after the startof superfusion and the new value for the slow efflux component (k'_2) calculated (see Fig. 5).

N.D. = not determined.

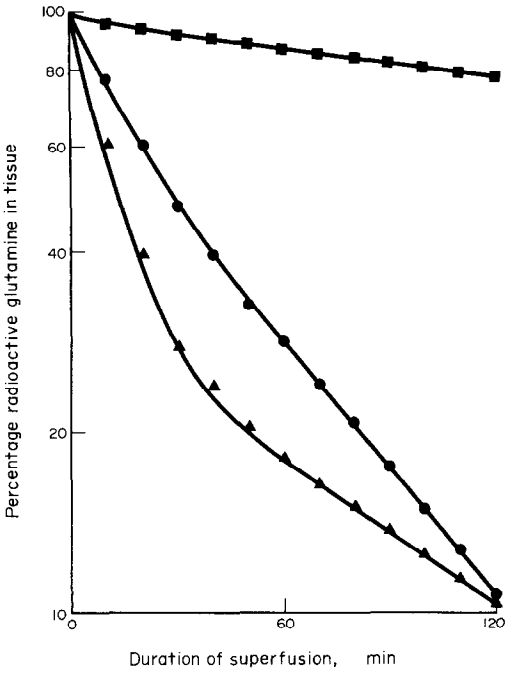

Fig. 2. Time-course of the release of glutamine from superfused retinae. Experimental details are shown in Fig. 1. The figure shows the release of radioactive glutamine labelled from glucose (●—●), acetate (▲—▲) and exogenous GABA (■—■). Each curve is the average of at least six experiments.

the experiment, although this early phase continued for a longer time and eventually accounted for a greater depletion of the tissue radioactive glutamine content, amounting to a loss of 27% of the glutamine with glucose and 69% with acetate as the substrate. The remaining amino acid continued to efflux at a fairly fast rate and after 120 min superfusion scarcely any radioactive glutamine (about 10%) was detected in the tissue.

Glutamate efflux. The efflux of glutamate formed from GABA was found to be a monophasic process, whilst the efflux of glutamate synthesised from either glucose or acetate was biphasic (Fig. 3 and Table 1). The two fast phases both accounted for the disappearance of roughly 10% of the tissue radioactive glutamate content and both lasted for about 60 min. Judging from the widely different values obtained for k_2 with the different precursors it would appear that they do not label the same tissue pool of glutamate.

Aspartate efflux. The efflux of aspartate was always found to be essentially monophasic in nature (Fig. 4 and Table 1). The efflux curves deviated from linearity at the beginning of each experiment, but this phase always accounted for such a small amount of the tissue radioactive aspartate content (less than 1%) that it was considered to be a possible residual extracellular washout and hence ignored for the purposes of the present study. Figure 4 and Table 1 both plainly show that when this amino acid was synthesised from GABA it was bound very tightly by the retinae, as only 2·5% escaped into the medium during the 120-min period of superfusion. On the other hand, slightly larger amounts of the aspartate derived from glucose

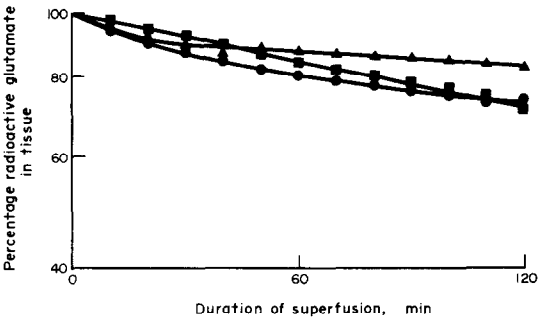

Fig. 3. Time-course of the release of glutamate from superfused retinae. Experimental details are the same as for Fig. 1. The figure shows the release of radioactive glutamate prepared from labelled glucose (●—●), acetate (▲—▲) and exogenous GABA (■—■). Each curve is the average of at least six experiments.

(11·5%) and acetate (16·2%) were lost under these conditions. The rates at which these effluxes took place are shown in Table 1.

