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ESTIMATION OF ADENINE NUCLEOTIDES IN BRAIN BY ENZYMIC AND ION-EXCHANGE CHROMATOGRAPHIC METHODS

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Abstract—A spectrophotometric assay for the adenine nucleotides using AMP deaminase, myokinase (MK) and apyrase has been examined and found to give erroneously high estimates of AMP, ADP and ATP in rat brain extract. The AMP deaminase preparation used is liable to contain creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) activities. However, it is suggested that the discrepancy may be due to the presence of yet another enzymic contaminant. Some aspects of nucleotide extraction from brain tissue are investigated.

IT HAS been suggested by Lewis and coworkers that a correlation exists between the behavioural effects and the changes in the brain ATP/ADP ratio induced by a wide range of centrally acting drugs.[1–6] This hypothesis was based on measurements of AMP, ADP and ATP levels in the rat brain using the Kalckar[7] method. This utilises the decrease in absorbance (at 265 mμ) occurring on conversion of AMP to IMP by AMP deaminase (see Fig. 1). After this reaction, addition of MK to the reaction

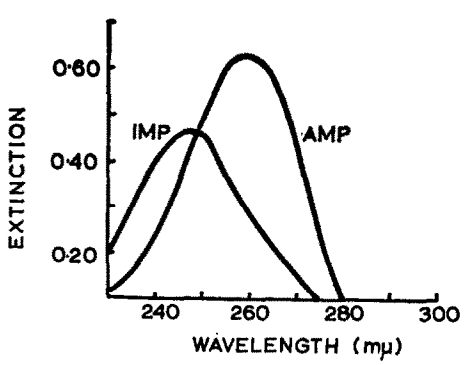

FIG. 1. Spectral absorption curves of AMP and IMP (after Kalckar[7]).

mixture causes dismutation of ADP to ATP and AMP, leading to further deamination and a fall in absorbance proportional to half the amount of ADP present. Finally, conversion of ATP to AMP by apyrase produces another absorbance decrease proportional to the amount of ATP present. This paper presents a critical re-examination of the adaptation of the Kalckar method used by Lewis and coworkers.

Considerable variation still exists amongst values reported for the *in vivo* levels of

adenine nucleotides in rat brain,[1,2,8–10] partly due to the different extraction procedures employed. Some aspects of nucleotide extraction are evaluated.

METHODS

Male Wistar rats (75–95 g) were killed by immersion in liquid nitrogen and the whole brain removed and weighed as described by Lewis and Van Petten.[1]

Extraction of brain (modified from that used by Lewis and Van Petten[1]). The frozen brain was pulverised[11] in a 50-ml stainless steel centrifuge tube embedded in solid CO_2. The tube was then placed in a beaker of wet crushed ice and ice-cold 0·3 M $HClO_4$ was immediately added from a rapid delivery pipette. The volume of 0·3 M $HClO_4$ employed for extracting each brain was sufficient to give a final volume of 10 ml brain extract. This was calculated from the water content of rat brain tissue (determined as 79·4 per cent, by weighing three brains before and after freeze-drying) thus:

$$\text{volume of 0·3 M } HClO_4 \text{ required} =$$

$$10 - (0{\cdot}206 \times \text{brain weight in g}) \text{ ml.}$$

The suspension was immediately homogenised at approximately 2000 revs/min for 2 min using an ice-cold loosely fitting plastic pestle. This procedure ensured rapid penetration of the tissue by the acid. The suspension was centrifuged (2200 *g*, 0°, 6 min) and the supernatant filtered at 4° into a test tube.

Assay of adenine nucleotides in brain extract

(a) For the estimation of AMP, ADP and ATP by the Kalckar method, 1 ml of brain extract was added to 0·67 ml 0·3 M $HClO_4$ and 8·33 ml water to give a final $HClO_4$ concentration of 0·05 M. This solution was then buffered and assayed as described by Lewis and Van Petten,[1] except that the MK used was the MK 15355 preparation supplied by the Boehringer Corporation (London) Ltd.

