Adenine Derivatives as Neurohumoral Agents in the Brain

THE QUANTITIES LIBERATED ON EXCITATION OF SUPERFUSED CEREBRAL TISSUES

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Adenine nucleotides of guinea-pig neocortical tissues were labelled by incubation with [^{14}C]adenine and excess of adenine was then removed by superfusion with precursor-free medium. Adenine derivatives released from the tissue during continued superfusion, including a period of electrical stimulation of the tissue, were collected by adsorption and examined after elution and concentration. The stimulation greatly increased the ^{14}C output, and material collected during and just after stimulation had a u.v. spectrum which indicated adenosine to be a major component. The additional presence of inosine and hypoxanthine was shown by chromatography and adenosine was identified also by using adenosine deaminase. Total adenine derivatives released from the tissue during a 10 min period of stimulation were obtained as hypoxanthine, after deamination and hydrolysis of adenosine and inosine, and amounted to 159 nmol/g of tissue. This corresponded to the release of approx. 7 pmol/g of tissue per applied stimulus. The hypoxanthine sample derived from superfusate hypoxanthine, inosine and adenosine was of similar specific radioactivity to the sample of inosine separated chromatographically, and each was of higher specific radioactivity than the adenine nucleotides obtained by cold-acid extraction of the tissue.

Tissues of the mammalian brain contain 2–3 μmol of acid-soluble adenine derivatives/g, of which ATP preponderates; these can undergo reaction in their phosphate moiety at 0.1–1 $\mu\text{mol/s}$ per g on electrical excitation (see McIlwain & Bachelard, 1971). Present attention is directed to quantitatively minor components in these changes, which result in formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and output of adenosine and some of its derivatives (Kakiuchi *et al.*, 1969; Sattin & Rall, 1970; Pull & McIlwain, 1971, 1972). These processes occur on electrical excitation at rates of less than 1 nmol/s per g and are interconnected to some degree, for adenosine added independently of electrical excitation can also augment the tissue cyclic AMP, and theophylline inhibits each process (paradoxically, and in contrast to other of its actions in relation to cyclic AMP). The adenine derivatives of which output is augmented by excitation are preponderantly adenosine, inosine and hypoxanthine, but the compound first appearing may be an adenine nucleotide, from which the compounds named can be derived by enzymes of known cerebral occurrence (McIlwain, 1971; Pull & McIlwain, 1972).

Characteristics of the output of adenine derivatives on excitation suggest a neurohumoral status for adenosine (McIlwain, 1971, 1972; Pull & McIlwain, 1972; and see the Discussion section). Because of the small quantities of material involved, these characteristics have been determined largely through

measurement of chromatographically characterized ^{14}C -labelled derivatives. We now report measurements of the molar quantities of material involved. Combined with ^{14}C -labelling results these measurements give information also on compartmentation of the adenine derivatives, and on the concentration of liberated adenine derivatives: such information is necessary in appraising the biological status of the liberation processes.

Experimental

Tissues

Samples were prepared from the neocortex of guinea pigs, and were incubated, superfused and electrically stimulated, as described by Pull & McIlwain (1972) unless specified otherwise. The standard glucose – bicarbonate – saline contained: NaCl, 120 mm, KCl, 30 mm, KH_2PO_4 , 1.2 mm, MgCl_2 , 1.2 mm, CaCl_2 , 0.75 mm, NaHCO_3 , 25 mm, and glucose, 10 mm, in equilibrium with $\text{O}_2 + \text{CO}_2$ (95:5). [^{14}C]Adenine, when added, was 3.3 μM and 59 Ci/mol.

Separation and measurement of adenine derivatives in superfusates

Adenine derivatives released from the tissue were recovered from the effluent perfusion medium by adsorption on to charcoal and elution with aq. 10%

(v/v) pyridine. The pyridine was freshly distilled from KOH and kept in the dark. The procedure was as previously described (Pull & McIlwain, 1972) except that added carrier compounds were omitted when molar amounts of the released derivatives were to be measured. The eluates were evaporated to dryness under reduced pressure and any residual pyridine was expelled by the addition of 3 ml of 2M-NH₃, which was also removed by rotary evaporation. The u.v. spectra of the residues dissolved in 2 ml of 0.1M-HCl were examined against a blank obtained by similar treatment of an equal volume of fresh perfusion medium. T.l.c. separation of the compounds was then performed (Pull & McIlwain, 1972) and u.v.-absorbing regions seen after development, together with corresponding blank areas of the gel, were eluted with 1.3 ml of 0.1M-HCl. The u.v. absorbance and radioactivity of these eluates were determined. Extinction coefficients given by Burton (1969) were used to determine the eluted compounds.