Effect of AOAA on efflux of amino acids labelled from [1-^{14}C]GABA. The experiments with [1-^{14}C]GABA were repeated with AOAA (10^{-4} M) present in both the washing and superfusion media. At this concentration AOAA is known to inhibit completely the activity of the enzyme 4-aminobutyrate aminotransferase, thereby reducing considerably the metabolism of accumulated GABA [19]. The results are shown in Table 2. There did not appear to be any marked differences in the rates of output of any of the four amino acids in the presence of AOAA (Tables 1 and 2), and the levels of radioactivity recovered in the amino acids present in the retinae at the termination of superfusion were practically identical with those found in control tissues. The values for k_1 for the efflux of GABA and glutamate from retinae prelabelled with [^{14}C]GABA were approximately 24% lower after AOAA treatment (Table 2) than in untreated tissues (Table 1). However, because k_1 describes the output of only very small quantities of these radioactive amino acids (about 1%), these changes are not considered to be significant. These findings imply that although the breakdown of GABA and its metabolites must be taking place in untreated retinae, this process does not appreciably interfere

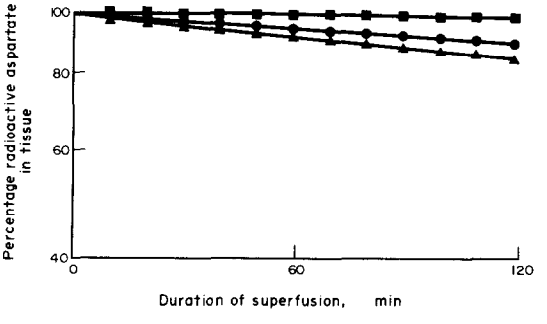

Fig. 4. Time-course of the release of aspartate from superfused retinae. Experimental details are given under Fig. 1. The figure shows the release of radioactive aspartate prepared from labelled glucose (●—●), acetate (▲—▲) and exogenous GABA (■—■). Each curve is the average of at least six experiments.

Table 2. Effect of AOAA on the release of amino acids from retinae prelabelled with [1-^{14}C]GABA

Amino acid	Efflux rate constants ($min^{-1} \times 10^{-3}$) k_1	k_2	Percentage amino acid remaining in tissue after superfusion
GABA	301	0·59	90·1
Glutamine	274	1·84	71·6
Glutamate	—	2·81	68·4
Aspartate	—	0·15	97·8

Retinae were incubated with radioactive GABA in the usual way (see Fig. 1) but were then washed and subsequently effluxed in a medium containing AOAA (0·1 mM). Efflux curves were constructed as before and the new rate constants evaluated. Each result is the mean of five experiments.

with the measurements of the rates at which the amino acids normally leave the tissues for the medium.

Effect of exogenous GABA on amino acid efflux. In these experiments retinae were first incubated with the different radioactive precursors and superfusion commenced in the usual way. After 120 min superfusion the normal medium was replaced by one containing 0·4 mM non-radioactive GABA and the superfusion continued for a further 60 min and the new efflux rate constants (k'_2) determined. Figure 5 illustrates the effect of such treatment on the output of GABA. With each of the three substrates the efflux of GABA was steady up to the point of changeover of the medium. After the addition of cold GABA to the superfusion fluid the outflow of GABA was accelerated in each case. This is indicated by the ratios of the efflux rate constants, k'_2/k_2 (Table 1). The potentiation was greatest following prelabelling with glucose (7·1-fold increase) and GABA (9·5-fold increase), but was less noticeable in tissues preincubated with acetate (1·8-fold increase).

From Table 1 it can be seen that the release of glutamine was also increased by exposing the retinae to a high external concentration of GABA, more especially when the glutamine was derived from glucose than from either acetate or GABA. By contrast, the egress of glutamate was invariably suppressed under these conditions, particularly when glucose was the precursor. Aspartate output, on the other hand, did not appear to be modified by this procedure.

DISCUSSION

The above results show that the rates at which radioactive amino acids pass out of retinae into a bathing medium depend on the nature of the isotopic precursor from which they are labelled. The interpretation of these findings is rendered difficult by the variety of possible factors which can influence the shape of the efflux curves from which the kinetic data are derived. For example, it is important to consider the effects of changes in the pool sizes of the amino acids which can occur during incubation. It is a relatively simple matter to determine the total tissue concentrations of the free amino acids and previous experiments have indicated that although the levels of glutamate, aspartate and glutamine, but not the level of GABA, are usually markedly reduced in incubated retinae, the levels stabilise after about 60 min incubation [18]. Unfortunately, it is not so easy to follow changes in the pattern of distribution of amino acids among the various intracellular pools [2], although it seems likely that the balance of the equilibrium between the contents of these metabolically (and possibly morphologically) distinct pools would contribute significantly to the availability of their metabolites for release. Thus while the importance of this factor is realised, further discussion of this point is beyond the scope of the present paper.

In all but one case the analysis of the kinetic data revealed that amino acid efflux consisted of more than one component. This could mean that the outflow of the amino acids occurred from at least two distinct pools [4]. However, according to Cohen [6], only the slower of these two components (depicted by k_2) may truly represent efflux from an intracellular compartment, as the faster phase (depicted by k_1) can be attributed to a loss from less accessible extracellular spaces. The present data would seem to support this idea, since the amounts of radioactivity accounted for by the fast component were frequently too small (1–2%) to be seriously considered a major intracellular tissue pool.