(b) For the estimation of the adenine nucleotides by ion-exchange chromatography (IEC), as large a volume of brain extract as possible was measured into a beaker and brought to pH 6·5–7·0 with 18 N KOH using a Pye pH meter with glass electrode assembly. The alkali was delivered slowly and accurately by means of an Agla Micrometer Syringe, while the solution was agitated by a magnetic stirrer. The resulting precipitate of $KClO_4$ was removed by cooling in ice for 5 min followed by centrifugation (2200 *g*, −2·5°, 8 min). The supernatant was withdrawn and its volume noted (about 7 ml). It was then applied to a 1 × 10 cm column containing Dowex 1-X8 resin (Bio-Rad AG1-X8; 100–200 mesh; formate form) which had been washed with 50 ml of 90% (v/v) H.COOH then with water (250 ml) till neutral. The unadsorbed materials in the sample were washed through the column with 35 ml of water and the nucleotides were eluted using a gradient elution technique.[12] Gravity feeding of the eluent was commenced at a flow rate of 1·2 ml/min with about 3 ml of water above the top of the resin bed. The airtight mixer vessel initially contained 500 ml of water and the fluid escaping at the bottom of the mixer was replaced by concentrated eluent from the reservoir (an open 250-ml flask) through a siphon, unlike the positive pressure system utilised by Hurlbert *et al.*[12] The volume of fluid contained in the mixer remained unchanged throughout the gradient elution. Successive ranges of

eluting power were obtained by substituting reservoir flasks containing more concentrated solutions (see Fig. 2).

From fraction 18 onwards, the flow rate was increased to 2·0 ml/min. The automatic fraction collector metered each 10 ml fraction in a glass siphon and this volume was checked accurately for one fraction from each important peak. After collection of fraction 75, the mixer was disconnected from the reservoir and allowed to drain through the column.

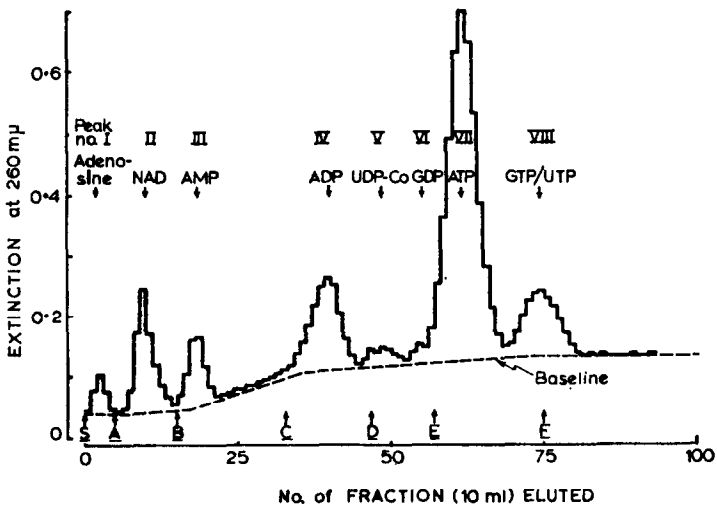

FIG. 2. Chromatography of brain extract on a column of Dowex-1 formate. Sample + water were applied to the column at S. The following concentrated eluents were placed in the reservoir at points indicated ↑ : A, N H.COOH at fraction 6; B, 8·5 N H.COOH at fraction 15; C, 0·5 M $H.COONH_4$ + 4N H.COOH at fraction 33; D, 1 M $H.COONH_4$ + 4 N H.COOH at fraction 47; E, 2 M $H.COONH_4$ + 4 N H.COOH at fraction 57; F (none) at fraction 76. Peaks were identified as shown ↓. UDP-Co = UDP-coenzymes.

Extinction readings against air at 260 mμ on a Hilger H.700 Uvispek spectrophotometer typically gave a chromatogram as in Fig. 2. The position of the baseline was determined from the absorbance of the fractions in the first trough and of fractions 90–100, and from histograms resulting from the chromatography of blank extracts (i.e. 10 ml of 0·3 M $HClO_4$ treated as described above). The identification of the components of the peaks I–VIII was carried out by comparison of the chromatograms with those previously published for brain extract[10,13] and of the ratios of extinction values at 275 and 260 mμ (E_{275}/E_{260}) with published figures.[14] Confirmation was obtained by running a descending paper chromatogram of the freeze-dried, pooled eluate from each peak using the solvent isobutyric acid:NH_4OH:H_2O (66:1:33, by volume) and examining the dried paper by visual u v. quenching.