Trial experiments with superfusates of 60–100 ml from approx. 400 mg of tissue, preincubated with [¹⁴C]adenine, showed that 5 mg of charcoal was the minimum adequate to adsorb all of the ¹⁴C-labelled compounds in the effluent media. The optimum time for adsorption of the ¹⁴C to charcoal was found to be 10 min with the 5 mg of activated charcoal, used as supplied. The charcoal was then collected on a membrane filter and washed with 10 ml of water; only 3–5% of the initial radioactivity was present in the filtrate and washings.

Use of adenosine deaminase

Calf intestinal enzyme was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., as a 2 mg/ml suspension in (NH₄)₂SO₄ with an activity of 200 i.u./mg, and was diluted with 5 mM-potassium phosphate buffer, pH 7.4, to a protein concentration of 0.2 mg/ml before use. Adenosine present in the purine concentrate obtained from charcoal was identified and measured by reaction with the enzyme (Mollering & Bergmeyer, 1963). For this purpose a sample of the concentrate was dried again by rotary evaporation, dissolved in 1 ml of 5 mM-potassium phosphate buffer, pH 7.4, and transferred to a spectrophotometer cuvette. Adenosine deaminase (2 µg) was added and the extinction change at 265 nm noted. In one experiment authentic adenosine was then added to verify enzyme action. The deaminated samples were hydrolysed in 1M-HCl at 100°C for 1 h and the resulting hypoxanthine was obtained for spectrophotometry and radioactivity determination after separation by t.l.c. In another experiment, samples treated with the deaminase were divided into two portions, one of which was hydrolysed with 1M-HCl. Both samples were supplemented with marker compounds and chromatographed, and

the distribution of ¹⁴C among the individual samples was determined. Deamination of adenosine to inosine and hydrolysis of inosine to hypoxanthine was found to be virtually complete.

Other analyses and measurement of radioactivity

Tissues were removed from their holders at the end of superfusion and promptly homogenized in 6% (w/v) trichloroacetic acid. The deposits after centrifugation were further extracted with trichloroacetic acid and the combined supernatants made to 5 ml. A measured sample was extracted with diethyl ether, dried by evaporation and the residue hydrolysed in 1M-HCl at 100°C for 1 h. Adenine was separated by t.l.c. and eluted for spectrophotometry and determination of radioactivity. Carrier adenosine (50 µg) was added to other samples of the tissue extract, which were then extracted with diethyl ether and dried. The residue was dissolved in water, the adenosine separated by t.l.c. and eluted for the determination of radioactivity and absorption at 258 nm, from which the recovery of the added carrier adenosine was calculated. Radioactivity of samples of superfusates, tissue extracts or acid extracts from chromatography was determined as previously described (Pull & McIlwain, 1972).

Results

Identity of adenine derivatives yielded on excitation

Four experiments were carried out, each of which used approx. 0.5 g of neocortical tissue from two guinea pigs. The tissue samples were incubated first in glucose–bicarbonate–salines containing [¹⁴C]-adenine under conditions described by Pull & McIlwain (1972). Superfusion of the tissues was then commenced, which first removed excess of [¹⁴C]-adenine, and then yielded superfusates, collected each 2 min, of small and constant ¹⁴C content; results from one experiment are shown in Fig. 1. At this point, with continued superfusion, the tissues were electrically stimulated and displayed the previously reported (Pull & McIlwain, 1972) increase in output of ¹⁴C. Superfusion was continued for a further 20 min and on the basis of ¹⁴C measurements samples were pooled to form the four groups *a* to *d* shown in Fig. 1. These were then subjected to more detailed analysis.