It is known that glucose is a better precursor of GABA than is acetate and it has been suggested that whereas glucose labels a large, probably neuronal pool of GABA, acetate feeds into a smaller GABA pool which is located in glia [2, 3, 17, 24]. If this is

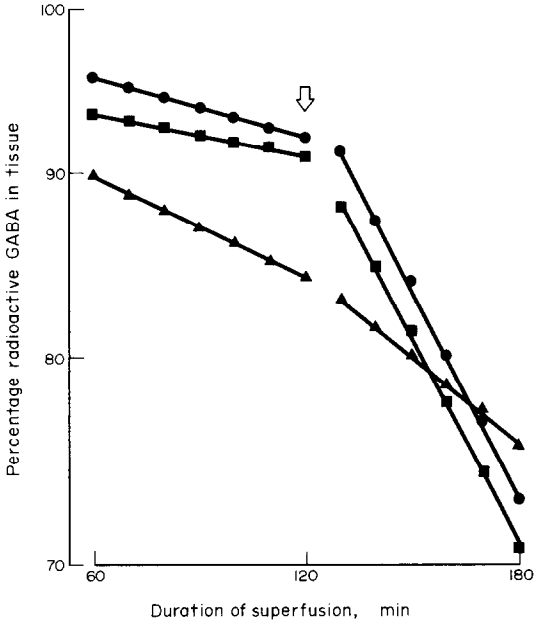

Fig. 5. Effect of addition of cold GABA to the superfusion medium on the output from retinae of radioactive GABA derived from various radioactive substrates. The experimental procedure was the same as for Fig. 1 except that after 120 min superfusion the medium was changed for one containing 0·4 mM non-radioactive GABA (shown by the arrow). The figure shows the release of radioactive GABA prepared from labelled glucose (●—●), acetate (▲—▲) and exogenous GABA (■—■). Each result is the mean of at least six experiments.

true, then the substantial differences observed in the rates of output of amino acids derived from these substrates probably reflect the release of these compounds from separate anatomical sites within the tissue. The results of autoradiographic studies indicate that exogenous GABA is taken up and metabolised almost exclusively in the glial cells of the rat's retina [15], hence one might have predicted a close similarity between the patterns of efflux of amino acids formed from acetate and GABA. It came as a surprise, therefore, to find that the efflux patterns were entirely different. For instance, both glutamine and aspartate derived from GABA were bound far more strongly to intracellular structures than when they were synthesised from acetate. From these findings it would seem that GABA is metabolised at a different site from either glucose or acetate.

The kinetic analysis of the above data did not take into account the losses of amino acids occurring as a result of ongoing metabolism, which may thus have given low estimates of the efflux rate constants. However, it was considered valid to make comparisons between amino acids prelabelled from different substrates because the view was taken that no matter what compound was used to label a particular pool, its constituent amino acids should nevertheless be subject to the same influences controlling their metabolism and release. Using metabolic inhibitors in an attempt to overcome this difficulty can also present problems, because such compounds seldom possess the desired degree of selectivity. For example, using AOAA to halt the metabolism of GABA showed that the output of the amino acids already formed from GABA was not substantially altered, suggesting that the continuing metabolism of GABA did not interfere with the efflux of its metabolites. However, these results have to be reconciled with the findings that AOAA is capable of reducing, quite markedly, the total retinal pool sizes of some free amino acids, such as glutamate and glutamine, which would therefore be expected to modify radioactive amino acid efflux accordingly [18].

The second approach used in this study was based on the supposition that homoexchange between cold GABA in the medium and radioactive GABA in the tissue will not take place with equal facility in each of the GABA pools. The above results bear out this notion, inasmuch as the pool entered by exogenous, radioactive GABA resembled more closely that labelled by glucose than by acetate, simply because the latter was far less susceptible to exchange diffusion with cold GABA than were either of the other two. However, apart from this similarity between GABA and glucose, the evidence of these experiments indicates that exogenous GABA does not label a tissue pool of GABA in the rat's retina that can readily be identified with either of those which are normally entered by carbon derived from glucose or acetate.

The effect of a high concentration of GABA in the medium on the outflow of other amino acids is also interesting. The efflux of glutamine, particularly that derived from glucose, was always enhanced. The reason for this is not known but it may be due to heteroexchange rather than to a metabolic effect. On the other hand, the reduction in the rate of output of radioactive glutamate, which again was most pronounced with glucose as the precursor, could be due to isotopic dilution by freshly synthesised, non-radioactive glutamate. Why aspartate was not affected similarly is not known.

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