(c) The Boehringer Test Combinations[15,16] for the assay of ATP (TC-J) and ADP/AMP (TC-K) in blood were adapted for brain extract analysis. It was confirmed that, while their estimates of ATP and ADP include (GTP + UTP) and (GDP + UDP), respectively, that of AMP is specific. For both methods the solutions containing buffers, NADH and reactants were made up according to the manufacturer's instructions and the procedures were modified to give assays of maximum sensitivity.

(i) The "ATP" assay relies on the phosphorylation (by phosphoglycerate kinase, PGK) of 3-phosphoglycerate to 1,3-diphosphoglycerate, the latter entering the indicator reaction in which formation of glyceraldehyde-3-phosphate (by glyceraldehyde phosphate dehydrogenase, GAPDH) accompanies NADH oxidation, which is followed spectrophotometrically at 340 mμ. The reaction mixture consisted of 0·4 ml of 0·3 M $HClO_4$ brain extract, added to 2·4 ml of buffer and 0·02 ml of NADH solution in a cuvette.

(ii) The "ADP"/AMP assay utilises the reactions catalysed by MK, for AMP, and pyruvate kinase (PK), for ADP, coupled to the oxidation of NADH by pyruvate (LDH-catalysed indicator reaction). Perchlorate was precipitated from 2·0 ml of the brain extract by mixing with 2·0 ml of deionised water and 1·0 ml of buffer/K_2CO_3 solution, cooling to 0° for 10 min and filtering. 2·3 ml of filtrate was then combined with 0·15 ml of phosphoenolpyruvate and 0·03 ml of NADH solutions in a cuvette.

The extinction of the solutions was observed every minute and the decreases in absorbance due to the addition of 0·04 ml of GAPDH/PGK mixed suspension (for the "ATP" assay) or 0·02 ml each of LDH, PK and MK, successively (for the "ADP"/AMP assay), were noted. Each decrease was corrected both for the change due to dilution of the optically dense reaction mixture by the enzyme suspension and for the slow background decrease due to non-specific oxidation of NADH.

Spectral studies of the Kalckar method. The action of the rabbit muscle AMP deaminase preparation on brain extract, prepared as in (a) above, was compared with that on AMP solution, similarly prepared, but substituting AMP solution (0·05 mM in 0·05 M $HClO_4$) for the 0·05 M $HClO_4$ brain extract. The extinction changes between 230 and 270 mμ following the addition of the deaminase preparation (0·02 ml) to 3·0 ml of each of these solutions were observed. These changes were repeatedly examined at 265, 250 and 240 mμ in a number of brain extracts and AMP solutions In all cases at least 30 min was allowed for completion of the reaction.

CPK activity of deaminase preparation. This was determined using a commercial. kit (TC-V, Boehringer Corporation (London) Ltd.), in which the rate of ADP formation, from the phosphorylation of creatine, is measured by utilising the reactions described in (c)(ii) above.

0·01 ml of deaminase preparation was diluted to 1·0 ml with deionised water to give a suitable sample. The test was carried out according to the manufacturer's instructions, except that extinction readings at 2-min intervals were continued for 16 min. The difference between the absorbance of the sample and blank solutions was plotted against time to give a straight line graph. From this was calculated the CPK activity of the sample (see manufacturer's literature).

LDH activity of deaminase preparation. This was detected by substituting 0·02 ml of deaminase preparation for the LDH normally added at the start of the Boehringer "ADP"/AMP assay (see (c)(ii) above). The usual procedure employing LDH was carried out as a control.

Extraction of brain nucleotides

(1) The effect of allowing the pulverised brain (at −78°) to approach 0° by placing in ice before addition of 0·3 M $HClO_4$ was investigated by extending this "thaw" period from zero (<5 sec) to 1, 2, 3 and 4 min. Extracts thus obtained were analysed by the Boehringer assays.