Each of these pooled superfusion fluids was adsorbed and eluted by the procedure described in the Experimental section and the u.v. absorption of the eluted material between 220 and 280 nm was determined. Material from the first and last periods, when least ¹⁴C was being released, was of lower absorbance than was material collected in the intervening periods. Samples *a*–*c*, but not *d*, showed absorption maxima in the region of absorption maxima given

Fig. 1. Output of adenine derivatives from guinea-pig neocortex

Cortex (446mg) was preincubated with [¹⁴C]adenine in each of four vessels as described in the Experimental section. After 15min superfusion at 3.5ml/min (zero time in the Fig.) the flow rate was diminished to 1.5ml/min and collections of superfusate were made each 2min. Stimulating pulses were applied at 40/s (20Hz) during the period shown by the dark bar. On the basis of the ¹⁴C measurements the superfusates were pooled to give the four groups *a* to *d*.

Table 1. Absorptiometric characteristics of materials from tissue superfusates

Superfusate specimens (see Fig. 1) were adsorbed and eluted as described in the Experimental and Results sections. T.l.c. of superfusates (periods *b* and *c*) was carried out on silica gel, and material from u.v.-absorbing regions of the stated *R_F* values was examined. Extinction measurements were made in 0.1 M-HCl. Specimen (1) from t.l.c. is described further in Table 4.

Specimen		Wavelength (nm)		Extinction ratio	
Origin	<i>R_F</i> value	Max.	Min.	<i>E</i> ₂₅₀ / <i>E</i> ₂₆₀	<i>E</i> ₂₈₀ / <i>E</i> ₂₆₀
Superfusate, period <i>a</i>	—	244	235	1.13	0.59
Superfusate, period <i>b</i>	—	254	230	1.01	0.53
Superfusate, period <i>c</i>	—	252	246	1.05	0.52
Superfusate, period <i>d</i>	—	—	—	1.17	0.61
Difference spectrum periods (<i>b</i> + <i>c</i>) – (<i>a</i> + <i>d</i>)	—	258	—	0.87	0.42
Absorbing regions from t.l.c. (1)	0.38	246	227	1.38	0.23
Absorbing regions from t.l.c.	0.60	246	<240	1.49	0
Absorbing regions from t.l.c.	0.68	260	240	0.60	0.34
Absorbing regions from t.l.c.	0.68	258	<240	0.88	0.29
Reference compound, inosine	0.38	250	222	1.46	0.18
Reference compound, hypoxanthine	0.60	248	217	1.44	0.05
Reference compound, adenosine	0.68	258	230	0.82	0.22

by hypoxanthine, inosine and adenosine, previously (Pull & McIlwain, 1972) identified as present by chromatography and measurement of ¹⁴C. The *E*₂₈₀/*E*₂₆₀ ratios and wavelengths of minimum extinction showed the additional presence of compounds other than the adenine derivatives, so that difference

spectra based on the absorption values for samples (*b* + *c*) – (*a* + *d*) were examined. Properties of the difference spectra resembled those of adenosine (Table 1), indicating this to be the principal material appearing during periods *b* and *c* as a result of stimulation. Inosine and hypoxanthine are also present, and this

Table 2. *Purine derivatives from neocortical tissue on stimulation*

Three experiments are reported, in each of which four slices of guinea-pig neocortex were separately incubated for 40 min in 5 ml of medium containing $1 \mu\text{Ci}$ of $[8-^{14}\text{C}]\text{adenine}$ (59 Ci/mol) before superfusion at 3.5 ml/min for 20 min. The flow rate was then decreased to 1.5 ml/min and collections of superfusate were made each 2 min before samples were pooled as shown in Fig. 1 to give samples *a* to *d*. These were adsorbed separately to charcoal and eluted with pyridine as described in the Experimental section. Absorbance of the dried residues was measured (Table 1) and values at 260 nm were expressed as adenosine, after applying recovery quotients (see Table 3) of 70–80%. All tissues were stimulated during period *b*.

Expt. no.		'Adenosine' calculated from E_{260} (nmol/g)				Increment due to stimulation, i.e. $(b+c)-(a+d)$ (nmol/g)
Period	...	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	
1		47	62	72	37	50
2		78	107	122	82	69
3		72	90	128	99	47
Mean ...						55 ± 12 (s.d.)
		Apparent specific radioactivity of samples (Ci/mol of 'adenosine')				Apparent specific radioactivity derived from increments, i.e. $\frac{(b+c)-(a+d) \text{ in nCi/g}}{(b+c)-(a+d) \text{ in nmol/g}}$
Period	...	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	
1		0.38	1.35	1.57	0.76	3.00
2		0.42	1.23	1.45	0.51	3.37
3		0.54	1.42	1.30	0.42	4.50
Mean ...						3.62 ± 0.78 (s.d.)