(2) Aqueous 0·3 M $HClO_4$ extraction was compared with that using 10% (w/v) trichloroacetic acid in acetone at −78°, as described by Minard and Davis.[8]
(3) Extraction of pulverised frozen brain was compared with that of frozen brain which had been freeze-dried by breaking into chips and placing in a Speedivac-Pearse Tissue Drier at −60° for 24 hr. The tissue was then pulverised and extracted as usual, using 10 ml of 0·3 M $HClO_4$ since this was the final volume of extract required.

The extracts for both (2) and (3) were analysed by IEC.

RESULTS

Comparison of adenine nucleotide assays. The estimated adenine nucleotide concentrations in aliquots of the same pooled brain extracts are shown in Table 1. The

TABLE 1. COMPARISON OF KALCKAR AND ION-EXCHANGE ESTIMATES OF ADENINE NUCLEOTIDES IN RAT BRAIN EXTRACT

Assay method	Concn (μmoles/g frozen brain)			
	ATP	ADP	AMP	ATP + ADP + AMP
Kalckar	2·33 ± 0·11	0·74 ± 0·03	0·74 ± 0·03	3·79 ± 0·15
Ion-exchange	*1·99 ± 0·03	†0·51 ± 0·02	†0·24 ± 0·02	†2·73 ± 0·05
Boehringer	[2·58 ± 0·06]	[0·66 ± 0·02]	†0·19 ± 0·01	[3·42 ± 0·05]

Aliquots of the same brain extract (pooled from two brains) were assayed simultaneously by all three methods. Square brackets [ ] indicate the results where ATP includes GTP + UTP and ADP includes GDP + UDP. Each value is a mean (± S.E. of mean) of nine determinations (nine duplicates in the case of the Kalckar method). Significance of difference from the result of the Kalckar method (Student's *t*-test): *0·02 > P > 0·001; †0·001 > P.

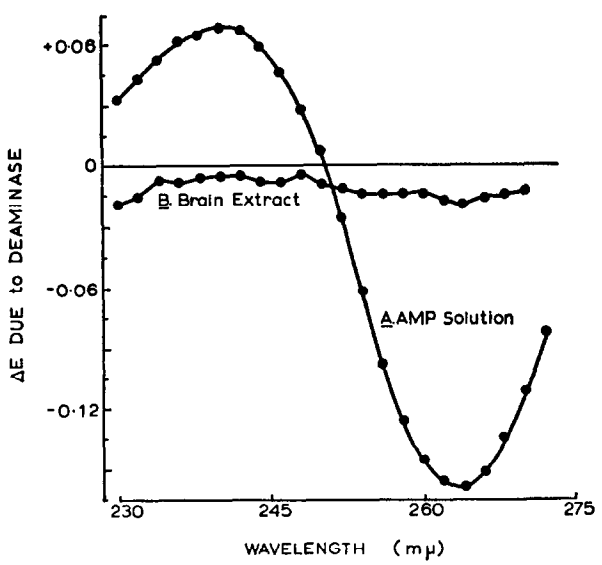

FIG. 3. Spectral changes accompanying the action of the deaminase preparation on AMP solution (0·05 mM in 0·05 M $HClO_4$) and brain extract, both buffered as described by Lewis and Van Petten. The extinction changes (ΔE) shown have been corrected for the rise in absorbance due to the enzyme itself. At least 30 min was allowed for completion of the enzymic reaction. Each point is a mean of two determinations.

Kalckar method gave significantly higher results for all three nucleotides than did the ion-exchange assay. The former procedure indicated an AMP concentration more than three times higher than the ion-exchange estimate, but this low AMP level was concurrent with the Boehringer result. Earlier experiments[17] on the Kalckar method have shown that it is relatively reliable in assaying standard adenine nucleotide solutions and in measuring recovery of nucleotides added to brain extract.

Spectral studies of the Kalckar method. From the spectra of AMP and IMP (Fig. 1) the absorbance changes (ΔE) expected on deamination are a maximum decrease at 265 mμ, no change at 250 mμ and a maximum increase at 240 mμ. Such changes were observed to follow the action of the deaminase preparation on standard AMP solution (Fig. 3, curve A). On brain extract, however, the deaminase preparation caused decreases in extinction at 250 and 240 mμ also (Fig. 3, curve B). This discrepancy in the spectral changes occurring in brain extract was repeatedly observed (Table 2).