resulted in the amounts of adenosine derived from the difference spectra (Table 2) being over-estimated. Calculations, assuming absorbances of the different compounds to be additive, showed that inosine and hypoxanthine in amounts approximately one-quarter of the total nucleoside present could be contributing to the observed extinctions of the samples at 250 and 260 nm. T.l.c. in silica gel showed adenosine, inosine and hypoxanthine in samples *b* and *c*, and these compounds were also characterized by their spectra after elution (Table 1). Inosine was consistently obtained in amounts equivalent to 20–38 nmol/g of tissue, but adenosine and hypoxanthine showed considerable variation, between 24–87 nmol of adenosine/g and 7–145 nmol of hypoxanthine/g.

Quantities in superfusates after excitation

Absorbance results from three experiments similar to that of Fig. 1 are summarized in Table 2, and on the basis of the foregoing results are expressed in terms of adenosine, though some inosine and hypoxanthine are also present. In Table 2, appreciable absorption is shown by solution *d*; this was noted above to contain little ^{14}C from adenine and to be without the absorption maxima of the identified adenine derivatives. The absorption by solution *d* is variable,

whereas the increment in absorption due to stimulation found in solutions *b* and *c* was more consistent: a mean additional output of 55 nmol/g of tissue, as adenosine, was concluded on this basis to result from 10 min stimulation in the three experiments.

Adenosine was measured in material from the superfusates of three experiments by using adenosine deaminase (Table 3). Little was found during periods *a* and *d*, but stimulation resulted in the appearance of up to 25 nmol/g during each of periods *b* and *c*. Output in one experiment is quoted in Table 3. The deaminase treatment, followed by hydrolysis, was employed also in one experiment to convert adenosine and inosine in supernatants into hypoxanthine, so obtaining a collective value for the three compounds. This indicated (lines 2 and 5, Table 3) an output per g of tissue of 159 nmol of hypoxanthine plus its precursors during periods *b* and *c* when the output was augmented by stimulation.

Output of ^{14}C and specific radioactivities of adenine derivatives

Measurement of output of ^{14}C , noted above as contributing to the way in which samples were grouped for analysis, was employed also to determine the percentage recovery during fractionation of the

Table 3. *Adenosine and related compounds from superfusates of neocortical tissue*

Purine derivatives present in superfusate samples pooled as shown in Fig. 1 were adsorbed on charcoal and eluted with pyridine as described in the Experimental section and their adenosine content was measured by deamination. After deamination, the samples were hydrolysed in 1M-HCl for 1 h and the total hypoxanthine, including that derived from adenosine and inosine, was separated by t.l.c., eluted and its absorbance and radioactivity were determined (measurements 2 and 4). Values in the Table have not been corrected for the recovery factors (5); values in Table 4 have been so corrected.

Measurement	Period of tissue superfusion (min) with conditions			
	Unstimulated (0–10)	Stimulated (10–20)	Washout (20–30)	Unstimulated (30–40)
(1) Adenosine by deaminase (nmol/g)	0	22.2	25.9	1.2
(2) Hypoxanthine, inosine and adenosine determined as hypoxanthine (nmol/g)	<15	67	60	<15
(3) Total ^{14}C of superfusates (nCi/g)	26.5	103.1	137.6	30.4
(4) ^{14}C in hypoxanthine, inosine and adenosine (nCi/g)	14.8	88.1	118.9	25.9
(5) Recovery of total ^{14}C on adsorption and elution from charcoal (%)	80	78	78	77
(6) Specific radioactivity of adenosine plus metabolites, obtained as hypoxanthine, from rows (2) and (4) (Ci/mol)	>0.9	1.3	1.98	>1.7

Table 4. *Quantities and specific radioactivities of adenine derivatives of superfusates and tissues*

The superfusate materials are those released by stimulation and collected during periods *b* and *c* in the experiments of Tables 1–3, where experimental details are stated; mean values $\pm$ S.D. are given where appropriate, with the numbers of determinations in parentheses. The adenine derivatives of the tissues were determined at the end of the experiments: 95–99% of the ^{14}C of tissues was as nucleotides; mean content 3.04 ± 0.23 (4) $\mu\text{mol/g}$ of specific radioactivity 1.33 ± 0.08 (4) Ci/mol. The values derived from Table 3 have had the recovery factors there quoted applied (see the text). The significance of the difference from the specific radioactivity of the tissue nucleotides (by using Student's two-tailed *t* test) was * $P < 0.01$; ** $0.1 > P > 0.05$.

Compound and origin	Superfusate material	
	(nmol/g of tissue)	(Ci/mol)
Adenosine by difference spectra (1)–(3); see Table 2	55 ± 12 (3)	3.62 ± 0.78 (3)*
Adenosine by deaminase; see row (1), Table 3	61	—
Inosine by t.l.c., see Table 1	28.6 ± 7.6 (3)	2.16 ± 0.17 (3)**
Hypoxanthine <i>per se</i> and from adenosine and inosine; see row (6), Table 3	159	1.75

superfusates (Table 3). This showed 77–80% recovery, a value which is taken to be sufficiently high for it to be applied to an individual compound (adenosine) as well as to the mixture of ^{14}C -labelled compounds. Superfusate analyses so calculated are included in Table 4. The ^{14}C results further enabled the specific radioactivities of some of the superfusate [^{14}C]adenine derivatives to be calculated. These values, together with those for specific radio-

activities of the tissue nucleotides determined at the end of incubation, are also given in Table 4 and are discussed below.

Discussion

Output of adenine derivatives

The adenosine yielded to superfusates on excitation is estimated (Table 4) as 60 nmol/g of tissue, with

adenosine plus its metabolites inosine and hypoxanthine at 160 nmol/g. The volume of extracellular fluid associated with 1 g fresh wt. of neocortical tissue is 0.25–0.3 ml, becoming 0.4–0.5 ml during incubation *in vitro* (Harvey & McIlwain, 1968). If the amount of adenosine, alone or plus metabolites, now observed to be released *in vitro* accumulated in the tissue as adenosine *in vivo*, its concentration would therefore reach 150–400 μM . Added adenosine can cause a 30-fold increase in the cyclic AMP of cerebral tissues, and the half-maximal concentration for this action was 30 μM ; 10 μM -adenosine caused a fourfold increase (Sattin & Rall, 1970). The adenosine released would therefore be expected to show marked effects on cerebral cyclic AMP even after diffusion or metabolism had diminished its concentration to 3% of the estimated value.

Suggestions about processes of release of adenine derivatives on excitation were discussed previously (Pull & McIlwain, 1972); they included the possibility of nucleotide or nucleoside release as or in association with neurotransmitting agents. This possibility prompts a calculation of the molar quantity released per stimulating pulse. The 60–160 nmol of adenine derivatives calculated above as being released per g of tissue on excitation resulted from the application of $10 \times 60 \times 40$ pulses and thus corresponded to 3–7 pmol/g of tissue per stimulating pulse. During investigation of transmitter substances released from non-adrenergic nerves of the gastrointestinal tract, Burnstock *et al.* (1970) concluded that ATP was released as an inhibitory transmitter by the toad stomach in quantities of 1.5 pmol/0.25 g of neural tissue per stimulating pulse. The release per g is thus interestingly similar to that now found from cerebral tissue, though further results from these and other neural systems are needed to judge the status and significance of this similarity.

Origin of the superfusate [^{14}C]adenine derivatives

[^{14}C]Adenine taken up by cerebral tissues in circumstances similar to those of the present experiments has been found to the extent of nearly 90% as trichloroacetic acid-extractable adenine nucleotides (Santos *et al.*, 1968; Shimizu *et al.*, 1969). In the present experiments, 95–99% of the ^{14}C of tissue trichloroacetic acid extracts was as nucleotides and of the remainder, about 0.6% was found as adenosine. [^{14}C]ATP added to cerebral tissues yielded adenosine, inosine and hypoxanthine (Pull & McIlwain, 1972) and during the present experiments the ^{14}C which appeared as the three purines in tissue superfusates on excitation averaged about 6% of the trichloroacetic acid-extractable adenine nucleotides of the tissue. The three purines are thus likely to be derived from the adenine nucleotides. Their specific radioactivities were found (Table 4) to be similar for

inosine and for the group inosine, hypoxanthine and adenosine.