CPK activity of deaminase preparation. Two batches of deaminase preparation were tested in duplicate. The CPK activities found were:

batch 1 3·08, 2·85} units/ml of undiluted
batch 2 2·20, 2·02} deaminase preparation.

LDH activity of deaminase preparation. The absorbance changes occurring at 340

TABLE 2. SPECTRAL CHANGES ACCOMPANYING THE ACTION OF THE DEAMINASE PREPARATION ON AMP SOLUTIONS AND ON BRAIN EXTRACTS

Solution (in 0·05 M $HClO_4$) used	No. of expts.	ΔE at (mμ)			Ratios	
		265	250	240	$\Delta E_{250}/\Delta E_{265}$	$\Delta E_{240}/\Delta E_{265}$
AMP (0·02 mM)	2	−0·062 −0·061	0·000 0·000	+0·033 +0·032	0·00 0·00	−0·53 −0·52
AMP (0·05 mM)	2	−0·150 −0·161	+0·006 +0·005	+0·067 +0·071	−0·04 −0·03	−0·45 −0·45
		(Means)			(Means ± S.E. of mean)	
Brain extract	10	−0·029	−0·017	−0·009	+0·54 ±0·05	0·30 ±0·05

The Kalckar assay for AMP was carried out on the solutions shown. The extinction changes (ΔE) at each wavelength were observed at least 30 min after the addition of the deaminase preparation.

TABLE 3. CONTAMINATION OF THE DEAMINASE PREPARATION BY LDH

ΔE_{340} following addition of	1st enzyme: LDH	1st enzyme: deaminase preparation
1st enzyme	0·041	0·040
PK	0·075	0·075
MK	0·055	0·051

The deaminase preparation was tested for LDH activity by substituting it for LDH in the Boehringer "ADP"/AMP assay.

mμ following the addition of the usual enzymes during the Boehringer "ADP"/AMP assay are shown in Table 3 to be similar to those resulting from the addition of the deaminase preparation in place of LDH.

Extraction of brain nucleotides

(1) The pattern of nucleotide triphosphate breakdown observed in the brains which were allowed to "thaw" before extraction is shown in Fig. 4.

(2) The concentration of ADP extracted by 10% (w/v) trichloroacetic acid in acetone at −78° was significantly (0·001 > P) lower than was found in the aqueous 0·3 M $HClO_4$ extract (Table 4) and this difference was reflected in the significant (0·01 > P >

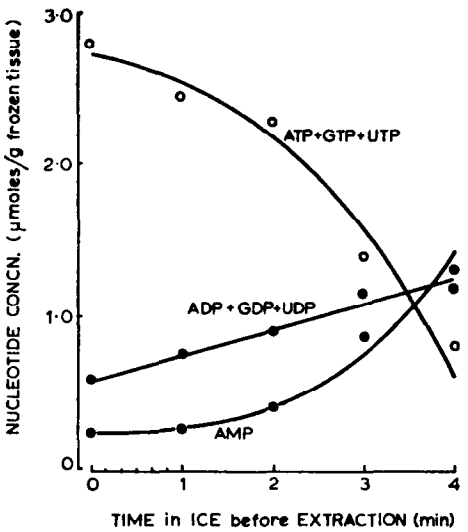

FIG. 4. Nucleotide degradation resulting from "thawing" pulverised frozen brain by placing in wet ice before extraction with 0·3 M $HClO_4$.

TABLE 4. COMPARISON OF AQUEOUS AND NON-AQUEOUS EXTRACTION OF BRAIN

Nucleotide	Concn (μmoles/g frozen brain) after extraction by:	
	Aqueous 0·3 M $HClO_4$ at 0° (9)	10% (w/v) trichloroacetic acid in acetone at −78° (4)
AMP	0·31 ± 0·03	0·26 ± 0·03
ADP	0·51 ± 0·03	†0·33 ± 0·01
ATP	2·10 ± 0·05	2·08 ± 0·03
AMP + ADP + ATP	2·92 ± 0·07	*2·66 ± 0·03

Significance of the difference between the ion-exchange chromatographic analyses of extracts produced by the two methods of extraction:

* 0·01 > P > 0·001; † 0·001 > P.

Concentrations are means (± S.E. of mean) of the number of estimations shown in brackets ( ).

0·001) difference which occurred also in the total adenine nucleotide concentrations. No significant differences occurred in the AMP or ATP concentrations of the two types of extract.

(3) The concentration of ATP in extracts from freeze-dried brains was significantly ($0{\cdot}01 > P > 0{\cdot}001$) higher than in those prepared from frozen brain in the usual way (Table 5). No significant changes were observed in the concentration of AMP, ADP or the total adenine nucleotides, as a result of freeze-drying before extraction.

TABLE 5. COMPARISON OF EXTRACTION OF FROZEN AND FREEZE-DRIED BRAIN TISSUE

Nucleotide	Concn (μmoles/g frozen brain) found by extraction of:	
	Frozen brain (6)	Freeze-dried brain (4)
AMP	0·25 ± 0·04	0·24 ± 0·09
ADP	0·51 ± 0·03	0·49 ± 0·01
ATP	2·07 ± 0·03	*2·30 ± 0·04
AMP + ADP + ATP	2·83 ± 0·04	3·04 ± 0·13

Significance of the difference between the ion-exchange chromatographic analyses of extracts produced by the two methods of extraction: *$0{\cdot}01 > P > 0{\cdot}001$. Concentrations are means (± S.E. of mean) of the number of estimations shown in brackets ( ).

DISCUSSION

It is clear from the comparison of assay procedures that the Kalckar method is unsatisfactory as an assay for the adenine nucleotides in brain extract. Its estimate of AMP is particularly high. This may be due to enzymic contaminants in the deaminase preparation, which is obtained from rabbit muscle by a very simple purification.[18] The spectral changes which result from its addition to brain extract are not those which would be expected from the deamination of AMP. A brief search for enzymic contaminants in the deaminase preparation revealed CPK and LDH activity. Neither of these enzymes would themselves be likely to cause the erroneously high AMP estimates. LDH would be unlikely to cause any optical changes at 265 mμ since no brain NADH would survive oxidation during the acid extraction. If CPK interfered with the Kalckar assay of ADP and ATP, at pH 6·1 it would probably convert ADP to ATP.[19] Preliminary work[17] suggests that the presence of added PC in the Kalckar reaction mixture does not alter the observed estimates of ATP, ADP or AMP. It does seem likely, however, that some enzymic contaminant present in the deaminase preparation is responsible for the discrepancies observed.

Whatever the cause(s) of the errors in the results of the Kalckar method, it is clearly unsuitable for use on brain extract. The reports by Lewis and coworkers[1–6] that many centrally acting drugs alter the *in vivo* levels of the rat brain adenine nucleotides in a consistent manner, thus come under suspicion. Some of these drug effects have been re-examined using reliable methods of analysis and the results will be published elsewhere.

The experiments on the extraction of brain indicate that a very brief period (<5 sec)

of exposure of the pulverised brain to temperatures just below 0° does not introduce an appreciable error due to nucleotide triphosphate degradation. This is confirmed by the comparison of the extraction method with the two other procedures designed to obviate such breakdown. The non-aqueous acid, while yielding less ADP, did not yield a correspondingly higher ATP concentration than the aqueous $HClO_4$; the latter is thus the preferable extractant. Extraction of freeze-dried brain similarly failed to achieve significantly less degradation since ADP and AMP concentrations were unchanged, although the ATP concentration was increased by this procedure. The latter finding has been previously reported[9,20] although total adenine nucleotide levels as high as 3·0 μmoles/g were not observed, possibly because the extractions were carried out with 50% ethanol/0·3 M $HClO_4$.