The superfusate purines were, however, of higher specific radioactivity than the collective value for the acid-extracted adenine derivatives of the tissues (Table 4). This is reminiscent of the findings by Sattin & Rall (1970), Shimizu *et al.* (1970) and Krishna *et al.* (1970) that in cerebral tissues labelled under conditions similar to those now used, the cyclic AMP formed by added reagents could also be derived from high-specific-radioactivity pools of ATP. In Sattin & Rall's (1970) experiments the relative specific radioactivities (cyclic AMP)/(ATP+ADP) were 2–5, in those of Krishna *et al.* (1970), 1.25–3, and those of Shimizu *et al.* (1970) imply values of 8. As ATP is the only known source of cyclic AMP, it was concluded that small ATP pools of at least three to eight times the mean ATP specific radioactivity existed in the tissues and formed a preferential source of cyclic AMP.

Results on adenosine output during the present experiments (Table 4) when relative specific radioactivities (adenosine metabolites)/(adenine nucleotides) were 1.4–2.7, suggest again that pools of ATP of high specific radioactivity contribute to the output. The proportion of the total tissue ATP that was collected as adenosine plus metabolites during the present experiments was greater than the proportion giving cyclic AMP in the other experiments cited, and it is presumably variable contributions from high-specific-radioactivity pools of [^{14}C]ATP that contribute to the variation in specific radioactivity of the adenosine metabolites reported in Table 4. The observed sequence of specific radioactivities, adenosine > inosine > hypoxanthine, indicates that not all superfusate inosine derives from the released adenosine, nor all hypoxanthine from the superfusate inosine. Granted local tissue pools of more highly labelled adenine nucleotides, many hypotheses proposing local occurrence of degrading enzymes or localized release of ^{14}C -labelled and unlabelled nucleotides could account for the observed sequence.

A further conclusion may be drawn from the superfusate adenosine of the present experiments showing a specific radioactivity relative to ATP which is much lower than that of cyclic AMP in the experiments just cited. This is that the adenosine, in augmenting the cyclic AMP of tissues to which it is added, is not acting solely as precursor for the cyclic AMP. The alternative or supplementary mechanism for the augmentation is an adenosine-activated adenylate cyclase, for which other evidence has been adduced (Sattin & Rall, 1970; Shimizu *et al.*, 1970). Such a mechanism requires the substrate for the cyclase to be a high-specific-radioactivity pool of ATP, originating, for example, from localized uptake of [^{14}C]adenine, and this possibility is supported by the present results.

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References

- Burnstock, G., Campbell, G., Satchell, D. & Smythe, A. (1970) *Brit. J. Pharmacol.* **40**, 668–688
- Burton, K. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliot, D. C., Elliot, W. H. & Jones, K. M., eds.), 2nd edn., pp. 169–179, Oxford University Press, London
- Harvey, J. A. & McIlwain, H. (1968) *Biochem. J.* **108**, 269–274
- Kakiuchi, S., Rall, T. W. & McIlwain, H. (1969) *J. Neurochem.* **16**, 485–491
- Krishna, G., Forn, J., Voight, K., Paul, M. & Gessa, G. L. (1970) in *Role of Cyclic AMP in Cell Function* (Greengard, P. & Costa, E., eds.), pp. 155–170, Raven Press, New York
- McIlwain, H. (1971) in *Effects of Drugs on Cellular Control Mechanisms* (Freedman, R. B. & Rabin, B. R., eds.), pp. 281–302, Macmillan, London
- McIlwain, H. (1972) *Biochem. Soc. Symp.* **36**, 69–85
- McIlwain, H. & Bachelard, H. S. (1971) *Biochemistry and the Central Nervous System*, pp. 43–54, 80–82, 380–383, Churchill-Livingstone, London
- Mollering, H. & Bergmeyer, H. U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 491–494, Academic Press, New York and London
- Pull, I. & McIlwain, H. (1971) *J. Physiol. (London)* **221**, 9P–10P
- Pull, I. & McIlwain, H. (1972) *Biochem. J.* **126**, 965–973
- Santos, J. N., Hempstead, K. W., Kopp, L. E. & Miech, R. P. (1968) *J. Neurochem.* **15**, 367–376
- Sattin, A. & Rall, T. W. (1970) *Mol. Pharmacol.* **6**, 13–23
- Shimizu, H., Daly, J. & Creveling, C. R. (1969) *J. Neurochem.* **16**, 1609–1619
- Shimizu, H., Creveling, C. R. & Daly, J. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 1033–1040