TABLE 6. COMPARISON OF *IN VIVO* LEVELS OF ADENINE NUCLEOTIDES IN RAT BRAIN AS FOUND BY VARIOUS WORKERS

Reference	No.*	Concn (μmoles/g frozen brain) AMP	ADP	ATP	AMP + ADP + ATP
Present work	55	0·28	0·53	2·03	2·84
8	4	0·07	0·24	2·06	2·34
9		0·02	0·13	1·90	2·04
10	3	0·06	0·34	1·58	1·97
13	2	0·18	0·47	1·46	2·11
20	4	0·03	0·14	2·05	2·22
21	7	0·25	0·60	1·76	2·61
22	11†	0·23	0·55	1·77	2·55
23	2	0·17	0·39	1·99	2·56

* Number of determinations on which means are based.
† Rats lightly anaesthetised before sacrifice.
Method of extraction: refs. 13, 21–23 and present work, aqueous denaturant at approx. 0°.
refs. 8–10 and 20, non-aqueous denaturant at −78°.
Method of assay: refs. 8–10, 13, 20, 23 and present work, ion-exchange chromatography.
refs. 21, 22, paper chromatography.

Thus rapid extraction of the adenine nucleotides from brain tissue using aqueous 0·3 M $HClO_4$, as described in the present paper, satisfactorily prevents breakdown of ATP. Table 6 shows a comparison of *in vivo* levels of adenine nucleotides in rat brain as reported by some other workers.

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REFERENCES

1. J. J. LEWIS and G. R. VAN PETTEN, *J. Pharmac. exp. Ther.* **136**, 372 (1962).
2. J. J. LEWIS and G. R. VAN PETTEN, *Br. J. Pharmac. Chemother.* **20**, 462 (1963).
3. C. L. KAUL and J. J. LEWIS, *J. Pharmac. exp. Ther.* **140**, 111 (1963).
4. C. L. KAUL and J. J. LEWIS, *Biochem. Pharmac.* **12**, 1279 (1963).
5. C. L. KAUL, J. J. LEWIS and S. D. LIVINGSTONE, *Biochem. Pharmac.* **14**, 165 (1965).
6. J. J. LEWIS, A. P. RITCHIE and G. R. VAN PETTEN, *Br. J. Pharmac. Chemother.* **25**, 631 (1965).
7. H. M. KALCKAR, *J. biol. Chem.* **167**, 445 (1947).
8. F. N. MINARD and R. V. DAVIS, *J. biol. Chem.* **237**, 1283 (1962).
9. S. EDEL, C. JUDES, M. RAMUZ and P. MANDEL, *C.r. hebd. Séanc. Acad. Sci., Paris* **261**, 3695 (1965).
10. I. PEČHÁŇ, *Biochem. Pharmac.* **14**, 1651 (1965).
11. R. F. FURCHGOTT and T. DE GUBAREFF, *J. biol. Chem.* **223**, 377 (1956).

12. R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM and V. R. POTTER, *J. biol. Chem.* **209**, 23 (1954).
13. K. KANIG and W. KORANSKY, *Arch. exp. Pate. Pharmak.* **241**, 484 (1961).
14. Pabst Laboratories, Milwaukee 5, Wisconsin,Circular OR-10 (1956).
15. H. ADAM, *Methods of Enzymatic Analysis.* (Ed. H.-U. BERGMEYER) p. 539. Academic Press, New York (1963).
16. H. ADAM, *Methods of Enzymatic Analysis.* (Ed. H.-U. BERGMEYER) p. 573. Academic Press, New York (1963).
17. W. S. WILSON, Ph.D. Thesis, Glasgow University (1968).
18. H. M. KALCKAR, *J. biol. Chem.* **167**, 461 (1947).
19. S. A. KUBY, L. NODA and H. A. LARDY, *J. biol. Chem.* **210**, 65 (1954).
20. S.-O. BRATTGÅRD, H. LØVTRUP-REIN and M. L. MOSS, *J. Neurochem.* **13**, 1257 (1966).
21. E. GERLACH, H. J. DÖRING and A. FLECKENSTEIN, *Pflügers Arch. ges. Physiol* **266**, 266 (1958).
22. H. J. DÖRING, A. KNOPP and T. MARTIN, *Pflügers Arch. ges. Physiol.* **269**, 375 (1959).
23. R. N. LOLLEY, W. M. BALFOUR and F. E. SAMSON, JR., *J. Neurochem.* **7**, 289 (1961).